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Interfacial stabilization of aqueous two-phase systems: a review

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Aqueous two-phase systems (ATPS) are useful in various applications, from purification and separation of biomolecules to wastewater treatment. While they have great utility on their own, there is great interest in discovering how their emulsions, comprising droplets of one aqueous phase dispersed in the other aqueous phase, might be stabilized to enhance their functionality and applications. There are several examples of these systems, but the two most common systems found in the literature are PEG–dextran and complex coacervate ATPS. In this Review, we discuss these systems, their utility, and many different approaches for stabilizing their water/water (w/w) emulsions. We highlight examples wherein interfacial stabilizers such as liposomes, polymers of diverse architectures, colloids of varied shapes and morphologies, and even whole cells have been employed. These stabilization approaches for both PEG–dextran and complex coacervate ATPS are discussed. We conclude with a discussion of the applications of these ATPS and how they can benefit from the creation of corresponding w/w emulsions with stabilized droplets.

Introduction

Aqueous two-phase systems (ATPS), consisting of two immiscible aqueous solutions, are routinely encountered in biology, chemistry, chemical engineering, and food science.[1–5] One of

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the first demonstrations of the utility of these systems was using dextran and methylcellulose for the separation of proteins by Albertsson in 1958.[6] Since then, ATPS have been utilized for the separation and purification of biomolecules,[1,5,6] in drug delivery,[7–9] as protocells and bioreactors,[10,11] detection of drug residues in food, drink, and water samples,[12,13] sequestration of precious metals,[14–16] and even for wastewater treatment.[1,3,17–19] These systems provide benign, biocompatible, and cheaper alternatives to oil–water systems, making them attractive materials systems for environmentally friendly manufacturing and processing techniques.[1,20–24]

The two phases in ATPS can consist of two immiscible aqueous polymer solutions, aqueous solutions of a polymer

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and a salt, two aqueous salt solutions, or a complex coacervate phase comprising oppositely charged polyelectrolytes in equilibrium with a polyelectrolyte-lean supernatant phase.[4,25–27] ATPS can be categorized as segregative or associative systems, depending on the thermodynamic drivers of phase separation.[11,25–27] In segregative systems, the incompatibility between the aqueous solutions of the (macro)molecular solutes results in aqueous phases enriched in one solute (macro)molecule or the other.[1–3,25] A common, widely employed system is the mixture of polyethylene glycol (PEG) and dextran, which form a segregative ATPS with two phases comprising either PEG or dextran chains (Fig. 1A).[5,28] In contrast, associative ATPS form upon attractive interactions-driven complexation of (macro)molecules. Common examples of such systems include polyelectrolyte complex coacervates (Fig. 1D) and membraneless cellular organelles.[29–32]

Segregative polymer–polymer ATPS have proven effective for separating many biological compounds and thus has become a familiar and frequently-used method in biotechnological settings.[6,21,33–37] The utility of ATPS for extracting biomacromolecules has been enhanced further by influencing the biomacromolecule to partition into either phase.[38,39] This can be done by affinity partitioning,[38–46] where the polymers themselves may be modified with affinity groups that are specific for biomacromolecules of interest[39–42,44,45] or by modulating the molecular weight of the polymers.[4,6,46–49] The physicochemical properties of the polymers, such as superficial electrochemical charge and hydrophobicity, can also affect the partitioning of biomacromolecules.[3,44,47,50] Polymer–polymer ATPS have been utilized not only for the separation and purification but also for the eventual extraction and encapsulation of proteins,[51] nucleic acids,[52] viruses,[53] antibodies,[39,54] and even whole cells.[43,45,55–60] These examples describe the diverse utility of polymer–polymer aqueous two-phase systems.

Polymer–salt systems are another common way to create segregative ATPS, wherein salts with high ionic strengths, such as phosphates, sulfates, and citrates, are most often used in combination with PEG and result in coexisting salt-rich and polymer-rich phases (Fig. 1B).[4,34,61] These systems are often used for cell encapsulation using microfluidic platforms, where droplet sizes and behaviors can be precisely manipulated.[57] Polymer–salt systems have also been used to separate proteins,[62–64] DNA,[65] and virus-like particles[66,67] in systems such as PEG–sodium sulfate or phosphate, in addition to the encapsulation of cells[57] and purification of antibodies and biopharmaceutical products.[23,35,37,68] Salt–salt systems or ionic liquid–salt ATPS have also emerged in recent years as segregative ATPS (Fig. 1C).[4,61,69,70] These systems are formed upon the co-dissolution of a salt with strongly charged ions with another salt or ionic liquid comprising low-charge density ions.[4,61,70] Greater tunability of polarity enables these systems to overcome some of the issues, such as slow phase separation, found in polymer–polymer ATPS.[4,61] Therefore, extraction and purification abilities can be enhanced in salt–salt ATPS, encouraging their use for extraction and purification of biomolecules when in solution with secondary compounds or contaminants.[61,71,72]

Associative ATPS, in contrast, are driven by the complexation of (macro)molecules *via* attractive interactions and include systems such as polyelectrolyte complex coacervates and membraneless cellular organelles.[29–32,73,74] In the former, oppositely charged macroions (typically polyelectrolytes) undergo complexation, creating a macroion-rich complex coacervate phase separating from a macroion-lean supernatant phase (Fig. 1D).[29–32] In the latter, intrinsically disordered proteins (IDPs), or proteins with intrinsically disordered regions (IDRs), undergo phase separation in the cytoplasmic milieu, forming membraneless organelles such as nucleoli, stress granules, and P-bodies.[74,75] While attractive electrostatic interactions and the entropy gains from counterion release are the primary drivers for complex coacervation, the formation of membraneless organelles also benefit from hydrophobic collapse, hydrogen-bonding, π–π stacking, van der Waals interactions, and other short-range attractive intermolecular interactions to drive phase separation.[73,74,76–78]

Complex coacervates comprising oppositely charged polyelectrolytes are fascinating systems with immense potential for many different applications ranging from their use as biomaterials for cartilage mimics, adhesives for wound healing, and drug delivery vehicles to their use as colloidal bioreactors in cell-free biocatalysis,[79,80] encapsulants in cosmetics,[81,82] and sorbents in wastewater treatment processes.[17,83,84] Concomitantly, a significant body of research investigating the thermodynamics of complexation as well as on the influence of diverse intrinsic properties (such as the polyelectrolyte chemistry,[85,86] length,[31,87] architecture,[32,86] charge density,[88–90] *etc.*) and extrinsic properties (ionic strength,[32,87] pH,[32,86,91] solvent quality,[85] temperature,[92] *etc.*) on the composition and properties of the coacervate and supernatant phases has emerged.[93] The crowded interiors of the coacervates and their ability to sequester charged (bio)macromolecules from their surroundings have

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Fig. 1 An overview of various aqueous two-phase systems (ATPS) comprising water–water interfaces. (A) Segregative polymer/polymer ATPS, comprising immiscible solutions of incompatible polymers (depicted as green and purple chains). A common example is aqueous poly(ethylene glycol) (PEG)/dextran solution. (B) Segregative polymer/salt ATPS, comprising immiscible solutions of incompatible polymer and salt (depicted as purple chain and orange dots, respectively). A common example is aqueous solution of PEG and magnesium sulfate. (C) Segregative salt/salt ATPS, or ionic liquid/salt ATPS, comprising immiscible solutions of incompatible salts (depicted as orange and navy dots). Aqueous solution of lithium chloride and lithium bis(trifluoromethanesulfonyl)imide is an example of such a system. (D) Associative ATPS, comprising polyelectrolyte complexes (PECs), which are composed of a complex phase of polyanions (depicted in blue chains) and polycations (depicted in red chains) in equilibrium with the polymer-lean supernatant phase. Common examples of polyanions include poly(acrylic acid), poly(styrene sulfonate), succinyl amylose, poly(glutamic acid), poly(aspartic acid), and DNA. Common examples of polycations include poly(diallyldimethylammonium)chloride, poly(allylamine), poly(4-vinylyridine) (P4VP), quaternized amylose, poly(lysine), and poly(arginine).

promoted their utility as encapsulants and stabilizers in biological,[79,94] industrial,[95–97] environmental,[17,19,83] and cosmetic applications.[81,82]

Stabilization of the aqueous two-phase systems, including those such as polymer/polymer, water/ionic liquid,[98,99] and polyelectrolyte complex systems,[80] at the micrometer length scale or smaller, can be expected to enhance their utility even further by enabling the implementation of ATPS in a host of novel applications.[1,10,25,26,100] The canonical two-phase mixture of oil and water derives its significant utility from the stabilized mixtures in the form of emulsions and micellar solutions. These have inspired efforts for stabilizing water–water (w/w) interfaces, resulting in progressively increasing research attention. While the design of the interfacial stabilizer depends on the nature of the w/w interface and the composition of the two aqueous phases, a few broad themes can still be identified. As such, the energetically favorable coalescence (that reduces the interfacial area) must be superseded to stabilize droplets of one phase in another. This is typically accomplished by adding a third component that accumulates at the interface, reduces the interfacial area, and stabilizes the droplets.[101,102] The energy barrier that stabilizes the droplets is estimated as the product of interfacial tension and the area taken up by the stabilizing components and is referred to as the trapping energy. In w/w interfaces, the interfacial tension is low, and correspondingly the trapping energy is significantly lower as compared to oil–water systems. Therefore, unique design approaches that go beyond employing surfactant-like molecules (with one anchoring point at the interface) for stabilizing the w/w interfaces must be developed. At the same time, the design criteria need to encode an affinity for both phases of the specific ATPS in the different parts of the stabilizer to promote its interfacial assembly.

The effectiveness of typical w/w interfacial stabilizers is typically ascertained by microimaging (to monitor the timespans over which droplets resist coalescence) and turbidimetry (to monitor the temporal evolution of the emulsion turbidity as a proxy for emulsion stability). The temporal evolution of the droplet size distribution can also be monitored to ascertain droplet stability. It can also provide insights into how the size distribution may affect the ability of the droplets to be stabilized. However, it is more often that researchers consider and modify the volumes of polymer or salt solutions or the size of stabilizing particles instead, which can ultimately affect the size of the droplets.[80,103,104] For example, it has been found that native proteins are generally too small for stabilization purposes (radius of ∼ 2 nm).[101] It has also been reported that droplets stabilized by triblock polymers, discussed in detail in the following section, can experience changes in stability based on droplet size. In this case, smaller droplets with diameters of a few micrometers are more stable than those with sizes of the order of tens of micrometers.[105] It may be noted that the coalescence time varies significantly for different systems. The coalescence time can be affected by Brownian diffusion or the height of the energy barrier to form a hole in the stabilizing shell, which is influenced by the extent to which the droplet is covered.[106] Additionally, while the interfacial energy is minimized in these systems, it is not zero. Thus, the droplets will eventually coalesce to reach their lowest energy state.

In this Review, we assess recent progress in creating and stabilizing ATPS (micro)emulsions, specifically focusing on PEG–dextran and complex coacervate (micro)emulsions. We highlight different stabilization methods for PEG–dextran and complex coacervate (micro)emulsions and their prospective applications. These systems provide unique avenues for separation and encapsulation, using compounds that are commonly used, are generally cheap, and are environmentally friendly. As a result, they will likely continue to expand and become ubiquitous materials in research and industry.

Stabilization strategies for PEG–dextran emulsions

Aqueous mixtures of PEG and dextran are one of the most common systems that undergo segregative liquid–liquid phase separation to produce aqueous two-phase systems (ATPS) and the corresponding water–water (w/w) interfaces (Fig. 1). Numerous examples of this system being particularly useful in separating and purifying biomolecules have appeared in the literature.[35,39,107,108] The incompatible phases both generally contain 70–95% water, and the interfacial tension between them is low (10^{-4}–10^{-1} mN m^{-1}); significantly lower than oil–water interfaces (~ 25 mN m^{-1}) or alkane–water interfaces (~ 50 mN m^{-1}).[100,109–112] This ultimately leads to high mass transfer and facilitates the partitioning of sensitive biomolecules, often into the dextran phase, in a non-destructive manner.[59,100,109,113]

Proteins, in particular, can be extracted and purified using PEG–dextran systems. A notable benefit of this approach is that protein denaturation can be diminished in these systems, as polymers typically used in these systems (PEG, dextran) can stabilize both the structure and the activity of proteins and enzymes.[28,114,115] Additionally, depending on the protein, it is possible to separate a protein into either the PEG or dextran phase, and this partitioning can be modulated based on the molecular weight of and modifications to the polymers and the proteins' hydrophobicity and surface properties.[3,39,46,68,116]

These systems are not only gentle enough to maintain protein structure and function, but they can also support enzyme function in bacterial cells. Dextran, in particular, can have stabilizing effects on microbial cells.[60,107,117] At the same time, the partitioning of cells can be modulated by altering the molecular weights and concentration of the polymers.[118] For example, if the molecular weight of dextran is lowered, more cells partition into the dextran-rich phase.[119] As such, PEG–dextran ATPS have been employed to partition complex cell lines, such as hybridoma cells (mouse/mouse hybridoma cell line BIF6A7), as well as other cell types and cell particles.[28,58–60,107,120] These hybridoma cells were supported for long-term growth.[59] Additionally, different cell types can be separated from each other using ATPS[43,45] or sequestered.[56] Thus, this system allows for a wide range of biological applications and affords excellent opportunities for optimizing the system for specific proteins and other biomacromolecules of interest.[52,118,119]

Stabilizing the water–water interface against coalescence and subsequent emulsification and formation of PEG-in-dextran or dextran-in-PEG emulsions can further enhance the utility of PEG–dextran ATPS. These emulsions have both been investigated extensively, but there is no clear consensus on which is more stable.[104,121] However, in either form, these emulsions are particularly exciting because they allow for compartmentalization, typically achieved in oil–water emulsions, without using organic solvents and hydrophobic fluids. The emulsion droplets can be stabilized in several ways by employing materials such as block copolymers, colloids, and liposomes.[1,25,26,100] These different methods and some specific examples are highlighted below.

The use of block polymers containing a hydrophobic middle block and two hydrophilic ends stands out as one of the most intuitive approaches to successfully stabilize PEG–dextran interfaces. Self-assembly of an ABC triblock copolymer, where A is hydrophilic and has a specific affinity for one polymer phase, B is hydrophobic, and C is hydrophilic and has a specific affinity for the second polymer phase, in water can form a polymersome (Fig. 2A).[105] Such polymersomes have been effective in stabilizing PEG/dextran ATPS, in some cases, for over eight months.[105] In particular, it has been reported that more stable systems tend to have smaller droplets and vice versa; increasing the hydrophobic block length correlates linearly with droplet stability against coalescence.[105] Jin and coworkers have simplified this system by using two block copolymers to form polymersomes that stabilize PEG–dextran ATPS, where two amphiphilic diblock copolymers co-assemble to form polymersomes with an aqueous cavity. In this case, one block copolymer has a hydrophilic block that has an affinity for one polymer phase and a hydrophobic block, and the other block copolymer has a hydrophilic block that has a specific affinity for the second polymer phase and a hydrophobic block (Fig. 2B).[122] These polymersomes self-assemble and enable facile modifications and tunability, presenting a versatile method for creating PEG–dextran microemulsions.

Liposomes, commonly consisting of phosphatidyl glycerol and phospholipids, form capsules that can accumulate at the PEG–dextran w/w interface, stabilizing the PEG–dextran phases (Fig. 2C).[123,124] They have also been employed to stabilize PEG/Ficoll and PEG/sulfate aqueous two-phase systems.[123] Moreover, lipids can be modified such that the liposomes achieve selectivity for the transport of different solutes across the liposome membrane.[124] These modified lipids are combined with unmodified lipids to form multifunctional liposomes. Additionally, liposome concentration has been shown to influence the size of the droplet, with greater liposome concentrations leading to smaller droplet sizes. Permeability of these liposome capsules has also been shown to be tunable, where only molecules of specific sizes can pass through, and this has been successful using a (sub)monolayer coverage. Here, only part of the droplet interface is stabilized by liposomes, meaning there are gaps in between, and excitingly, both transport and stabilization can occur simultaneously.[124] This dual utility expands upon the initial goal of stabilization, and the opportunities for modification provide greater tunability for a specific system or compound of interest. Combined with their biodegradability and biocompatibility, this approach presents an exciting avenue for using ATPS microemulsions in biomedicine and biotechnology.

Similar to polymersomes, colloids can assemble at the PEG–dextran interface to form colloidosomes, which have also been investigated as stabilization methods. Colloidal particles stabilize PEG–dextran emulsions as they are readily trapped at the interface and lower the interfacial energy, forming Pickering emulsions.[25,101,103,109,121,125–131] For instance, microgel
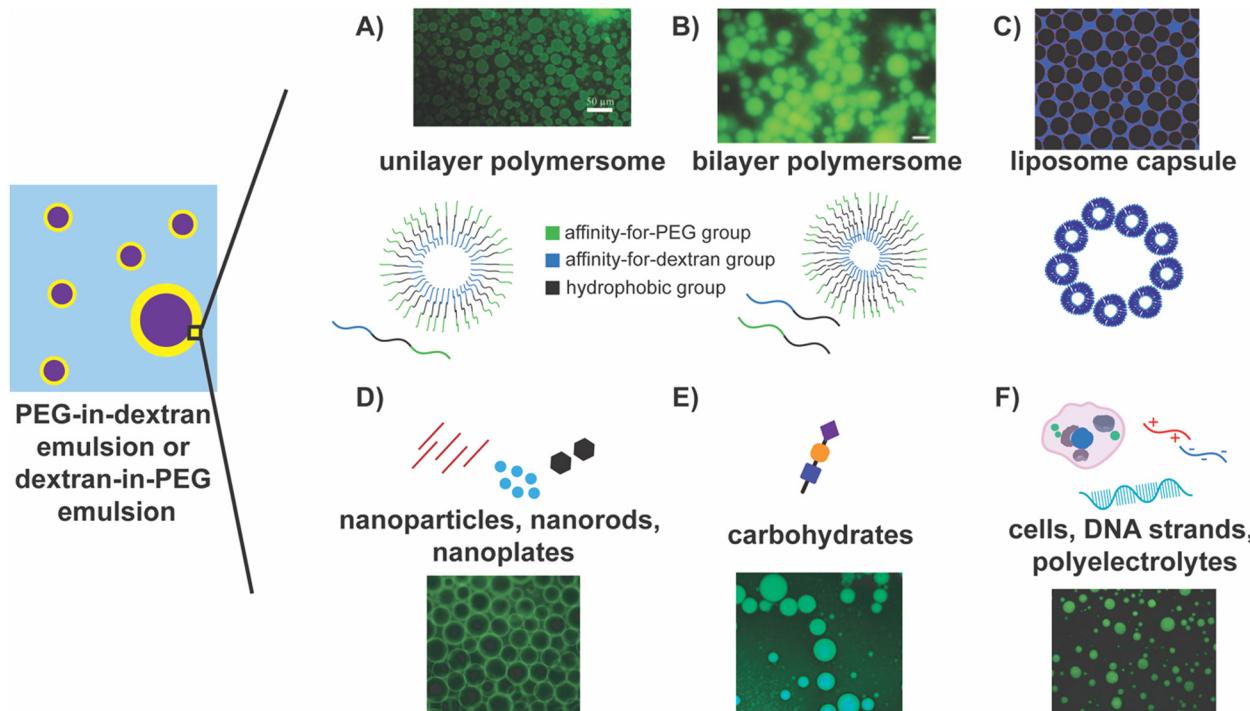
Fig. 2 Highlights of approaches for stabilizing PEG–dextran ATPS emulsions. In the schematic on the left, the PEG and the dextran phases are depicted by the blue and the purple regions, respectively, or *vice versa*, and the yellow region depicts the interface between them. (A) Unilayer[105] and (B) bilayer polymersomes[122] composed of tri- and diblock copolymers, respectively, stabilizing dextran-in-PEG emulsions. Reprinted (adapted) with permission from D. M. A. Buzza, P. D. I. Fletcher, T. K. Georgiou and N. Ghasdian, *Langmuir*, 2013, **29**, 14804–14814. Copyright 2013 American Chemical Society. Reprinted from Journal of Controlled Release: Official Journal of the Controlled Release Society, 147, Yulong Zhang, Feik Wu, Weien Yuan, and Tuo Jin, Polymersomes of asymmetric bilayer membrane formed by phase-guided assembly, 413–419, Copyright 2010, with permission from Elsevier. (C) Liposome capsules stabilizing dextran droplets in PEG phase.[123] Reprinted with permission from *Soft Matter*, RSC. (D) Nanomaterials, including nanoparticles, nanorods, and nanoplates, stabilize the w/w interface by assembling at the interface. The fluorescent image depicts PEG-in-dextran emulsions stabilized by nanoplatelets, where dextran is labelled with fluorescein.[128] (E) Carbohydrates, namely chitosan, assemble at the interface to stabilize the w/w interface. The fluorescent image shows chitosan stabilizing dextran-in-PEG emulsions.[103] Reprinted with permission from *Carbohydrate Polymers*, Elsevier. (F) Cells, DNA strands, and polyelectrolytes as stabilizers of w/w interfaces. The fluorescent image depicts DNA stabilizing the dextran-in-PEG interface.[102] Reprinted (adapted) with permission from Y. Wang, J. Yuan, S. Dong and J. Hao, *Langmuir*, 2022, **38**, 4713–4721. Copyright 2022 American Chemical Society.

particles, comprising a crosslinked polymer network, can serve as interfacial stabilizers while imparting stimuli responsiveness to the stabilization.[121,126,129] In these systems, droplets have been shown to remain stable for at least one week. At the same time, a dramatic increase in microgel diameter was noted as the pH of the system approached the pK_a of the particles.[121] Thus, droplets were quickly destabilized upon raising the pH from 7.2 to 8.0, exemplifying the system's sensitivity to pH changes.[121] Latex particles have also been employed as they can be trapped at the interface. Alternatively, colloidosomes can be formed from multilayered protein fibrils that form fibrillosomes.[127,132,133] Similarly, amphoteric protein particles (created by heat treating and salt exposure), as opposed to native, intact proteins, can form a monolayer that maintains the droplet form while lowering the interfacial tensions significantly.[104,125] Nanoparticles, such as nanocrystals, nanorods, nanoplates, and metal (gold and silver) nanospheres and nanowires, some equipped with DNA, have all been shown to stabilize the PEG–dextran w/w interface successfully (Fig. 2D).[14,128,134–137] Once self-assembled, they readily form a layer around the droplet. In fact, nanorods and

nanoplates can be more effective stabilizers, as they can lie flat along the interface and increase the covered surface area.[128] Complexation of oppositely charged polyelectrolytes (PEs) or nanoparticles to form a shell around the droplet that maintains high rigidity and permeability can also serve as a stabilization mechanism.[56,130,138–140] This approach accomplishes both goals of droplet stability and permeability while using inorganic materials.

Biomolecules, such as proteins, carbohydrates, DNA, and even whole cells, have also been employed to stabilize PEG–dextran emulsions. Carbohydrates, specifically chitosan, have found great success, and in some cases, the resultant droplets remained stable for at least one week (Fig. 2E).[103] In this setting, increasing the PEG and dextran concentration increased the viscosity of the phase-separating polymer solutions, ultimately allowing for tuning of the stability and size of a droplet. Chitosan has also been used in conjunction with cellulose nanocrystals to form a solid, membranous interfacial layer *via* electrostatic complexation, stabilizing the interface.[134] Carbohydrates can also be combined with protein microgels to

form complexes that spontaneously form a layer around the droplets.[129] DNA (both single and double-stranded) of different lengths have been shown to stabilize droplets and modulate droplet size (Fig. 2F).[102] Different cells have also been used as stabilizers, including red blood cells (RBC), NAMRU mouse mammary gland epithelial cells (NMuMG cells), and BIF6A7 hybridoma cells (Fig. 2F).[58,60] Under varying proportions of PEG and dextran solutions, different cells could be seen at the interface, and in cases where the interface had a higher tension/surface energy, RBCs, in particular, helped to reduce the surface energy.[58] The ability to use live cells as stabilizing agents will be helpful moving forward, opening avenues for novel biological applications.

Overall, while these techniques vary in their approach and chemistries, they all serve and seek to stabilize the w/w interfaces between PEG–dextran solutions, maintain their droplet form, and mitigate droplet coalescence. These systems have immense potential, as they can be used for various applications, imparting little to no detrimental effects on biological compounds and the ability to separate and purify a wide range of compounds from many different (biological, chemical, environmental) settings. It is also important to note the ability of these strategies to simultaneously lower the interfacial tension and stabilize the droplets, often by self-assembly, meaning that little to no input of energy or pre-treatment is required. The opportunities for simultaneous modulation and tuning of the stability of these systems and controlling the transport of molecules across the stabilizing membranes[124] make them vital assets that will undoubtedly lead to exciting discoveries in the future.

Stabilization strategies for complex coacervate emulsions

Complex coacervation of oppositely charged polyelectrolytes (polyanions and polycations) is attributed to the increase in entropy of the counterions and water when they are released from the vicinity of their corresponding polyelectrolyte chains upon the latter's complexation as well as the gains in the attractive electrostatic energy of the two polyelectrolytes.[32,141,142] The subsequent phase separation results in the formation of a polymer-rich complex coacervate phase which remains in equilibrium with the polymer-lean supernatant phase.[29,31,32] These phases exhibit very low interfacial tension ($\sim$100 mN m^{-1}), nearly 1000 times lower than water.[27,32,143–146] The lack of any membrane at the interface also allows for rapid transport of small molecules between the two phases. However, this lack of a stabilizing membrane also suggests that the droplets of one phase in another can grow magnanimously via coalescence or Ostwald ripening until they macro-phase-separate.[147]

Due to the low interfacial tension and the ability for rapid transport, these systems have found great utility as cellular mimics,[148–152] bioreactors,[80,134,150,152–160] and carriers.[95,97] Because these systems mimic membraneless organelles, they have been used to study cellular processes outside of a cellular

environment, not least of which are enzymatic pathways and cascades, as well as synthetic processes such as nanoparticle catalysis.[152,155–159,161] They have also been used to encapsulate scents for perfumes[81,82] and hold great promise for other encapsulation strategies and applications.

Stabilization strategies for coacervate droplets have gained increasing research attention as the applications for these systems have become increasingly diverse. While the unstable coacervate systems have broad utility, some of which are listed above, it is in their stabilized droplet forms that their potential uses can be greatly expanded. Recent attempts to stabilize complex coacervates have been inspired by strategies adopted to stabilize hydrophobic phases in water. Conjugation of the polyelectrolytes with a neutral block restricts the coarsening of the coacervates at the nanoscale, resulting in complex coacervate micelles that have been successfully employed as delivery vehicles for charged biomacromolecules, including nucleic acids and proteins.[29,162,163] However, their small size and relatively low loading capacity for biomolecules have limited the use of coacervate micelles as viable bioreactors. At the same time, efforts to stabilize micrometer-sized coacervate droplets have relied on the introduction of additional interfaces around the droplets comprising amphiphilic fatty acids,[164] amphiphilic terpolymers,[94,149,150,155,165,166] and phospholipid vesicles.[167,168]

ABC triblock copolymers, or terpolymers, have been employed to create unilamellar polymersomes to stabilize coacervate droplets (Fig. 3A).[94,149,150,155,165,166] The vast design space wherein each block can be specifically designed for droplet stabilization is a key advantage of this approach.[166] The first block of the triblock polymer extends toward the aqueous phase. Its length can be modulated but must avoid internalization by the complex coacervate and ensure successful assembly on the surface.[166] This is also conjugated with a hydrophobic middle block, forming the membrane around the droplet.[166] The third block of the polymer is often charged at neutral pH and thus provides anchoring to the charged surface of the coacervate microdroplets.[166] Following successful demonstrations of the prevention of the coalescence of coacervate droplets, the structure of these polymers has been further explored through various analytical techniques, including MALS, TEM, and confocal microscopy, and refined to enable the transport of selective molecules such as proteins, RNA, and small molecule substrates through the membrane.[155]

Similarly, unilamellar lipid vesicles have been employed to stabilize coacervate droplets as they readily accumulate at the coacervate–supernatant interfaces, ultimately forming liposomes (Fig. 3B).[154,169] Unfortunately, in the earliest studies with coacervates composed of a modified RNA (RNA (polyuridylic acid)) and short polyamines (spermine), such vesicles were not shown to provide sufficient stabilization against coalescence due to the apparent neutralization of adsorbed spermine.[169] Subsequently, lipid vesicles were modified with PEG groups and were negatively charged to prevent aggregation. They have even been made pH-responsive such that the coacervate dispersion's stability can be reversibly tuned.[170] These modifications have improved droplet stabilization while
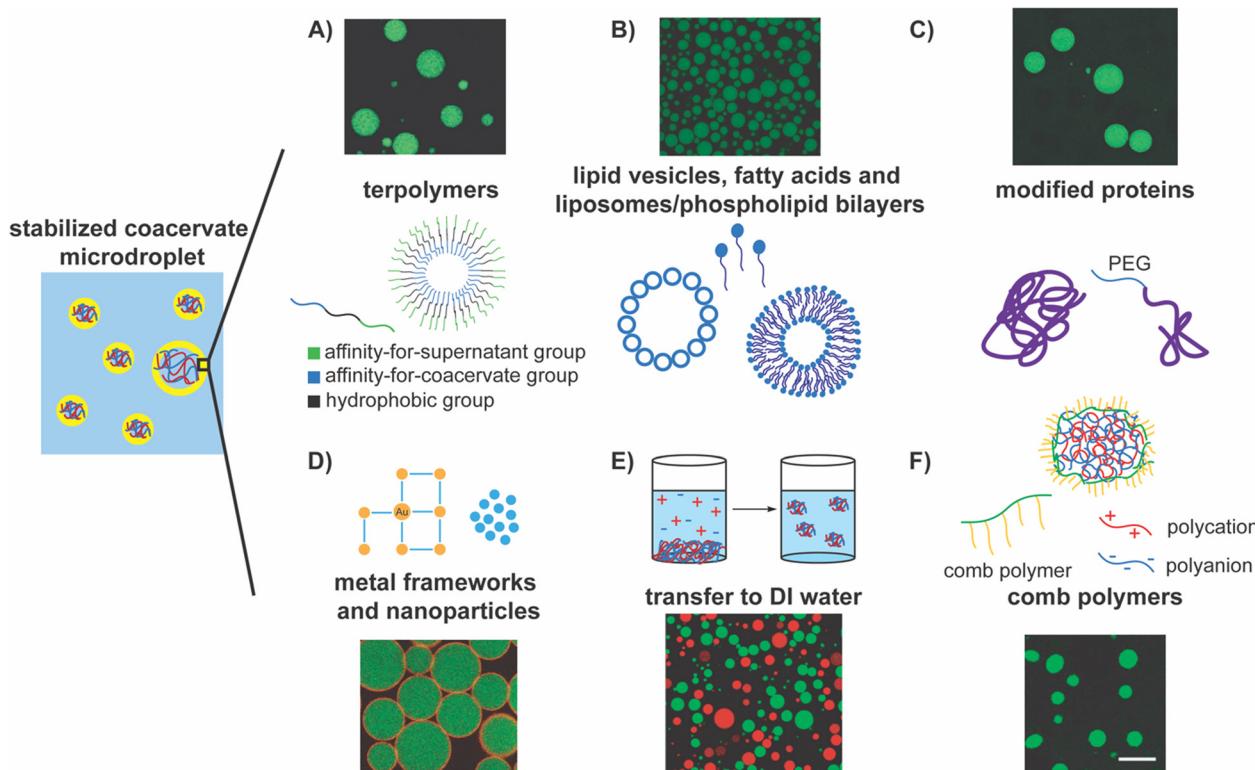
Fig. 3 Highlights of the approaches for stabilizing complex coacervate droplets. In the schematic on the left, polyanions are depicted as blue chains, polycations are depicted as red chains, the polymer-lean supernatant phase is depicted by the blue region, and the yellow region depicts the interface between the coacervate and the supernatant phases. (A) Terpolymer, or triblock copolymers, form polymersomes that stabilize the coacervate droplets. In the schematic, the green block has an affinity for the supernatant group, the blue block has an affinity for the coacervate, and black blocks is hydrophobic. The fluorescent image shows stabilization of droplets using this kind of terpolymers.[166] Reprinted with permission from *Methods in Enzymology*, Elsevier. (B) Different self-assemblies of lipids, including lipid vesicles, fatty acids, liposomes, and phospholipid bilayers can serve as stabilizers of coacervate droplets. The fluorescent image shows stabilization of coacervate droplets by phospholipid vesicles.[154] Reprinted (adapted) with permission from F. Pir Cakmak, A. T. Grigas and C. D. Keating, *Langmuir*, 2019, **35**, 7830–7840. Copyright 2019 American Chemical Society. (C) Modified proteins can serve as stabilizers for the coacervate/water interface. The fluorescent image shows stabilization of coacervate microdroplets by mPEG-modified BSA accumulated at the interface.[173] Reprinted with permission from *Small*, Wiley. (D) Nanoparticles and metal frameworks can assemble at the coacervate/water interface and stabilize them. The fluorescent image shows gold/PEG nanoparticle stabilization of the coacervate/water interface.[177] Copyright 2022 American Chemical Society. Available under the Creative Commons Attribution 4.0 International Public License. No modification to the material was made. (E) Transfer of coacervate droplets from supernatant phase to DI water as a method of stabilizing coacervate droplets. The fluorescent image shows stable coacervate microdroplets in DI water.[178] Copyright 2022 A. Agrawal, J. F. Douglas, M. Tirrell, and A. Karim. Available under the Creative Commons Attribution 4.0 International Public License. No modification to the material was made. (F) Comb polymer stabilization of polyelectrolyte complexes *via* interfacial assembly. The fluorescent image shows stable coacervate droplets.[80] Reprinted (adapted) with permission from S. Gao and S. Srivastava, *ACS Macro Lett.*, 2022, **11**, 902–909. Copyright 2022 American Chemical Society.

retaining their biodegradability and biocompatibility, as they can now remain stable for at least one day.[170]

In a similar vein, fatty acids and phospholipid bilayers have been used as stabilizers, as these molecules can readily self-assemble at the coacervate–water interface (Fig. 3B).[167] Drawing inspiration from bilayer cell membranes, lipids that have been extracted from erythrocyte cells *via* hemolysis have been used to encapsulate coacervate protocells, in some instances providing stabilization for at least 90 minutes.[79] Similarly, yeast cell wall fragments have also been shown to assemble at the interface and stabilize the coacervates droplets.[171] The biocompatibility of these stabilizers provides exciting avenues to explore for biological and biomedical applications.

Researchers have taken nature-inspired stabilization a step further and modified enzymes such that they decorate the surface of coacervate droplets.[172] For example, proteins and enzymes have been modified with PEG and cationized to promote assembly at the interface (Fig. 3C).[173] As such, proteins are known to assemble at the interface as self-assembled filaments (actin filaments) or as unfolded globules (*e.g.*, bovine serum albumin), though neither were explicitly shown to stabilize the interface.[157,174] Huang and coworkers capitalized on the coacervates' negatively charged surface to motivate the migration of proteins to the interface and deemed the subsequent shell formation a proteinaceous membrane.[173] Other groups have explored this encapsulation method, resulting in proteinosome vesicles.[175] This biocompatibility expands our toolbox, offering new stabilization methods and the opportunity to broaden the scope of applications.

Polyoxometalate frameworks have also been used to form and stabilize coacervate droplets (Fig. 3D).[176] This method

successfully prevented the coalescence of coacervate droplets, and the resulting emulsions could even be lyophilized and rehydrated without losing their overall structure. Similarly, tannic acid–protected gold (Au) nanoparticles have been used together with thioctic acid–modified PEG to slow coalescence.[10,177] Conjugation of modified PEG with the tannic acid–Au surface has been accomplished at the droplet interface by harnessing the affinity of the modified PEG and tannic acid–Au particles' affinity for the continuous and coacervate phases, respectively.[177] These conjugate particles pack closely and form a cage around the microdroplets, stabilizing them and creating Pickering emulsions. Using inorganic materials provides a new lens through which the stabilization of coacervate microdroplets can be viewed, and novel ways to synthesize and employ stabilizing materials can be envisioned.

Recently, a unique approach that harnesses the coupling of screening of electrostatic interaction by salt ions with the composition of the coacervate phase has been employed to garner stability to coacervate droplets by creating an elastic coacervate shell around them *via* interfacial crosslinking. When the coacervate droplets were transferred from the supernatant phase (moderate to high salinity) to DI water (low salinity), an interfacial viscoelastic region formed owing to a reduced screening of the interaction among the oppositely charged polyelectrolytes, rendering stability against coalescence (Fig. 3E).[178] Even after vigorous mixing, these droplets with elastic shells remain intact. At the same time, fluorescent proteins (dye-labeled bovine serum albumin) were readily transported into the droplets, confirming the porosity of the elastic shells. Droplets stabilized using this method were shown to be stable for up to a month in standard laboratory conditions.[178]

Unfortunately, while stabilizing droplets, many of these strategies may result in a loss of functionality by introducing a hydrophobic or elastic gel-like region around the droplets that hinder the transport of small, hydrophilic molecules across them. So far, very few viable strategies for the long-term stabilization of complex coacervate droplets while maintaining their membraneless interface have been demonstrated, slowing the development of complex coacervate-based enzymatic reactors. Recently, we introduced a new approach for stabilizing complex coacervate microdroplets while maintaining the coacervate–water interfaces by using comb polyelectrolytes as interfacial stabilizers (Fig. 3F). The anionic backbones of the comb polyelectrolytes are posited to anchor to the coacervate–water interface *via* electrostatic interactions while the neutral sidechains extend into the aqueous surroundings and provide steric repulsion between the droplets, minimizing their coalescence and providing long-term stability to these microdroplets. In effect, membraneless complex coacervate emulsions that are stable for at least four months have been created.[80] Moreover, this stabilization approach allowed for the successful spontaneous sequestration of proteins and enzymes into the coacervate microdroplets. A combination of macromolecular crowding in the coacervate environment and successful transport of small molecules facilitated by the large area of the stabilized microdroplet interfaces enhanced enzyme-mediated bioreaction rates substantially. Notably, the bioreaction rate enhancements persisted over extended periods.[80]

Given the increased interest and potential applications for coacervate microdroplets, it is no surprise that a wide range of stabilization techniques and methods have been explored. Although they differ in approach, they follow a common thread in that they all strive to stabilize coacervate microdroplets against coalescence. A future can be envisioned wherein such droplets are utilized as delivery vehicles for hydrophilic cargo, such as pesticides, scents, or drugs, and diverse ways in which their modularity can be harnessed and manipulated to suit the intended applications. In stabilized form, these droplets have the potential to profoundly influence approaches for targeted delivery in medicine, in consumer products, and in the environment.

Applications and utility of stabilized W/W emulsions

Stabilizing water–water interfaces introduces feasible paths to several exciting applications in biology, chemistry, chemical engineering, food science, and various other fields, examples of which are discussed in Fig. 4A–E.[37] For instance, extractive bioconversions involving the synthesis and separation of products from their biocatalyst soon after they are synthesized can be facilitated using PEG/dextran ATPS. The bioconversion can be run in one aqueous phase, while the product can be extracted into the other phase.[33,107,108,124] ATPS are particularly useful in such a case because they provide a mild environment for the biocatalysts and allow for the extraction of hydrophilic species, such as proteins.

These processes can also be scaled up to industrial levels, which is restrictive with *in situ* product recovery techniques. ATPS, as a separation technique, has been taken a step further in its use for growing animal cells (mouse/mouse hybridoma cell line BIF6A7), as it similarly provides a mild environment for cell growth.[59,60] Additionally, it has been used to partition out hybridoma PFU-83 and BIC-2 CHO cell lines into the same phase and, in other cases, to selectively separate and isolate different cell lines.[55,60] It can also be used as a cell encapsulation technique in its stabilized form, where cell viability could be tested.[56] ATPS have been used to facilitate the growth of corneal tissue for applications in tissue repair, where stabilization of the interface led to a greater accumulation of cells at the interface and more uniform distribution across the interface.[179] This accumulation of cells at the interface can also be used to form tissue constructs that provide convenience in constructing tissue models and may prove advantageous in regenerative medicine.[180] ATPS have also proven useful in immunostaining, circumventing common issues such as needing large amounts of expensive antibodies and providing the ability to detect multiple antigens in one sample.[181]

Enhanced and precise separation of ligands, conjugated with protein, has also been demonstrated by employing ATPS.
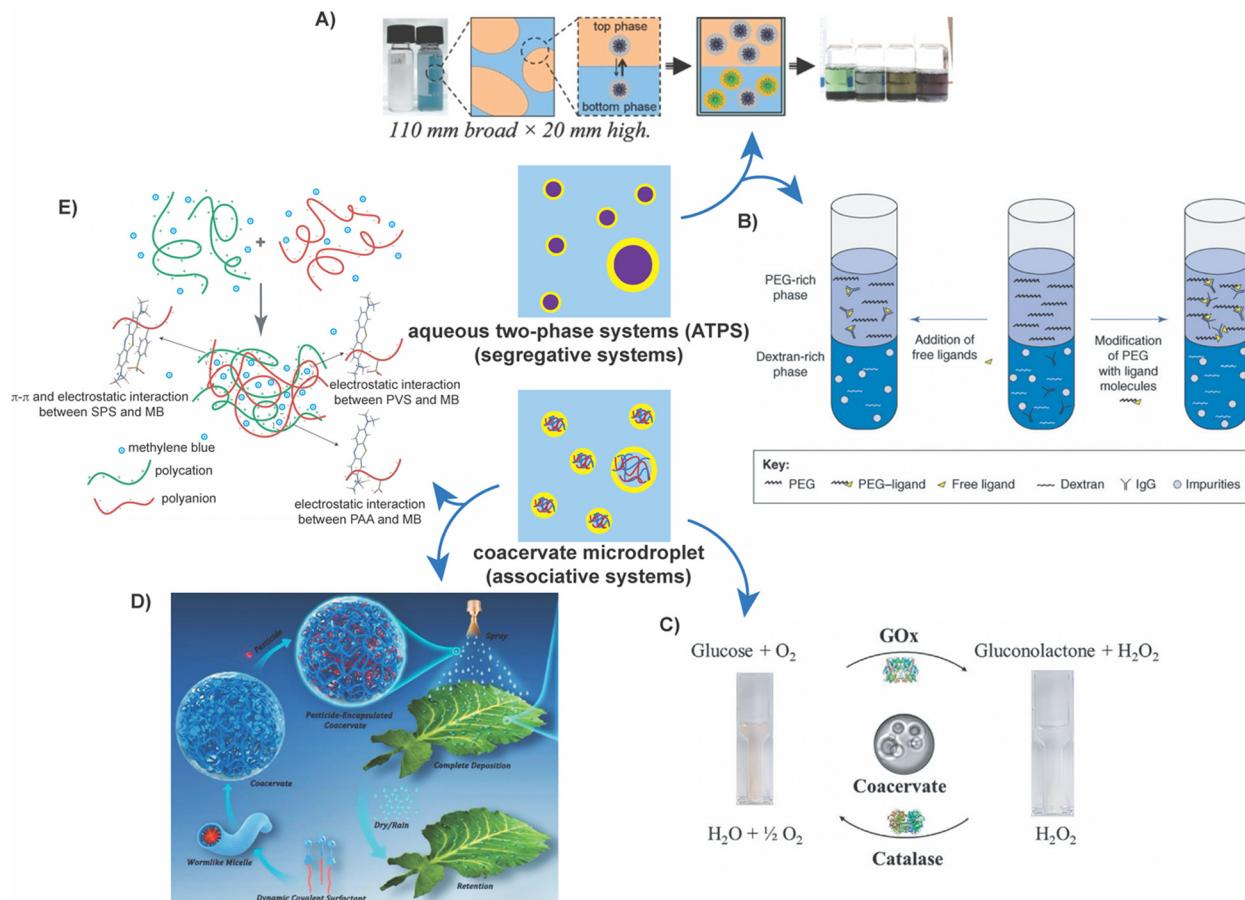
Fig. 4 Applications of stabilized ATPS and coacervate microdroplets. (A) ATPS separation of metal particles from solution. The figure shows the general procedure for isolating single semiconducting and metallic single-wall carbon nanotubes from a synthetic solution using a PEG/dextran ATPS.[185] Reprinted with permission from *Advanced Materials*, Wiley. (B) ATPS separation of impurities from antibodies. The figure shows methods by which an antibody can be influenced to partition into the PEG-rich phase, by introduction of free ligands or modification of PEG. In this case, a PEG/dextran ATPS was used.[35] Reprinted with permission from *Trends in Biotechnology*, Cell Press. (C) Enzymatic cascade reactions occurring in coacervate microdroplets. The figure shows the general cascade reaction that was successfully implemented in coacervate droplets.[115] Reprinted with permission from *Journal of Colloid and Interface Science*, Elsevier. (D) Pesticide application and release *via* sequestration and release from coacervate microdroplets. The figure shows the process of the pesticide, Buprofezin, being encapsulated in coacervates, sprayed on a leaf, and eventual deposition in a controlled fashion.[95] Reprinted with permission from *Advanced Functional Materials*, Wiley. (E) Sequestration of environmental contaminants in coacervate microdroplets. This figure portrays the encapsulation of methylene blue in coacervate microdroplets.[19] Reprinted with permission from *Macromolecular Rapid Communications*, Wiley.

Many works have cited ATPS to partition and purify proteins. In some cases, large molecular weight proteins have even been employed to selectively alter the partitioning of an affinity ligand, such as horseradish peroxidase (HRP) and human IgG, respectively.[38] In this specific example, this affinity ligand conjugated with HRP could then promote the successful recovery of rabbit anti-human IgG.[38] Additionally, these ligands may be chemically altered to influence which phase they partition into, ligands can be immobilized, and alterations can be made to the polymers that make up the aqueous solutions to influence partitioning. Stabilization of these systems may lead to better partitioning results.

Similarly, ATPS can isolate specific synthetic products from leftover reactants or side products in chemical syntheses or other reaction mixtures.[18,35,39–44,48,49,100,182–184] PEG/Na$_2$CO$_3$ ATPS can extract heavy metals from a sample containing many

other compounds.[182] A more involved example is the isolation of carbon nanotubes from a complex synthetic mixture.[185] This PEG/dextran ATPS technique, demonstrated by Zheng and coworkers, was reported to be rapid and robust, with scope for further tunability. Similarly, PEG/dextran ATPS has been used to select the most promising and ordered DNA-SWCNT (single-wall carbon nanotubes) structures of the many that are available, as the partitioning is strongly dependent on the DNA sequence and SWCNT structure.[186] This system can even separate SWCNTs based on chirality and subsequently purified from a synthetic mixture.[186,187]

Likewise, coacervate droplets provide new ways of pursuing drug and other small molecule delivery.[188] They can encapsulate proteins and have been shown to enhance the rates of enzymatic reactions occurring within the coacervate droplets.[80] Coacervate droplets can also be used as protocells and,

potentially, as bioreactors.[79,148,158,168] If stabilized, these reactors and protocells could be used for longer periods and enable the evolution of bioreactions and the accomplishment of enzymatic cascades.

Sequestration and sustained release of cancer drugs and other therapeutics within coacervate systems have been demonstrated.[8,9] As such, coacervates have garnered much attention in this realm and continue to be pursued as they also show enhanced drug solubility and protein stability.[7] They have been proposed as vehicles to deliver protein-based therapeutics essential for overall function[189] through oral delivery routes. Though the cited examples saw little to no need for a stabilizing agent, using a stabilizer could lead to greater selectivity and longer stabilization during delivery.

Proteins have also been shown to complex with polyelectrolytes to form coacervate droplets, especially after supercharging.[190,191] These protein–polyelectrolyte complexes are essential for the formation and function of membrane-less organelles.[32,163,192] In addition, the encapsulation of proteins often enhances their stability and activity and can help to maintain enzyme activity over time, providing a prime opportunity to use these droplets as protocells or synthetic cells and potentially provide information surrounding the origin of life.[80,115,174,193] Researchers have also created coacervates from peptides to understand their compartmentalization properties and allow for greater tunability of phase separation and selectivity for guest molecules. Other biomolecules have also been used, including ribonucleotide monomers and longer nucleic acids such as polyribonucleotides and tRNA, which can remain stable under varying pH and temperature changes.[169,194,195] Coacervate droplets were also used to study ribozyme catalysis, and faster ribozyme reactions (up to 12-fold enhancements) when paired with a polyanion were noted.[196] Enzymatic cascade reactions have also been performed in coacervate droplets, often deemed enzymatic reactors, where reaction rates have similarly been increased. If the coacervate droplets are stabilized, many more enzymatic cascades and pathways can be studied.

Coacervates have also been used in environmental applications to treat wastewater and extract potentially detrimental compounds from solutions.[17,19,84] The corresponding polyelectrolytes can also be modified such that they specifically sequester a dye or contaminant of interest. These contaminants are often organic compounds, such as dyes, that are regularly and readily released and can travel through water sources. Thus, coacervates provide an effective technique to clean these water supplies. They have also been used as soil stabilizers, as they can work to prevent erosion and desertification by strengthening the polymer–soil crust and forming a protective layer. They have even been used as pesticide applicators, where the pesticide particles are successfully encapsulated inside the coacervate droplets. Successful and enhanced deposition of the droplet, excellent erosion prevention by rainwater, and controlled release of the pesticide from the droplet have been reported. Stabilization may include precisely controlled release methods, as the stabilizers could be tuned to respond to only certain external stimuli or target specific plant areas.

They also find uses in cosmetics and can be used specifically in perfumes to help stabilize the scent for extended periods.[81,82] Protein/polysaccharide and other coacervate complexes are often used in food systems to enhance gellifying, viscosifying, foaming, and emulsification properties. In practical applications, this can lead to greater perceptions of creaminess in ice cream and sherbet and affect the structure of yogurts. By stabilizing these droplets, perfume scents would have the potential to last longer. Similarly, droplet stabilization may lead to longer-lasting emulsification in foods.

Conclusions

ATPS with water/water interfaces provide a unique materials platform that can be utilized in diverse applications. Thus, considerable research efforts have endeavored to find new routes for maintaining these phases in their droplet form by stabilizing their w/w interfaces against coalescence. In this Review, several of these approaches have been examined for stabilizing the two most common ATPS: aqueous PEG–dextran mixtures and complex coacervates. In both systems, stabilization approaches have witnessed steady development and optimization in the past decade. These approaches can be broadly classified into two categories, comprising either the formation of a hydrophobic membrane, in effect converting the water/water interface into a water/hydrophobe/water interface, accompanied by a crowded hydrophilic shell that prevents coalescence by steric hindrance or by localization of colloids or polymers, synthetic or bioderived, at the water/water interface, thus creating Pickering-like emulsions that minimize interfacial energy and dramatically slow down coalescence. Modular synthesis of terpolymers and modifications of different particles, biological compounds, and inorganic materials have allowed for even greater tunability, with the ability to selectively partition molecules of interest (such as proteins, nanoparticles, small molecules, etc.) or to regulate droplet stabilization.

Yet, there are significant gaps in our understanding of the composition and interfacial properties of the water–water interface. Moreover, investigations of how the stabilizers that have already been demonstrated as effective emulsifiers interact with and influence the interface as well as the thermodynamic equilibrium between the two aqueous phases are critical for the development of the next generation of interfacial stabilizers and expanding the utility of w/w ATPS emulsions. We envision that this field of research will continue to evolve as newer strategies for stabilizing ATPS emulsions and their applications in novel areas are investigated.

Author contributions

CF, ZK, and SS conceived the idea and contributed to the literature survey and writing of the manuscript.

Conflicts of interest

There are no conflicts to declare.

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