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Aqueous Two-Phase Systems and Microfluidics for Microscale Assays and Analytical Measurements

Tasdiq Ahmed,[1] Cameron Yamanishi,[1] Taisuke Kojima,[1] and Shuichi Takayama[1,2]

[1]Walter H. Coulter Department of Biomedical Engineering, Georgia Institute of Technology and Emory School of Medicine, Atlanta, Georgia 30332, USA; email: takayama@gatech.edu

[2]Parker H. Petit Institute of Bioengineering and Bioscience, Georgia Institute of Technology, Atlanta, Georgia 30332, USA

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Abstract

Phase separation is a common occurrence in nature. Synthetic and natural polymers, salts, ionic liquids, surfactants, and biomacromolecules phase separate in water, resulting in an aqueous two-phase system (ATPS). This review discusses the properties, handling, and uses of ATPSs. These systems have been used for protein, nucleic acid, virus, and cell purification and have in recent years found new uses for small organics, polysaccharides, extracellular vesicles, and biopharmaceuticals. Analytical biochemistry applications such as quantifying protein–protein binding, probing for conformational changes, or monitoring enzyme activity have been performed with ATPSs. Not only are ATPSs biocompatible, they also retain their properties at the microscale, enabling miniaturization experiments such as droplet microfluidics, bacterial quorum sensing, multiplexed and point-of-care immunoassays, and cell patterning. ATPSs include coacervates and may find wider interest in the context of intracellular phase separation and origin of life. Recent advances in fundamental understanding and in commercial application are also considered.

1. INTRODUCTION

1.1. History

Like many scientific discoveries, aqueous two-phase systems (ATPSs) were established in the pursuit of addressing a different problem (1). In the mid-1950s, Per-Åke Albertsson, then a graduate student, struggled to extract pyrenoids from green algae. Pyrenoids are subcompartments in algal chloroplasts, which his advisor suggested can be isolated using chromatography. Albertsson's dilemma was the apparent adsorption of chloroplasts onto the column grains, resistant to even high-molarity phosphate elution buffer. Mistakenly believing polyethylene glycol (PEG) to be a detergent, Albertsson mixed the phosphate buffer solution—containing the grains and organelles—with PEG, and after some time, complete separation was observed, the grains remaining in the buffer and the chloroplasts moving entirely into the top PEG layer. In one experiment, Albertsson would realize two major points: (*a*) polymer and salt can form an ATPS, and (*b*) ATPSs can have an immediate impact on biological research. Albertsson would continue in his graduate work to explore the polymer–polymer ATPS (establishing dextran-PEG as a popular pairing) and its ability to selectively partition proteins (2), organelles (3), and viruses (2). Later work would extend the repertoire to include nucleic acids (4), as well as bacteria and mammalian cells (5, 6), but not pyrenoids, much to Albertsson's dismay.

Although Albertsson is considered the father of the ATPS, he was not the first to discover this phenomenon. Indeed, in 1896, Beijerinck (7) reported that gelatin forms distinct phases in water with either agar or starch. Later work by Dobry & Boyer-Kawenoki (8) would show that polymers soluble in organic or aqueous solvents would generally phase separate. While this work suggests that polymers are incompatible in solution, each forming its own phase, other researchers had detailed polymer combinations in which one phase is polymer rich and the other polymer poor, summarized neatly by Bungenberg de Jong & Kruyt (9). They named this type of phase separation coacervation; the coacervation is complex when opposite-charge polymers make up the dispersed phase. Unlike Albertsson's polymer-demixed ATPS, coacervates are heavily influenced by factors such as pH and salt concentration.

The main interest in using an ATPS lies in its versatility and its broad compositional variability. As Albertsson demonstrated, many liquid-liquid systems are biologically inert and can be handled in physiologic conditions, meaning that proteins, nucleic acids, and cells may be separated without damage. However, extended exposure to PEG or osmotic effects resulting from high-polymer concentrations are factors to be considered when designing experiments, although these can be mitigated.

1.2. Theory

A theoretical understanding of the ATPS begins with an explanation of polymer partitioning (the process of phase separation) followed by target partitioning. Although the Flory-Huggins theory lumps the contribution of water polarity into the interaction terms, recent work examines water in more detail. Zaslavsky and coworkers (10) found that phase separation could be altered by replacing salts, introducing other additives, or adjusting temperature. They proposed that these changes do not affect polymer–polymer interactions, but rather polymer–water interactions. These considerations may also explain how micellar ATPSs phase separate with increasing temperature, while some polymer–polymer ATPSs form with decreasing temperature. A complete theory of phase separation with fewer exceptions remains to be developed, but there is a renewed interest (11) in light of the recent discovery of intracellular phase separation.

The primary use of ATPS has exploited its ability to purify and concentrate biomolecules. A biomolecule will partition, i.e., concentrate primarily into one phase, according to its solute chemical potential, which is a function of different solute physicochemical properties. Partition coefficients can be affected by numerous factors (3, 12). These properties include hydrophobicity, charge, molecular size and surface area, structure and conformation, and solute-polymer affinity. In turn, properties of the phase system that affect partitioning include polymer weight, polymer concentration, interfacial tension, ionic strength, potential across the interface, pH, temperature, hydrophobicity, density, and viscosity. Many of these parameters can be difficult to modulate independently, so empirical analysis will often be necessary to find an optimal ATPS for a particular application.

For biological cells, however, a more reliable method for optimization exists. The surface free energy g is the factor critical for determining if a particle will partition to the top, inter, or bottom phase. The differences in surface free energy Δg between the particle and the individual phases are related to the ATPS contact angle q via Young's equation, $\Delta\gamma = \gamma_{12}\cos(q)$, where γ_{12} is the liquid-liquid interfacial tension. Therefore, estimates of where a cell may partition can be guided by measuring the interfacial tension and contact angle independent of the cell (3, 13). Consideration of buoyancy and density is also important for cells and similarly sized particles.

1.3. Practicalities: Phase Diagrams

For any ATPS, it is necessary to determine the minimal concentrations of its phase-forming components. The collection of these data produces a phase diagram (**Figure 1**). The phase diagram for an ATPS is composed of a binodal (or coexistence) curve, a critical point, and tie-lines. Although the coexistence curve exists along many dimensions, the commonly used axes are (*a*) the mole fraction of each component for polymer–polymer or polymer–salt ATPSs, (*b*) concentration versus temperature for micellar ATPSs, and (*c*) concentration versus temperature or salt for coacervate ATPSs.

For the polymer-demixed ATPS consisting of components A and B, a phase diagram will report concentrations of A and B at the two axes; the component mostly comprising the top phase will be on the vertical axis, and the bottom phase–rich component will be on the horizontal axis. The binodal curve indicates the concentrations of each component where the system transitions from one phase to two phases. For a given initial condition (point a in **Figure 1*a***), the resulting top and bottom phases will have compositions b and c, respectively. The line that connects points a, b, and c is called the tie-line. Applying mass conservation, the relative sizes of the top and bottom phases can be determined by the ratio of the lengths between each segment (a–b versus a–c). This strictly translates to the mass ratio between top and bottom phases, and it also serves as a reasonable approximation of the volume ratio between the top and bottom phases.

Typical micellar ATPS phase diagrams (**Figure 1*b***) instead compare temperature versus surfactant concentration. In these systems, phase separation can be achieved by increasing temperature. The surfactant concentration that allows phase separation at the lowest temperature is also called the cloud point, as the solution becomes turbid (cloudy) as it phase separates. Similar to the polymer-demixed ATPS, a tie-line can be drawn at each temperature, indicating the resulting surfactant-rich and -poor compositions.

Coacervate phase diagrams (**Figure 1*c***) are often applied in more complex forms. The binodal curves are usually inverted, as the forming components will prefer to associate. In these systems, any parameter that influences interaction strength may be used as the y-axis. Temperature

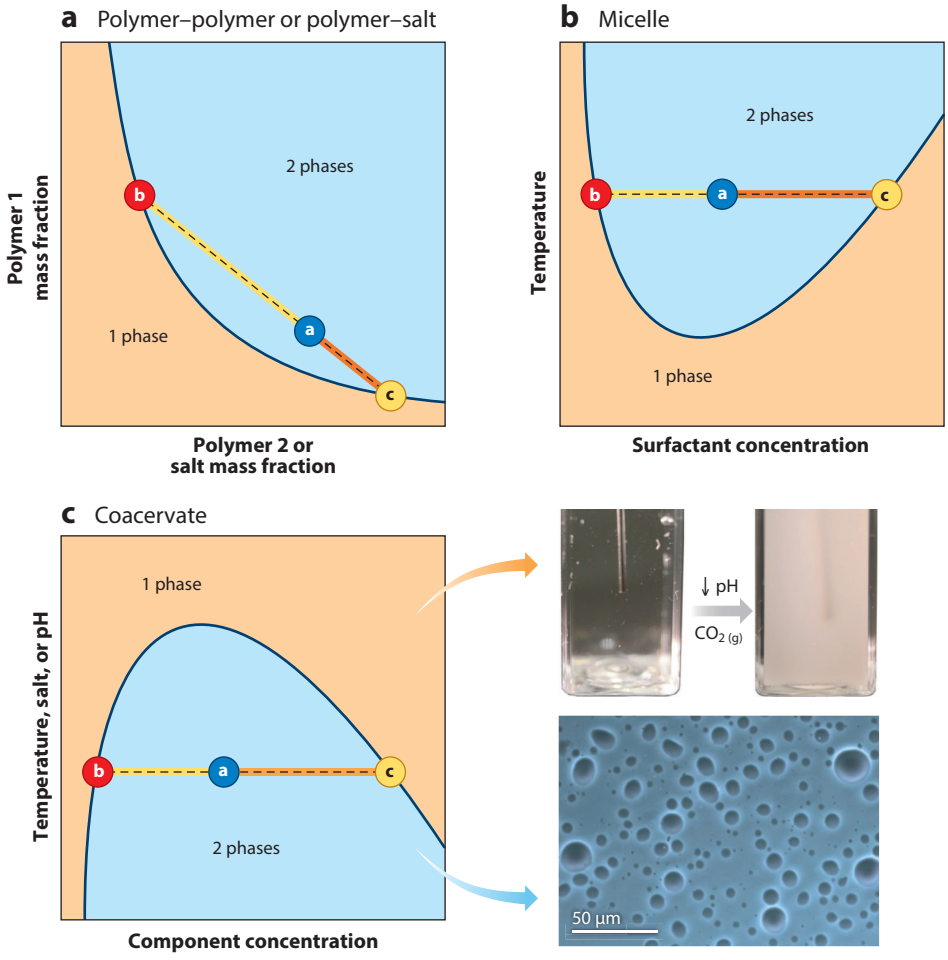

Figure 1

Phase diagrams for three different types of aqueous two-phase systems (ATPSs). (*a*) The most common ATPSs are polymer–polymer and polymer–salt. At high enough concentrations of both components, phase separation occurs. (*b*) Micellar ATPSs are temperature induced and result in surfactant (micelle)-rich and solvent-rich phases. (*c*) Coacervate diagrams are typically inverted in shape, as they are associative. Coacervation is more sensitive to effects such as pH change (*bottom right*). Panel *c* (*right*) adapted with permission from Reference 146; copyright 2011 Springer Nature.

and ionic strength (for polyanion–polycation systems) are frequently used, though pH can be more appropriate for some coacervate systems (**Figure 1*c***), particularly when proteins are the phase-forming components.

Phase diagrams and binodal curves can be found empirically. A common approach takes 10–30 solutions that form two phases, then dilutes them to find the point at which only one phase forms. Care must be taken that orthogonal dimensions (pH, temperature, etc.) are controlled, as variations can affect phase separation. Creating a binodal curve can be tedious, and several groups have sought to achieve facile or automated/rapid binodal determination. Our group has addressed this through inverse droplet dehydration (14) or electrowetting-on-dielectric manipulation (15). Others have created binodals from microfluidic drop formation (16) or simple volume/density

measurements (17, 18). One rather simple method is to prepare an array of concentration pairs and use microscopy to check which pairs demix (19).

1.4. Practicalities: Handling an ATPS

An important consideration is the compatibility of ATPSs with downstream analytical methods, in the event that the ATPS components are not removed. Virtually every popular technique [e.g., high-performance liquid chromatography (HPLC), mass spectrometry, enzyme-linked immunosorbent assay (ELISA), absorbance-based measurement, nucleic acid sequencing, polymerase chain reaction (PCR)] will work provided that diluted aliquots are prepared. The presence of polymers can interfere with mass spectrometry, which can be a limiting factor for protein studies. Polymerases may interact with polysaccharides like dextran, but PCR workflows usually involve sample dilution. Diluting is recommended in general. Specific considerations are provided in later sections.

There is a growing interest in using microfluidics with ATPSs. In a microfluidic device, the length scales are sufficiently small (<100 μm) such that there is Stokes flow (smooth, laminar streams), faster diffusive transport across streams, and a predominance of capillary action over gravitational pull. In a typical device, having two or three input streams produces a continuous flow regime that may be optimal for partitioning experiments. Examples using this system for cell isolation and patterning are presented in later sections. Greater focus in the past decade has been placed on generating ATPS droplets with microfluidics for applications such as forming microscale bioreactors (20), constructing biomaterials (21), or encapsulating cells (22). The extremely low interfacial tension in an ATPS means it often will not form droplets spontaneously. To overcome this, several active process methods have been developed, such as multilevel channel devices using computer-actuated Braille pins (23), controlled vibration of an immersed tube providing a jet stream (24) (**Figure 2*a***), and oscillating inlet pressures to form droplets (25). Passive methods have been more recently reported, in which weak gravity-driven hydrostatic forces create sufficiently low flow rates such that Rayleigh-Plateau instability results in streams breaking into droplets (26). Further work from this group showed that droplet size could be modulated with the addition of a second cross junction downstream (27), and the incorporation of a microneedle enhances throughput (28). A competing method is to use an adjacent oil stream that forms oil droplets to chop the dextran-phase stream into droplets (**Figure 2*a***); this method exhibits even greater control over and consistency in droplet formation (29). The flow-focusing geometry of these devices can also generate coacervate droplets that remain stable after several days (30).

The engineering of droplet emulsions is also a focus of ATPS research, aided by the use of droplet microfluidics (**Figure 2*b***). Typical double emulsions in the literature are water/oil/water, though some examples of all-water emulsions exist. The first study formed dextran/PEG double emulsions using a glass capillary microfluidic device (31). Further complexity of ATPS emulsions is possible, from Janus droplets to multiphase droplets capable of applications such as glucose sensing and drug delivery (32).

2. PARTITIONING OF SMALL MOLECULES, BIOMOLECULES, AND CELLS

2.1. Small Molecules and Polysaccharides

Chromatographic partitioning has long been used for purification and analysis of small molecules produced by synthesis or collected from nature. However, traditional methods often rely on an organic phase/aqueous phase system. Apart from their incompatibility with biomolecules, organic

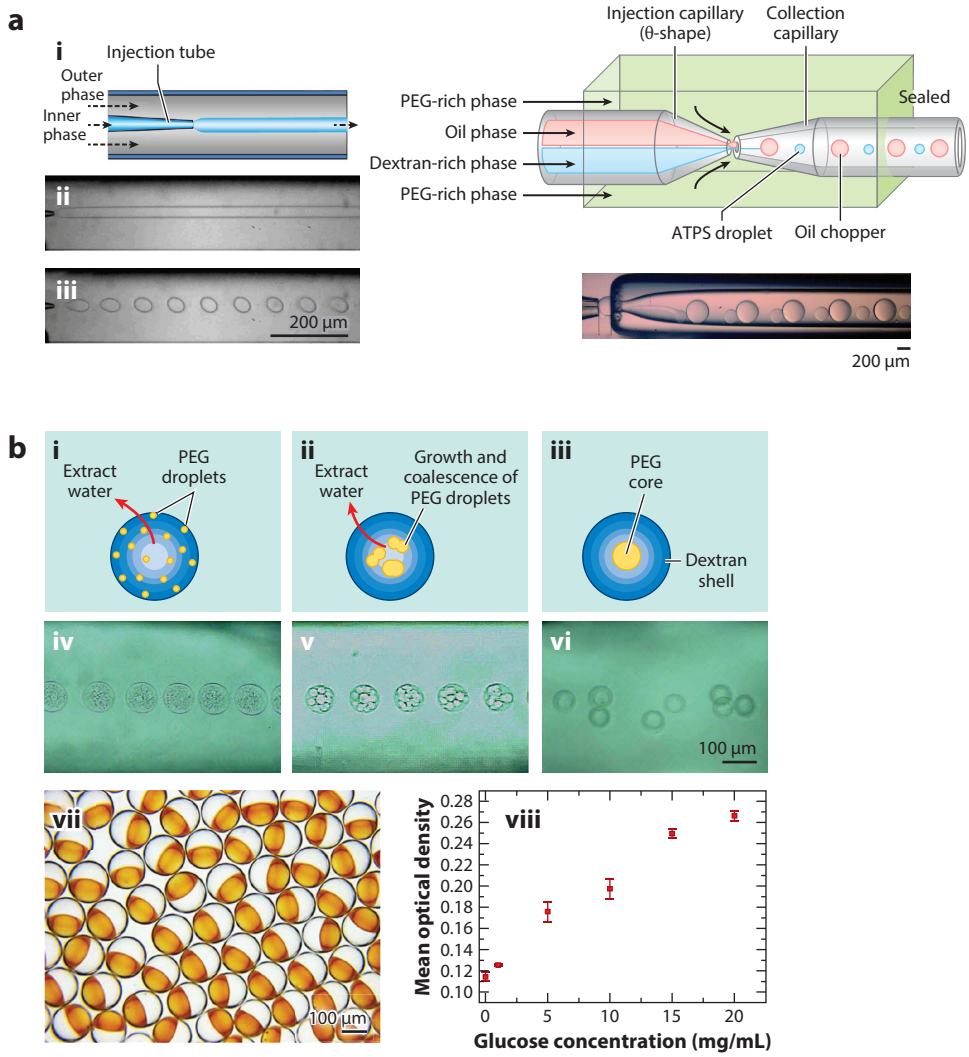

Figure 2

(*a*) Aqueous two-phase system (ATPS) droplets can be formed either actively (*left*) or passively (*right*). One example of an active process is to agitate a jet stream, such as in a glass capillary microfluidic device. Passive droplet formation is commonly done using a flow-focusing channel in which weak hydrodynamic forces generate inlet pressures that form a stream that eventually breaks into droplets; (*right*) the device uses an oil stream to improve consistency and formation rate. Panel (*left*) adapted with permission from Reference 24; copyright 2012 AIP Publishing and (*right*) adapted with permission from Reference 29; copyright 2017 Royal Society of Chemistry. (*b*) All-aqueous emulsions can be created, such as polyethylene glycol (PEG)-core/dextran-shell and Janus droplets that have many potential applications. In subpanels *i* and *iv*, initial contact with the outer PEG stream induces micro-separation of PEG within the emulsions. In subpanels *ii* and *v*, droplet dehydration occurs, shrinking the emulsions and driving the PEG droplets to coalesce. In subpanels *iii* and *vi*, PEG droplets have completely merged, forming a core-shell emulsion. (*vii*) Janus droplets composed of PEG and fluorescein isothiocyanate (FITC)-labeled dextran and loaded with concanavalin A, which quenches FITC-dextran; (*viii*) glucose can be sensed as it replaces dextran and recovers fluorescence. Panel *b*,*i–vi* adapted with permission from Reference 31; copyright 2012 American Chemical Society. Panel *b*,*vii–viii* adapted with permission from Reference 32; copyright 2017 American Chemical Society.

solvents can have environmental consequences by way of being toxic or volatile. ATPSs are quite favorable in this regard, with low environmental impact. Polymer-based ATPSs are still used for small-molecule separation, but the use of ionic liquid–based ATPSs has expanded in the past decade since its discovery in 2003 (33). Careful consideration of phase-forming component size is of special interest when working with molecules much smaller than typical ATPS polymers.

Charged and uncharged benzene derivatives can be separated with ATPSs, exhibiting similar partitioning to organic/water systems (34, 35). Dyes are heavily used in the textile industry but are prominent pollutants, and work toward green decontamination has materialized (36, 37). ATPSs allow for low-cost purification of commercially relevant molecules, such as vanillin (38), a phenolic extract from vanilla beans, and stevioside (39), a glycoside and natural sweetener. Although carbon nanotubes were used as an additive to enhance vanillin partitioning, they themselves can be rapidly isolated according to size using PEG/dextran (40, 41). Metal ions can also be purified in polymer-based ATPSs (42).

In recent years, research using ATPSs for antibiotics has increased. Notable antibiotics compatible with aqueous two-phase extraction include cephalosporin (43), vancomycin (44), and ciprofloxacin (45). However, antibiotics in food can be undesirable, as they may lead to resistance or adverse reactions in humans; methods relying on ATPSs for testing antibiotic presence have been developed, notably for tetracycline in honey (46) and fluoroquinolones and sulfonamides in milk (47). Insecticide detection has also been reported for the analysis of tea drinks (48).

Polysaccharides such as dextran and methylcellulose phase separate; indeed, together they form an ATPS. Rather than discovering new phase-forming sugar polymers, recent studies use ATPSs to isolate and purify polysaccharides (49, 50). There has been interest in using ATPSs for bioconversion of cellulose and related materials, which are among the most abundant sources of renewable energy (51). ATPSs have also been shown to aid the conversion of cellulose to glucose (52) or ethanol (53). In lieu of enzymes, bacteria cultured in ATPS may ferment cellulose to produce sugars and hydrogen (54).

2.2. Proteins

Proteins have been and continue to be a significant focus in the field of ATPS. We will briefly discuss the extraction of proteins, which started at the dawn of ATPS research and remains an active area as proteins find new clinical applications. It is not in our interest to review protein extraction in depth, as the principle remains the same for other molecules of interest. Instead, we focus more on using ATPSs for learning more about a protein.

2.2.1. Protein extraction. Extraction and purification have been demonstrated for amino acids (55, 56), polypeptides (57, 58), globular proteins (3, 57), and enzymes (3, 59). These processes can be scaled for commercial purposes (60). A recent case study is the extraction of monoclonal antibodies (mAbs), which are of growing interest since their realization as therapeutic molecules (61). Scaling up and accelerating production of existing mAbs, while reducing cost, are needed to meet global demand (62). Because these are pharmaceuticals, achieving complete purification is critical. Antibody purification is possible with PEG/salt ATPSs, notably PEG/citrate, which is more environmentally friendly at industrial scale (63). This system can be adapted to microfluidics for reduced process time (64). Additional research is needed to convince industry to change its preference from protein A chromatography (65).

2.2.2. Protein characterization. The biochemical and structural properties of a protein can influence its partitioning. Therefore, studying protein partitioning may in turn reveal or at least

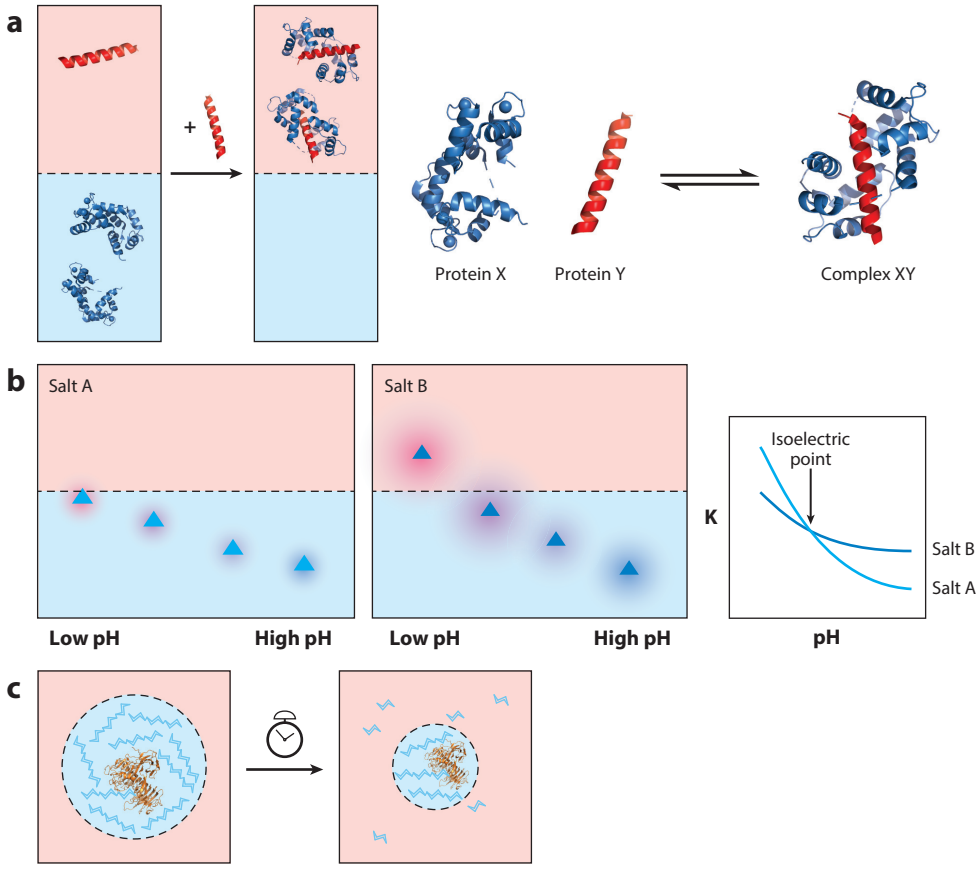

Figure 3

(*a*) Protein–protein and protein–ligand binding constants can be calculated from their partition coefficients, in which testing concentration ranges results in a partition versus concentration curve from which binding constants are extracted. Spectrin (*right*) binding to calmodulin (*left*) has been notably examined (Protein Data Bank: 2FOT). (*b*) Examining the salt effect on protein partitioning in relation to system pH can potentially identify the protein's isoelectric point. (*c*) Enzyme activity (e.g., dextranase; Protein Data Bank: 1OGM) can be assessed when the enzymatic substrate is one of the phase-forming components (dextran).

infer about those properties. In finding the concentration of a protein in the top and bottom phases of an ATPS, one may calculate the partition coefficient K and, in turn, assess the interaction of one protein and another, the influence of a ligand on a protein's structure, or even slight structural differences between closely related proteins. As discussed, the partitioning of any biomolecule in an ATPS can be swayed by the composition of the ATPS. Exploiting this principle will enable characterization.

2.2.2.1. Protein–protein and protein–ligand interactions. If two proteins interact, there may be a change in either protein's solubility, hydrophobicity, or surface charge, and thus, each partition coefficient K in an ATPS might change (66). Measuring any changes to K values could then reveal information about the interaction (**Figure 3*a***). To achieve this, one first measures the concentrations of two proteins (X and Y) independently in each phase of a chosen ATPS; it is important that the resulting K_X and K_Y are significantly different; e.g., protein X partitions mainly to the top

phase, protein Y to the bottom. If there is an interaction between X and Y, then less free protein is available to move, and so K_X and/or K_Y changes; subsequently, there exists a K_{XY}. To study the binding of X to Y, a concentration series of Y should be tested. K_{Xtotal} is a function of K_X and K_{XY}; K_{Xtotal} should equal K_X when $[X] \gg [Y]$ and equal K_{XY} when $[Y] \gg [X]$; adding Y incrementally should change K_{Xtotal}. Plotting the resultant K_{Xtotal} values and finding the best model fit will then allow calculation of an association constant. This method offers the simplicity and at-equilibrium state of the ATPS as well as its compatibility with any chosen buffer and a variety of methods for finding protein concentration (e.g., spectrophotometry, radioactivity, coupled enzyme reactions). However, unless the optimal ATPS is found (in which K significantly differs between X and Y), a lot of sample will be needed; trial and error in finding the ATPS that offers the highest sensitivity may be helpful.

The principle behind this method has been explored in different ways. One exhaustive study looked at pancreatic lipase and colipase interaction and the modulation of their complexation by system pH, bile salts, and lipid substrates (67). The method may be applied to studies of protein assembly, as shown by one group that examined tetramer–dimer dissociation in hemoglobin (68). Here, the researchers posited that polymers may affect the protein–protein equilibria; they showed that by plotting association constants against polymer concentrations of each phase, constants that agree with prior findings can be retrieved. An interesting but limited reworking of the principle can reveal information about the content ratio in initial mixtures to identify how much of a clinically relevant protein is present (69).

The same model for protein–protein interactions can be adapted for protein and ligand. Ligands such as ions (70) and nucleotides (71) will typically distribute evenly between two phases, in which case the procedure is made even simpler, as one would only have to measure the amount of free ligand for a range of protein concentrations to calculate K values and disassociation constants.

2.2.2.2. Protein conformational states and structure. When a ligand binds to a protein, there is a potential for a change in the structure and thus modulation of function. Imaging methods such as cryo-electron microscopy can provide atomic-resolution structural data important for drug design. However, this level of detail is not always needed, and research reports do not always cover multiple isoform variants, different posttranslational modifications, or conformational states. ATPSs can complement other methods to help address these shortcomings.

Any rearrangement of solvent-exposed residues, such as those due to ligand binding, should affect partitioning. An example of probing for structure changes can be seen in the study of hormone receptors; the case of the estrogen receptor (ER) is presented here. Partitioning of ER with steroid ligand is markedly different than for ER without ligand; substituting PEG-palmitate for PEG in a PEG/dextran ATPS enhances this difference by nearly ten times (72). The unoccupied ER prefers the PEG-palmitate phase, suggesting that upon hormone binding, the receptor surface hydrophobicity is significantly decreased; crystal structures have later confirmed this. Further work from this group examined the ER steroid-binding domain and found the same pattern, meaning the exposed hydrophobicity for ER is attenuated by a hormone-induced conformational change (73). The surface hydrophobicity of a protein may be characterized by log K or ln K, an analog to the well-known log P used for the quantitative structure-activity relationship (QSAR) with small-molecule drugs. It is suggested that compiling a database of ln K may guide computational modeling of protein folding or activity.

Protein–ligand binding is only one form of protein structural change. Other changes can result from enzymatic modifications, such as phosphorylation or glycosylation. In fact, a single phosphate group can dictate which phase a glycophosphatidylinositol (GPI)-anchored protein shows preference for (74). The reported PEG/dextran system with added salts would show distinct partitioning

patterns for alkaline phosphatase released from cell membranes by either phospholipase C or D. The marked differences were striking when compared to the inability for gel electrophoresis to tell apart the isoforms. The added benefit of using ATPS in this case was to overcome the known issue of lipoprotein forming micelles with detergents used in prior studies.

Many structural changes arise at the genetic level, such as through truncating or frameshift mutations that drastically alter the protein. Massive changes are not always present in diseases; indeed, point mutations resulting in closely related isoforms can drive pathophysiology. Zaslavsky et al. (75) carried out a large-scale investigation of the partitioning of variant isoforms in multiple ATPSs with different ion compositions, developing a structural signature based on K values in each system and forming a method they term solvent interaction analysis (SIA) in which proteins may be compared based on their signature. Several proteins were examined, but notably, human insulin different in size by one residue shows marked partitioning differences, and three sets of insulin that are alike except for two residues have distinct signatures that can even reveal lot-to-lot variation. A systematic study of the influence each amino acid has on partitioning is available (76). Modifying proteins through methods such as generating fusion proteins (77) is known to affect separation but may find greater use for extraction.

SIA is emerging as a new application of ATPS, but it has yet to find widespread use. Although it has been expanded to a greater set of proteins, comparing trends in signatures to explicit structural features (charged domains, water-filled cavities, etc.) (78), it may find use in the study of intrinsically disordered proteins (IDPs). IDPs have growing interest for their role in intracellular liquid-liquid phase separation (LLPS), but as they have no determinate structure (order), they can be difficult to characterize. Wild-type alpha-synuclein and three mutant forms were studied using SIA, with each variant showing distinct structural signatures (79). Further elucidation of residue/protein and solvent interaction is needed to reveal meaningful insights.

2.2.2.3. Isoelectric point. Another interesting case of deriving new information from the partition coefficient is the determination of the isoelectric point (pI) by cross-partitioning (**Figure 3*b***). Different salts can exhibit opposing effects on the K value of the same protein. By experimentally producing K value versus pH curves, it was seen that the curves from two ATPSs that differ only by salt composition cross at one point (80). Albertsson noticed that this cross-partition point marked a pH similar to that protein's known pI. The observed phenomenon could be explained by how at the pI, a protein's net charge is zero, and therefore the cross-point K value represents the charge-independent characteristic K value. Measured K values can, however, stray from expected values due to potential protein aggregation or polymer–protein interactions. The cross-point pH only sometimes agrees with the pI found by electrophoresis. One hypothesis is that disagreements result from polymer-mediated protein conformation changes; testing several ATPSs would resolve this (81). However, traditional models employ a linear equation to relate partition to pI, whereas linearity is not always observed in practice. These models do not account for the discovered roles that salt and solvent have on protein partitioning. More information is needed to produce a thorough theoretical basis and better representative model. While this method of cross-partitioning is not perfect and it does not offer the directness of using traditional electrophoretic methods (e.g., immobilized pH gradients), the low cost, ease of use, and general reliability may find use, especially for studying proteins stable at high salt concentrations, or even organelles such as mitochondria (82).

2.2.2.4. Enzyme activity. Proteins normally exist in the crowded environments of cells and have evolved to operate under these conditions. In vitro assays have shown that the addition of crowding agents such as PEG increases protein activity (83). Therefore, testing for enzyme

activity within an ATPS may provide better insight into intracellular function. Our group has designed a method for visualizing activity through droplet shrinking (**Figure 3*c***). Dextranase was used as a model enzyme, as its substrate is dextran. By placing the enzyme within a dextran droplet in PEG, shrinking of the droplet corresponds to dextranase activity (84). Further work in our lab sought to address the issue of substrate inhibition, as immersion within dextran can limit the reaction rate. Compartmentalizing the enzyme in a coacervate, forming a three-phase system of PEG/dextran/ATP:PDDA, overcomes this inhibition (85). Another example of monitoring activity involves a kinase within an ATP-based coacervate that also utilizes coupled reactions; this work is described further in Section 3.3. The general method of using an enzyme within a phase works well when the enzyme substrate is one of the phases, which limits the number of applications. A recent study looked at a cascade system of two enzymes that do not accept either of the phase polymers as substrates (86). In this work, activity was not found to be optimal, as substrate diffusion slowed, potentially as a result of polymer interaction. The result suggested that more complex features are at play in regulating intracellular enzyme activity.

2.3. Nucleic Acids

Nucleic acid partitioning depends on their molecular size and structure in addition to the phase-forming components, salt species, pH, and temperature. The majority of work has been performed on DNA. Partitioning of double-stranded and single-stranded DNA with the same molecular weight significantly differs in a PEG/dextran system (3). Notably, the DNA K values vary from 10^{-2} to 10^{2}, depending on electrolytes. This variance is perhaps attributed to the Hofmeister effect. The classical interpretation is that ions affect partitioning by interacting with the solvent H-bond network, thus either supporting or destabilizing the solute. However, newer models explain this effect by stating that certain ions (particularly anions) directly interact with the solute and its hydration shell (87). The exact mechanism of the Hofmeister effect is still unknown.

PEG/salt systems are often used for purification of plasmid DNA with high (80–100%) recovery rates that can be scaled up (88). Different types of plasmids can even be distinguished (89). These ATPSs can be applied to cell lysates, with efficient removal of impurities such as genomic DNA and RNA, but not with very high yields (89, 90). However, fragmented genomic DNA could be isolated using a micellar ATPS (91). This study did show slight separation of a 21-bp oligonucleotide, but it is generally expected that single nucleotides and short oligomers will distribute evenly in a polymer-based ATPS. In light of this, small DNA oligomers can be selectively partitioned by applying electric fields. Microfluidic devices can advance PEG/dextran systems with electric field–assisted manipulation of DNA partitioning (**Figure 4*a***) for step-wise accumulation, separation, and recovery (92, 93).

One area of interest is in the application of ATPSs toward PCR. Purification of PCR DNA amplicons has been demonstrated with a two-step process: a PEG/polyacrylate system for protein removal, followed by collecting the PEG phase and adding sodium sulfate to recover the amplicons (94). Performing PCR reactions within a two-phase reactor could be an interesting way to increase throughput and enable point-of-care testing. A recent report showed that an analogous DNA amplification method could operate in a micellar ATPS (95) (**Figure 4*b***). Here, a centrifuge tube containing amplification reagents, bacteria, and Triton X-100 is heated to 68°C. The heat induces phase separation and aids the surfactant in lysing the cells. The DNA partitions to the micelle-poor top phase alongside the reagents and the reaction is carried out, all at the same temperature and in the same vessel.

In contrast, relatively few studies have utilized ATPS for separation of RNA, likely because of significantly less-selective partitioning compared to DNA (96). Despite this, recent work has

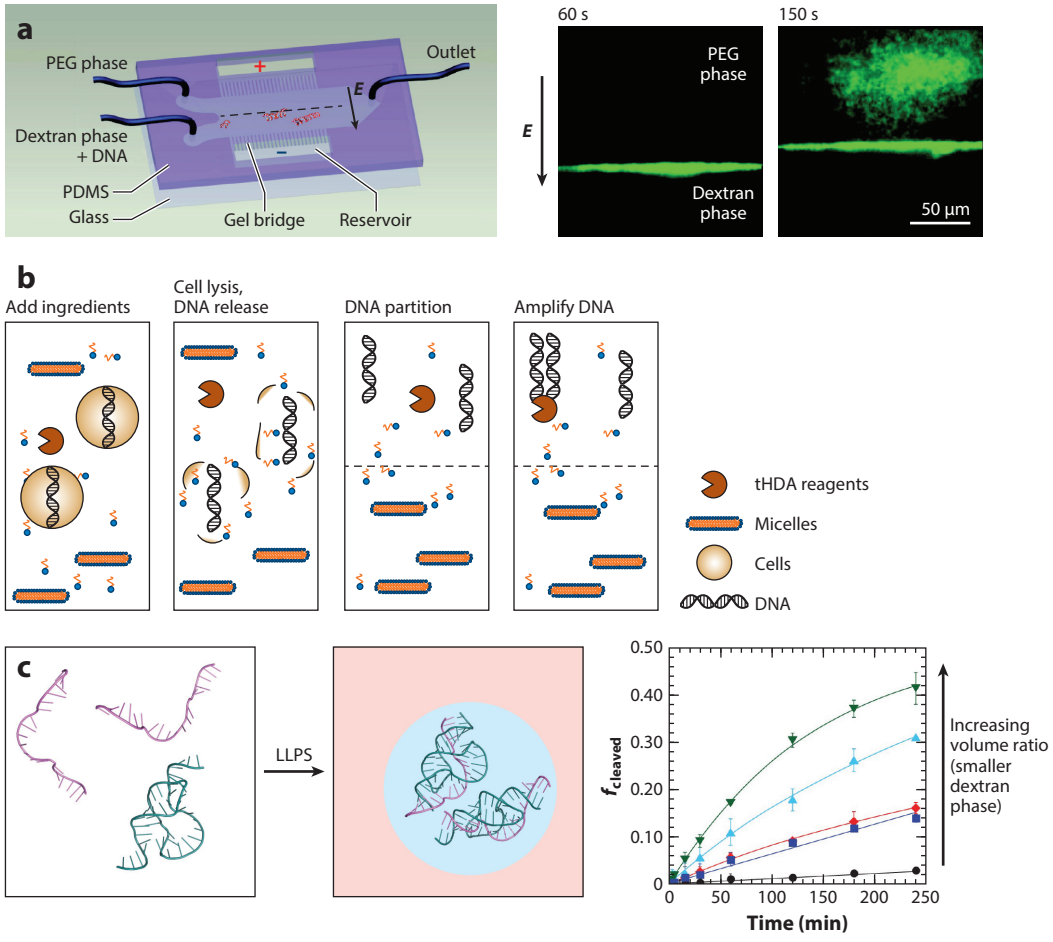

Figure 4

(*a*) A microfluidic device with an electric field applied across flow enables controlled partitioning of DNA with different sizes. Panel adapted with permission from Reference 92; copyright 2011 American Chemical Society. (*b*) By using a micellar system, cells are lysed, and their genomic content is compartmentalized with DNA-amplifying enzymes, resulting in an all-in-one reaction vessel. (*c*) When ribozymes (Protein Data Bank: 3ZP8) and their substrates are placed in a PEG/dextran aqueous two-phase system, they partition into the dextran phase, and the cleaving activity is enhanced. A further increase in activity is observed by adjusting the volume ratio such that the dextran phase shrinks. Panel (*right*) adapted with permission from Reference 97; copyright 2012 Springer Nature. Abbreviations: LLPS, liquid–liquid phase separation; PDMS, polydimethylsiloxane; PEG, polyethylene glycol; tHDA, thermophilic helicase–dependent amplification.

shown that volume ratio can have a strong impact on partitioning (**Figure 4*c***): Hammerhead ribozyme activity increases as the dextran-rich phase volume decreases (97). ATPS-based RNA purification has seldom been demonstrated to date. Smaller molecular size and lower stability of RNA compared to DNA perhaps hinder research progress.

2.4. Vesicles and Viruses

Extracellular vesicles (EVs) are membrane-bound nanoparticles that act as messengers between cells (98). As they contain biomaterial representative of their cell of origin, EVs are increasingly being studied for biomarker discovery as well as aiding in diagnosis. As such, high-yield EV

isolation is critical. ATPSs have been used to address this need, achieving rapid and relatively high-yield recovery through collecting intact, soluble EVs into the dextran phase and contaminants into the PEG phase (99). It is important to note that EVs are not precipitated in these systems. One major concern is the potential for dextran interference with PCR, but their compatibility is presented in Reference 99; however, the inability to perform mass spectrometry remains. Taking a step further, isolation of EVs from tissue or urine of prostate cancer patients followed by downstream gene and protein analysis demonstrates the potential for ATPSs in clinical diagnosis (100, 101). Because these developments are recent, further research is needed to expand the repertoire of bodily fluids compatible with ATPS-mediated EV isolation. The work performed so far does show the ease of use and efficiency of this method and that more focus may be placed on the analysis of retrieved biomarkers.

An interesting application of partitioning of liposomes is to probe surface interaction with blood plasma proteins and analyze if surface modifications affect these interactions (102). Work such as this can predict how drug-bearing liposomes will fare in vivo. Investigation into liposome cargo can shift perspective of drug delivery research away from membranes. A recent study designed liposomes to be loaded with PEG/dextran and doxorubicin (103). By using a drug that partitions mainly to the inner dextran core, slower and sustained drug release can be achieved. Results confirmed this, as greater uptake and significantly decreased IC_{50} was observed with ATPS-loaded liposomes compared with ATPS-free liposomes and free drug.

Viruses have been a cornerstone of biological research since their discovery in the 1890s. In fact, Beijerinck (104), whose initial ATPS work is mentioned above, also discovered the first virus and even redefined the term to its present meaning. Since the initial findings during the fin de siècle, work to understand viruses and their role in infectious diseases has been and continues to be of great importance. Low-cost, rapid, and scalable methods for diagnosing patients and detecting new viruses are crucial, and ATPS is one avenue for addressing this need.

While viruses are the most prevalent biological entity, they exist at very low concentrations (3), on the order of 10^7 per milliliter. Some of the early ATPS work was devoted to concentrating and purifying viruses. A plethora of examples have been published, but the method overall has yet to achieve wide adoption (3, 105). As noted in Reference 105, ultracentrifugation carries many risk factors, such as shearing of viral surface proteins necessary for cell latching and entry; loss of these markers not only reduces infectivity but can lead to poor or inaccurate quantification. These issues are avoided in ATPSs. An ATPS can concentrate virus by one to two orders of magnitude and achieve significant purification (3). The number of rounds of separation depends on the species of interest, but usually one is enough. Focus may need to be placed on polymer removal from isolated fractions; the addition of salt can induce polymer precipitation and thus permit removal. There is, however, a small likelihood of physical attachment of dextran sulfate, a polyelectrolyte, to viral membranes, potentially affecting their activity. This is prevented by using a PEG/salt system for extraction via precipitation (106).

The same principles guiding protein analysis have been adapted to the study of viruses. Miniscule changes in viral coat proteins, e.g., a single residue, can influence electrophoretic mobility; a PEG/dextran system at pH 8.0 can resolve four bacteriophage mutants of this kind, their partition coefficients forming a pattern similar to established electrophoresis data (107). Poliovirus partitioning can be affected by the introduction of a specific antibody, and their interaction kinetics can be evaluated purely by using a phase system (108, 109).

2.5. Cells

In the early work to test cell partitioning, different strains of bacteria were isolated using an ATPS (3). Beyond such preparative bacteria isolation applications, the ability of ATPS microdroplets to

confine bacteria has been used to print bacteria colonies and biofilms over other bacteria (110) and mammalian cell monolayers (111). The bacteria-over-bacteria bioprinting method demonstrated the ability of an antibiotic resistant bacteria to rescue nonresistant bacteria (110). The bacteria-over-mammalian cell method was used to screen for pathogenic bacteria that injure epithelial cells and for beneficial bacteria that rescue the epithelium from the attacking pathogenic species (111). In another bacteria study, droplets of quorum-sensing bacteria were printed with magnetic particles adjacent to a droplet of signal-sender bacteria (112). While the ATPS physically confines the sender and receiver bacteria in their respective droplets, the quorum-sensing signal diffuses freely through the ATPS, from the dispatching droplet to the receiving droplet whereby green fluorescent protein (GFP) expression is induced (112). The magnetic beads allowed the sensing bacteria–laden droplets to be moved. This capability helped to demonstrate that bacteria periodically relocate to maximize their quorum-sensing response in order to escape local oxygen depletion.

ATPSs are more often used for separating mammalian cells. Systems of affinity partitioning were developed to extract immune cells away from red blood cells; one example used a PEG-antibody conjugate (113). To target leukocyte subpopulations, tailoring an ATPS to cell-type surface charge has been demonstrated (114). Surface charge, however, is not the only determinant in cell partitioning (115). One high-profile application of using phase systems was recently shown for the identification and subtype characterization of sickle cell disease, on the basis of density differences (116) (**Figure 5*a***). Two systems were developed—one using PEG/Ficoll, the other PEG/dextran/poly(vinyl alcohol)—for rapid visualization of slightly denser, disease-indicative red blood cells.

In this century, researchers have focused on using microfluidics for efficient cell isolation and sorting. Continuous partitioning is achieved using microfluidics, in which the large surface-to-volume ratio mitigates the slow cell migration in stationary vertical setups. Channels can be designed such that two phases flow side-by-side with negligible effect from gravity during the time it takes for the liquids to flow through the channel. An early use of ATPSs with microfluidics was published in 2004, in which plant cell aggregates were partitioned in a device with PEG/dextran (117). Addition of sulfate reverses cell preference for the PEG stream, but more importantly, engineering the device to force cells to be at the interface increases partitioning efficiency. This was accomplished with a pinched microchannel segment near the inlets. Other attempts to increase interfacial area include the sandwiching of two dextran streams around a PEG phase, with cells from whole blood, to collect leukocytes (118). Encapsulating cells in all-aqueous droplets is another strategy (119) (**Figure 5*b***). In general, the low rate (20–30%) of cell capture means there is room for improvement.

3. RECENT MICROSCALE APPLICATIONS OF ATPS

3.1. Improving Immunoassays with ATPS

ELISA is a commonly used reproducible and highly sensitive method that reliably quantitates biomolecule concentration. Available sample is often limited, and for ELISA to guide clinical diagnosis, multiple biomarkers must be examined. These constraints call for a multiplexed ELISA. One issue that prevents successful multiplexing are false positives and negatives caused by off-target antibody binding. Confining detection antibodies within microdroplet dextran phases in a PEG/dextran system allows for otherwise incompatible antibody combinations to be used (120). In-laboratory preparation of ATPS-enhanced ELISA can be tedious, and later work demonstrated the ability to dehydrate dextran droplets containing both capture and detection antibodies (121). Manufacturers could then produce plates in which the end user will only have to rehydrate the

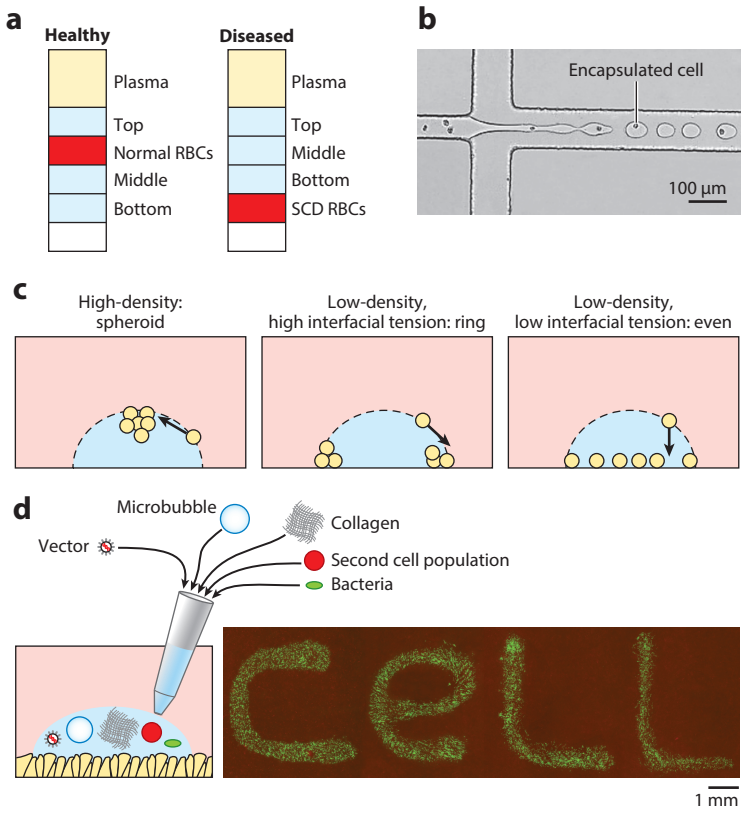

Figure 5

(*a*) A multiphase system composed of three polymers can differentiate between healthy and disease-indicative red blood cells (RBCs). Specific visual patterns that form can even identify disease subtype. (*b*) A flow-focusing microfluidic device can passively make single-cell salt droplets in a polyethylene glycol carrier fluid. Panel adapted with permission from Reference 119 under the terms of the Creative Commons Attribution 4.0 International License, **http://creativecommons.org/licenses/by/4.0**. (*c*) Cells can act as bioink printed onto desired substrates, with varying levels of control. High-density polymer solutions can promote cell buildup at the highest point of the liquid–liquid interface, forming spheroids. Low-density solutions can have different patterning modes based on the phase system's interfacial tension. (*d*) Cells can act as a bio-paper substrate for aqueous two-phase system–mediated delivery of experimental cargo. Uses include gene delivery and coculturing (*right*). Other abbreviation: SCD, sickle cell disease.

droplets with analyte/PEG mixture to perform the assay. Optimizing the polymer formulation for an ATPS–ELISA can create a test requiring just one washing step and one hour of assay time for the end user (122). Also, ATPS localization can spatially multiplex homogeneous immunoassays where all the reagents are in solution and otherwise not possible to array (123) (**Figure 6*a***).

ATPS has also been used to enhance the lateral flow immunoassay (LFA), an inexpensive point-of-care assay that flows the sample through a paper strip to detect targets using antibodies at specific locations on the paper strip. In exchange for simplicity, LFA suffers from relatively low sensitivity. To address that issue, Kamei and colleagues (124) used ATPS to preconcentrate a viral target prior to detection (**Figure 6*b***). To apply their system to proteins, which do not partition as well as viruses, they used antibody-coated gold nanoparticles to "fish out" proteins from a micelle-rich phase (125). These probes conveniently double as the visual markers in LFA. A 100-fold

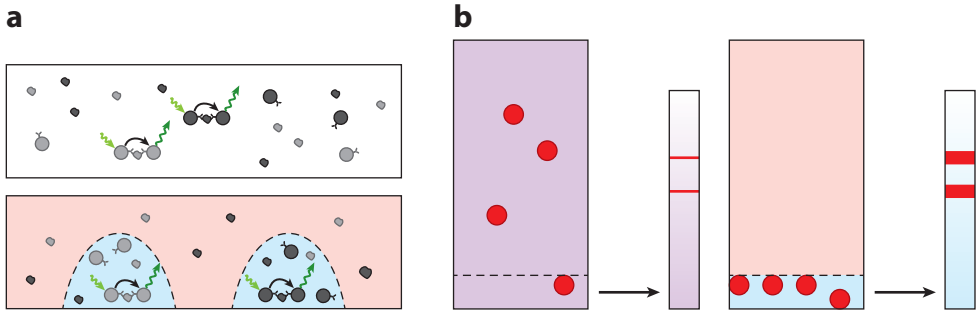

Figure 6

(*a*) Confinement of antibodies and reagents in a phase-separated microdroplet allows for multiplexing of immunoassays by confining each set of probes (*gray versus black*) to specific locations. Shown is the incorporation of droplets into a commercial AlphaLISA test. (*b*) Lateral flow assay signal readout can be faint or missing if analyte is present in low amounts. Partitioning the analyte in a two-phase system effectively increases concentration and improves detection limit.

improvement in detection limit could be achieved by modifying the nanoparticle surface chemistry such that they can be collected at the interface, resulting in a smaller volume (126). More recently, they have demonstrated that the phase separation step can be performed simultaneously with flow through the paper, drastically reducing the time to result (127).

3.2. Cell Patterning

Lentivirus-based vectors are of immense interest for their permanent delivery of genetic content and their enabling of gene therapies. However, their widespread use may be hindered by high viral production costs (128). Methods that maximize transduction and/or minimize vector amount are needed. Our group has addressed this issue with ATPS by performing lentiviral-mediated transduction using droplet patterned microarrays (129). The partitioning of GFP-encoded lentiviruses into dextran droplets housing cells resulted in an increased effective viral concentration that led to increased GFP expression. While another study (129) looks at viral transduction, it also demonstrates liposomal transfection in small groups of cultured cells. Indeed, ATPSs can enhance other transfection methods such as by improving electroporation efficiency (130) and enabling higher throughput of ultrasound-based sonoporation (131). Taking advantage of the ability to spatially target cells is not limited to the introduction of genetic material. One material that can be delivered with precision are antibodies. Site-specific, multiplexed immunostaining of cell monolayers and tissue sections could be performed in which only the primary antibody staining step utilizes a two-phase system (132).

Another material can be cells themselves. When cells are held inside a solution to be dispensed or printed onto a surface or paper substrate, the mixture can be thought of as a bioink. Normally, this ink would dissipate without some means of restriction; by containing cells in dextran, they are localized to their deposition sites. Some optimizing principles have to be considered here, particularly the interfacial tension, droplet contact angle, and partition coefficient (**Figure 5*c***). Cells normally partition to dextran from PEG, but dextran preference increases as contact angle and interfacial tension decrease, meaning different formulations may be needed. Remarkably, dextran droplets on cells do not keep a fully rounded shape but they remain stable. This is owing to surface heterogeneity, in which different surface energy states on a dense cell monolayer result in different dextran affinities along the surface (133). And so bioink may be drawn arbitrarily and with submillimeter precision.

In developing a bioink with controlled properties, a variety of applications has become available (**Figure 5*d***). The first use was with noncontact printing of embryonic stem cells onto support cells that induce differentiation (13). This has since been expanded to show that spacing between cell colonies can have a profound effect on differentiation (134). Tissue constructs can be formed using ATPSs, in analogy to cell sheets used in tissue engineering. At a high enough dextran concentration, the bottom phase exerts a buoyant force such that cells localize to the interface. Because of low interfacial tension in ATPSs, the interface is flat. Marangoni flows within the phases drive cells to interact and therefore create a planar tissue construct. This entire process occurs in minutes, and a cell sheet can be realized in a few hours. Overlaying a second set of cells would produce a two-layer construct. This method could create several different types of cell sheets; in particular, a keratinocyte sheet grafted onto a commercial decellularized matrix forms a skin-like structure in a couple of days (135).

Cells can also be cocultured with the aid of ATPS (**Figure 5*d***). Dextran droplets can rapidly form cell islands using Braille microfluidics (136) or by using an ultrasound transducer to eject droplets onto a plate (137); both methods are automated. Once islands are formed, different cells in a PEG solution can be added. Alternatively, a migration assay can be performed with dehydrated cell-free dextran droplets. When cells are added, they adhere around the dried dextran, and the medium rehydrates and washes away the dextran, leaving an unoccupied surface onto which cells move (138). The dextran phase can be formulated to have a higher density than cells such that cells partitioned within the dextran droplet float and collect at the apex of the droplet to form spheroids (139). The cells can also be printed with collagen to form collagen-embedded cell (140) or spheroid constructs (141) and could be expanded for drug screening assays (142).

3.3. Artificial Organelles and Protocells

Around the time coacervates were first discovered, Oparin (143) proposed that coacervation of small molecules and eventually biomacromolecules allowed for the development of living systems. This fundamental idea forms the research of artificial cells and organelles (note that artificial cells sometimes refer to encapsulated cells such as implantable pancreatic islets; protocell is a better term for a phase-separated, cell-like model). Because study in this area attempts to answer the key question of how life on Earth began, artificial organelles are of significant interest, as they can exploit the properties of ATPSs.

Phase separation in cells can be either nonassociative (as with polymer–polymer) or associative (as with coacervation). Polymer–polymer-based artificial organelles were the first type to be explored (144, 145), but their use has fallen out of favor for coacervate-based organelles, which have greater similarity to membraneless organelles. The first reported coacervate protocells were formed from single nucleotides and poly-L-lysine peptides (**Figure 1*c***), and they could sequester inorganic nanoparticles for catalytic oxidation (146). Within these droplets, macromolecular crowding and interior hydrophobicity were noted to enhance the activity of hexokinase, a key metabolic enzyme. In this case, the ATP/peptide coacervates do not shrink, as produced ADP replaces the spent ATP and maintains compartmentalization; activity is measured by a coupled reaction.

As mentioned before, placing ribozymes within a PEG/dextran droplet can potentially replicate intracellular conditions. Using a carboxymethyl-dextran/poly-L-lysine coacervate system to house ribozymes also shows support for catalytic activity (147). Both examples fail to examine RNA synthesis, a key origin-of-life question. However, introduction of transcription and translation machinery into coacervates for the purposes of in vitro gene expression has been recently shown (148) (**Figure 7*b***). An earlier example demonstrated the same but with two-phase and three-phase

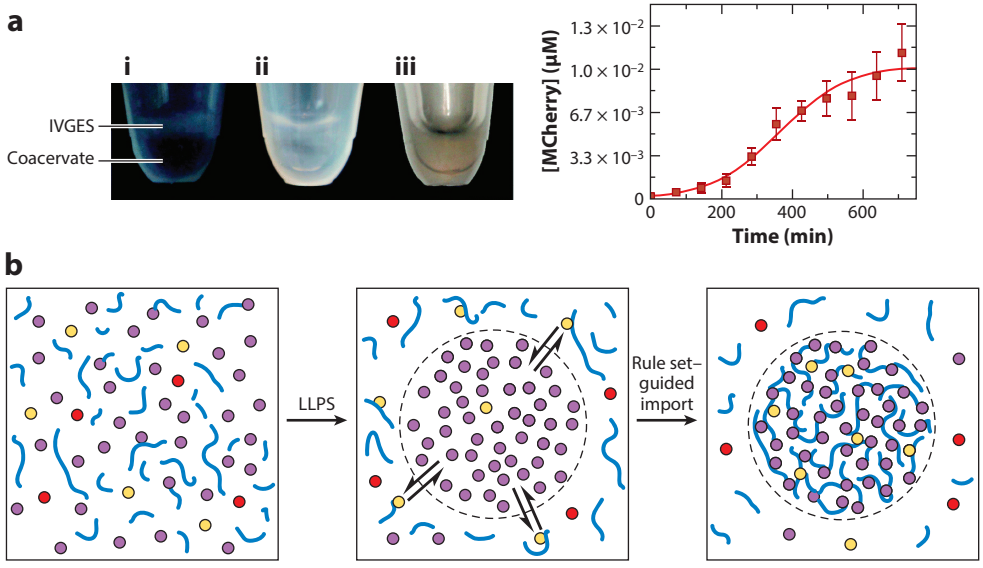

Figure 7

(*a*) An in vitro gene expression system (IVGES) can move into a coacervate phase (*i–ii*) and produce fluorescent protein (*iii*) within 24 h. Protein expression can be visually monitored; kinetic analysis reveals a sigmoidal process, plateauing due to excess protein-induced aggregation. (*b*) Disordered proteins spontaneously phase separate to form a biomolecular condensate, each having roles specific to their location within a cell. The present composition of a condensate can dictate what other molecules (proteins, nucleic acids) may enter. Panel adapted from Reference 148 under the terms of the Creative Commons Attribution-ShareAlike (CC BY-SA) License, **https://creativecommons.org/licenses/by-sa/3.0**. Other abbreviation: LLPS, liquid–liquid phase separation.

polymer systems forming water-in-oil emulsions (149). Having compartments allowed for neat partitioning of expressed protein away from process machinery.

Greater complexity can be achieved by mimicking biomolecular condensates. Many proteins have been identified as key drivers for condensate formation, such as nucleophosmin in nucleoli. One report looked at Ddx4-rich condensates and found that nucleic acid macrostructure is modulated by the droplet's interior hydrophobicity (150). Perhaps more relevant, the protein makeup of the condensate generates a set of rules that dictate what other proteins may enter; these rules are not just based on charge or size but also on the residue profile. The imported proteins then enhance nucleic acid partitioning (**Figure 7*b***). The findings of this work reveal that complexity can be achieved with simple starting material. Though research into coacervate protocells is in its early stage, the potential for engineering partition rules offers a clear advancement from traditional polymer–polymer ATPSs.

4. FINAL REMARKS

After more than a century of ATPS, it might be difficult to propose what the future of the field might be. But based on research over the past decade, at the time of writing, two major directions emerge: toward commercialization and toward fundamentals. Several large pharmaceutical companies, including Cytiva (formerly GE Healthcare, Marlborough, Massachusetts) and Boehringer Ingelheim (Ingelheim am Rhein, Germany), hold active patents for ATPSs to purify and separate antibodies. Kao Corporation (Tokyo, Japan), a general chemicals and cosmetics company, holds

patents for the purification and separation of a variety of proteins. Although information about the specific products associated with each ATPS technique has proven difficult to find from large pharmaceutical companies, smaller diagnostic companies feature their ATPS technology more prominently. Cleveland Diagnostics, Inc. (Cleveland, Ohio) has reported FDA Breakthrough Device Designation for the diagnosis of prostate cancer using SIA. The LFA technology has been brought to market for diagnostic sample preparation under the name Phase Diagnostics, Inc. (Garden Grove, California). They have recently had success using ATPS as an RNA extraction prep kit for COVID-19 diagnostic testing. Furthermore, they have received FDA emergency use authorization for their ATPS LFA diagnostic test, enabling patients to self-test at home. STEMCELL Technologies, Inc. (Vancouver, Canada) holds a patent for ATPS to generate and coat magnetic nanoparticles, with a variety of research applications potentially ranging from bio-assays to self-assembled biomaterials. In nonbiology fields, Schlumberger (Houston, Texas) has a variety of patents to use ATPS for sealing and/or cleaning subterranean oil wells. As researchers continue to search for applications that benefit from the unique advantages of ATPS, we anticipate that ATPS will become increasingly integrated into pharmaceutical manufacturing processes and diagnostics.

In parallel with the direct applications of ATPSs for industrial use, applications of ATPSs for basic science may unlock new paradigms for intracellular LLPS. Uncovering how these organelles form and what they regulate could provide insights into a variety of disease mechanisms and clues to develop new treatments. Furthermore, cross-partition analysis using ATPS enables the validation for updated thermodynamic models, which consider the polarity and orientation of water in crowded environments like the cytosol. Exciting possibilities abound to find creative uses for ATPS and the questions it provokes in new and unexpected fields.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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