

Exploring New Horizons in Liquid Compartmentalization via Microfluidics

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Cite This: *Biomacromolecules* 2021, 22, 1759–1769



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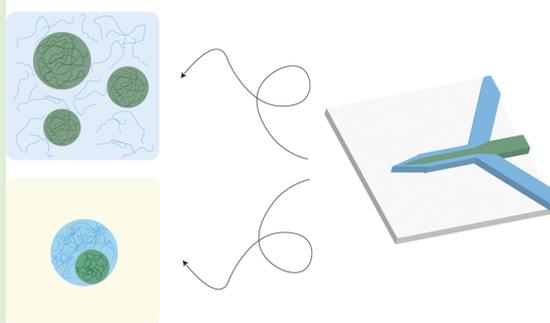
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ABSTRACT: Spatial organization of cellular processes is crucial to efficiently regulate life's essential reactions. Nature does this by compartmentalization, either using membranes, such as the cell and nuclear membrane, or by liquid-like droplets formed by aqueous liquid–liquid phase separation. Aqueous liquid–liquid phase separation can be divided in two different phenomena, associative and segregative phase separation, of which both are studied for their membraneless compartmentalization abilities. For centuries, segregative phase separation has been used for the extraction and purification of biomolecules. With the emergence of microfluidic techniques, further exciting possibilities were explored because of their ability to fine-tune phase separation within emulsions of various compositions and morphologies and achieve one of the simplest forms of compartmentalization. Lately, interest in aqueous liquid–liquid phase separation has been revived due to the discovery of membraneless phases within the cell. In this Perspective we focus on segregative aqueous phase separation, discuss the theory of this interesting phenomenon, and give an overview of the evolution of aqueous phase separation in microfluidics.

Compartmentalized membraneless systems



INTRODUCTION

Nature is very efficient in organizing its cellular processes. Cells are able to transport, localize, and concentrate specific compounds at their site of action, making them more readily available. This enables them to organize and regulate life's essential reactions, enhance their efficiency, and control in- and outgoing fluxes. Creating compartments is a mechanism to protect vulnerable processes in their own environment from external factors as well. This is achieved by membranes, such as the cell and nuclear membrane, and by the presence of liquid-like droplets within the cytoplasm, which are formed by liquid–liquid phase separation (LLPS).¹ A lot of research on the formation and functionality of these membraneless phases within cells is still ongoing and inspiring chemists for the realization of cell-like models with life-inspired functionality.²

Over many years, scientists have studied and designed micro- and nanocompartments, aiming to develop micro-environments that can mimic the functionality of the cellular architecture for catalysis³ or drug delivery purposes.^{4,5} Different strategies have been attempted to efficiently reproduce compartmentalization within systems, with every approach being unique. The first attempts to mimic the cell's compartment were to mimic the cell as close as possible by using lipids to form a membrane-based vesicle. Further approaches included the self-assembly of amphiphilic molecules in the broadest sense, including, but not limited to, liposomes,^{6–8} polymersomes,^{8,9} and dendrimersomes (Figure

1).^{10,11} Furthermore, scientists were able to obtain control over the morphology of these membrane assemblies, which allows for the generation of different shapes,¹² of which some contain multiple compartments, such as polymer stomatocytes.¹³ Compartmentalization is not exclusive to membrane-containing systems.¹⁴ It can also be achieved via a simpler method, namely via liquid–liquid phase separation. Two different phenomena are distinguished in LLPS, associative and segregative phase separation (Figure 1). The former occurs for oppositely charged polyelectrolytes, which form a polymer-rich phase and a polymer-poor phase, and these systems are called complex coacervates. The latter, also called aqueous phase separation (APS), is the phase separation of two neutral polymers or a polymer and salt into two phases, each enriched in one compound and depleted in the other.

APS has been known for centuries. It was first reported in 1896 by Beijerinck, who accidentally observed the phase separation of two aqueous solutions, that is, gelatin/agar and gelatin/soluble starch.¹⁵ This discovery remained trivial, until

Received: December 22, 2020

Revised: March 26, 2021

Published: April 9, 2021



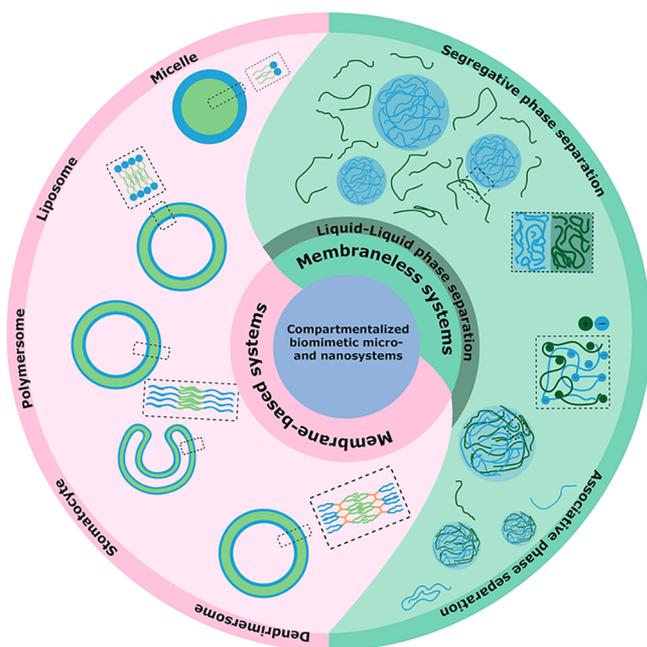


Figure 1. Different designs of micro- and nanoscale compartmentalization divided in membrane-based (left) and membraneless systems (right).

Per-Ake Albertsson rediscovered the formation of an aqueous two-phase system (ATPS) during chloroplast purification.¹⁶ The application of ATPS for the purification of biological materials inspired a generation of scientists. Later on, in fact, Johansson studied “interactions, charge, isoelectric point, hydrophobicity, purity and the presence of multiple forms of enzymes” using partitioning in ATPSs and demonstrated the importance of such systems for the separation and purification of biomolecules.¹⁷ Many different ATPSs were discovered, and systems up to six phases have been generated.¹⁸ The advantage of ATPSs over classic liquid–liquid extraction is the possibility to create a gentle aqueous environment using biocompatible and biodegradable polymers and salts.

For many years, ATPS has been used for the bulk extraction of biomolecules, such as proteins,^{19–21} enzymes,²² membranes,²³ viruses,²⁴ and nucleic acids,^{25,26} in biotechnological applications.²⁷ Recently, ATPSs are finally recognized for their versatility and flexibility,²⁸ as well as their suitability as a model system for mimicking the crowded environment within cells.²⁹ The emergence of microfluidic technologies opened up new possibilities to study ATPSs in more confined environments and utilize ATPSs for other exciting applications outside the scope of biomolecule extractions. Microfluidics, as well as ATPS, have evolved rapidly, starting from relatively simple setups generating water/water (w/w) jets to create complex multicomponent emulsions with diverse applications. Here, we focus on the use of APS in microfluidics to generate complex emulsions. First, we briefly explain the fundamentals of APS, after which we continue with APS in microfluidics, starting from simple ATPS jets and emulsions to complex multiphase droplets.

FUNDAMENTALS OF AQUEOUS PHASE SEPARATION

The most common APS systems consist of aqueous solutions of two or more incompatible polymers or polymer and salts above a critical concentration which is dependent on the compounds used. Other APS systems include ionic liquids, short chain alcohols, or even surfactants (Figure 2).¹⁸ These mixtures separate into two liquid phases, which are in equilibrium, each phase is enriched in one of the respective forming components. Water remains the main component, typically over 80% by weight,^{28,30} of both phases in ATPS, which ensures a biocompatible and gentle environment for separation and fractionation of biomolecules.

Polymer–Polymer ATPS. The phase separation in polymer mixtures is very common and based on steric exclusion, which is related to the concentration and molecular weight of the polymers.³¹ The demixing process, found in polymer–polymer solvent systems, is driven by the enthalpy related to the interactions of the solvent with the different components.³² Even though there is a loss in entropy due to phase separation, the gain in enthalpy is higher. Water, as a

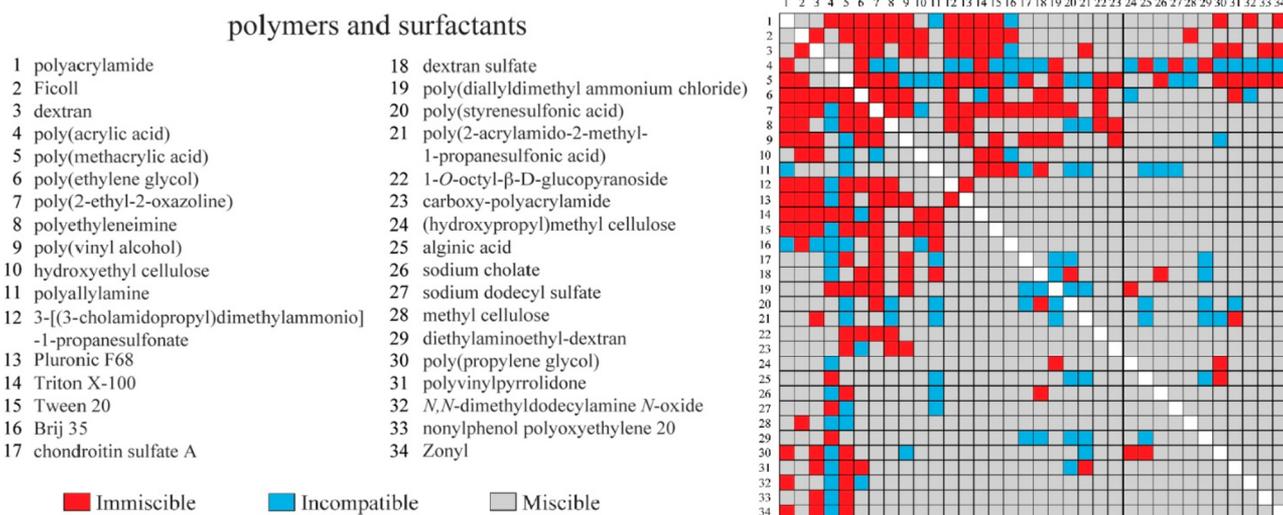


Figure 2. Overview of different compounds, including polymers, salts, and surfactants, that form aqueous-phase-separated systems. Reprinted with permission from ref 18. Copyright 2012 American Chemical Society.

solvent, has many noncovalent interactions with the polymers. Since these interactions increase proportionally with the size of the molecules, phase separation occurs at low concentrations for high molecular weight polymers.³³ As a result, polymers in a polymer–polymer system start forming aggregates and eventually separate in two different phases. The most frequently made biphasic polymer–polymer systems have been those of poly(ethylene glycol) (PEG) and dextran.

Polymer–Salt ATPS. Similar exclusion phenomena can be observed in polymer–salt systems, however, they are based on a different phenomenon.³⁴ In such a system, the salt absorbs large amounts of water that induce phase separation. This phase behavior is influenced by the concentration and the type of salt.^{35,36} In the most common polymer–salt systems, the salts are phosphates, sulfates, or citrates. Usually, an adequately high concentration of salt in these systems is necessary to induce phase separation, generating a salt-rich bottom phase coexisting with a polymer-rich top phase. The ability of the salt to promote phase separation follows the Hofmeister series, a classification of ions based on their salting-out ability, of which multivalent anions, such as HPO_4^{2-} and SO_4^{2-} , are the most efficient in inducing phase separation with PEG.³⁷ However, the exact mechanism through which salts influence the phase separation in ATPS is still poorly understood.

Single Polymer ATPS. An ATPS can even be formed from a single polymer by inducing thermoseparation. This happens when a polymer has a decreased solubility in water above a certain temperature. These thermoresponsive polymers have a lower critical solution temperature (LCST) above which they become increasingly hydrophobic. This induces aggregation of the polymer in globules and will result in a water-rich top phase and a polymer-rich bottom phase. Many thermoseparating polymers contain ethylene oxide and propylene oxide because of their low LCST.^{38,39} One of the most appealing thermoresponsive polymers is poly(*N*-isopropylacrylamide), (PNIPAM), which shows a sharp LCST transition in an aqueous environment near 32 °C. At the temperature-induced demixing transition, individual molecules of PNIPAM are highly sensitive to temperature changes due to their coordinated dehydration process during heating.⁴⁰ When passing the LCST, phase separation is caused by the partial dehydration of polymer chains that collapse and aggregate into polymer-rich domains undergoing a coil-to-globule transition in water. The model of reversible aggregation with variable attraction energy might explain the coordinated dehydration of PNIPAM molecules.⁴¹ According to this model, the association rate is larger than the dissociation rate at the gelation transition, which results in nonequilibrium and time-dependency. For this reason, the collapse of individual PNIPAM chains is relatively fast compared to the growth of the polymer-rich domains, which can take from minutes to hours. During this reversible transition, the amount of bound water decreases as a result of new intra- and interchain hydrogen bond formation. Due to its biocompatibility, its favorable LCST, which is close to body temperature, and its sharp transition, it is an often-studied polymer for biomedical applications.

Over the last decades there has been a growing interest in another type of thermoresponsive polymers, which are elastin-like polypeptides (ELPs). These ELPs are biologically inspired, stimulus-responsive polypeptides derived from human elastin, an extracellular matrix protein, with a LCST that can be controlled by the length and sequence of the polymer. Furthermore, they can be recombinantly synthesized with

complete control over polymer length and sequence, allowing for the generation of a monodisperse population, which is impossible for synthetic polymers.⁴² This makes them very attractive temperature-responsive materials to use in biomedical applications.^{42,43} Their LCST phase behavior is explained by their change in hydrogen bonds between the peptide and surrounding water. Upon temperature increase, the number of hydrogen bonds formed within the peptide itself increases, while less H-bonds are formed between peptide and water.⁴⁴

ATPS in a Phase Diagram. Each two-phase system can be characterized by its unique phase diagram that, like a fingerprint, shows the potential working area of the ATPS. A phase diagram indicates the point at which concentration the solution acts as a homogeneous mixture and at which concentration the solution phase-separates; this is unique for each system under specific conditions. The binodal curve distinguishes the homogeneously mixed region, below the binodal curve, from the phase-separated region, above the binodal curve (Figure 3). Above the critical concentration

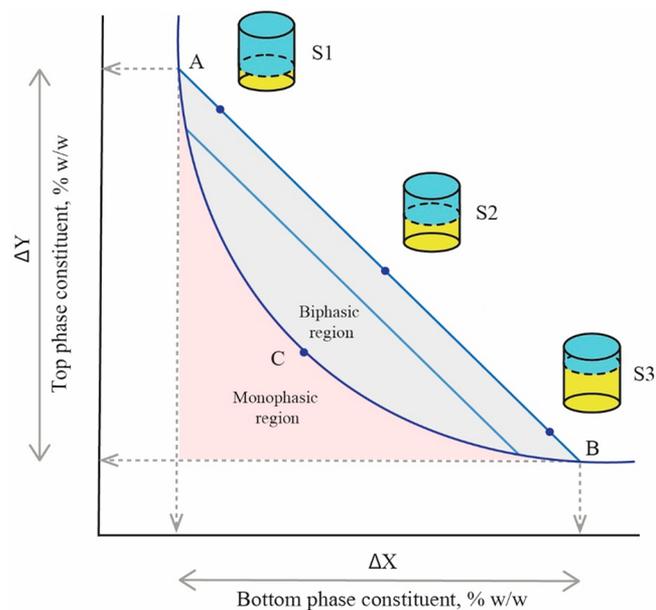


Figure 3. Schematic representation of phase diagram. Aqueous two-phase system is formed for concentrations above the binodal curve (ABC), while below the binodal curve, a homogeneous mixture is formed.

curve, two separate aqueous phases form, enriched in one material and deficient in the other. A tie line connects the two coexisting phases and represents the overall composition of the system. The intersection with the binodal curve for the top (A) and bottom (B) phase. All points on this line correspond to the same equilibrium composition of phases, but in different volume ratios. At the critical point C, the composition of both phases is identical, resulting in a single homogeneous phase. Close to the binodal curve, the system is sensitive to additives and changes in the environment, such as the addition of salt or change in temperature, which can affect the ATPS formation and composition. Besides concentration, molecular weight influences phase separation greatly. At higher molecular weights, steric exclusion will be stronger and lower concentrations are needed to induce APS. Moreover, the

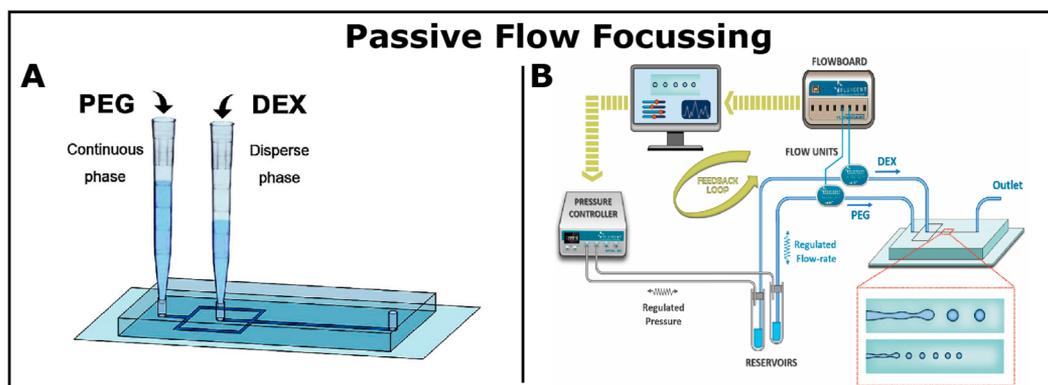


Figure 4. Schematic representations of all aqueous microfluidic set-ups. (A) Passive flow focusing microfluidic setup utilizing hydrostatic pressure via fluid filled pipet tips inserted in the inlets. Reprinted with permission from ref 59. Copyright 2017 Elsevier. (B) Passive flow focusing microfluidic setup utilizing air pressure to obtain low flow rates. Reprinted with permission from ref 60. Copyright 2018 American Chemical Society.

difference in molecular size between the two polymers affects the shape of the binodal curve. A larger difference will result in a more asymmetric phase diagram. Temperature has a great effect on phase separation and is therefore important to keep as constant as possible. Concerning polymer–polymer systems, phase separation occurs more easily at lower temperatures, while polymer–salt systems exhibit the opposite.^{45,46}

■ APS IN MICROFLUIDICS

ATPSs have been used extensively for batch extraction of biomolecules because of their mild conditions. A sample, such as enzymes, can be extracted from one aqueous phase by mixing it with another incompatible aqueous phase. By generating an emulsion, partitioning takes place due to the high surface to volume ratio. The lack of control over emulsion size and the resulting low efficiency of batch processing is a major drawback of this method. The emergence of microfluidic techniques allows for high-throughput processing and strict control over contact area of the immiscible phases. Microfluidics opens up many new possibilities for the design and use of ATPSs. Here, we discuss the generation of various ATPS systems, ranging from simple two-phase systems to complex multiphase systems.

All Aqueous Microfluidics. The simplest form of ATPS in microfluidics is the formation of an ATPS jet. These jets are used for high-throughput, continuous extraction of biomolecules.^{47–51} Two coexisting, immiscible phases are led through a microchannel, ensuring a large surface-to-volume ratio that is beneficial for partitioning. By decreasing the width and increasing the length of the channel, an even larger contact area is created, allowing for efficient and complete partitioning of a sample. Although w/w jets are easily formed, generation of monodisperse w/w emulsions proves to be more difficult. This is due to the ATPS's low interfacial tension values, reported to be, depending on the ATPS composition, between ~ 0.08 and $10 \mu\text{N m}^{-1}$,^{52–54} and is therefore in the same range of magnitude as membraneless organelles^{55,56} and several orders of magnitude lower than for typical w/o emulsions. The extremely low interfacial tension in ATPS's results in long w/w jets or uncontrolled breakups, generating polydisperse droplets.⁵⁷ Therefore, a different approach is needed to generate ATPS droplets. Until now, two types of techniques have been used, passive flow focusing and the application of external forces, respectively. In both traditional applications

and microfluidic set-ups, PEG and dextran ATPSs are widely used.

Passive generation of droplets uses traditional flow-focusing devices and techniques to generate stable flows. Due to the low interfacial tension of two aqueous solutions, droplet formation appears only at extremely low flow rates, which are impossible to generate using traditional pumps. To circumvent this problem, hydrostatic or air pressure can be used to generate these flows. The first example of passive microfluidics used hydrostatic pressure, generated by fluid-filled pipet tips, to load the solutions directly into the inlets (Figure 4A).^{58,59} This ensures very low flow rates, that is, $0.02\text{--}0.05 \mu\text{L}/\text{min}$, resulting in the frequent breakup of the dispersed phase by the continuous phase, often dextran and PEG, respectively. Monodisperse droplets are generated close to the junction, equivalent to relative high pressure for the continuous phase and low pressure for the dispersed phase. When dextran pressure is higher, or PEG pressure lower, the droplets are formed further from the junction, resulting in high polydispersity. When dextran pressure is too low, no droplets are formed, since the dextran phase flows back due to the relatively high pressure of the continuous PEG phase. The droplet size can be adjusted by changing viscosity, interfacial tension, and inlet height. Hydrostatic pressure and, thus, flow rates can be adjusted by the column height of the solution in the tip. Although this approach is extremely simple, it has one major drawback. Upon droplet generation, the solution level will drop and thus change the hydrostatic pressure. One of the main advantages of microfluidics is its high-throughput production. However, due to the low flow rates and the limited amount of solvent in the tip, this does not apply anymore for these systems. To compensate for this, parallel channels can be used to increase the output. A more sophisticated and controlled approach is the use of air pressure to drive the flow of solution (Figure 4B).⁶⁰ This method can reach flow conditions that are difficult to obtain using syringe pumps or pipet tips, which need to be on the lowest and highest limit of their abilities, respectively.

The other option is the generation of droplets by applying an external force to break up the stable flows. This method allows the use of normal pumps to control flow rates. Breakup of the aqueous jet can be obtained by many different external forces, the simplest, however, is by mechanical actuation on the tubing.⁶¹ Droplet size was found to be dependent on the

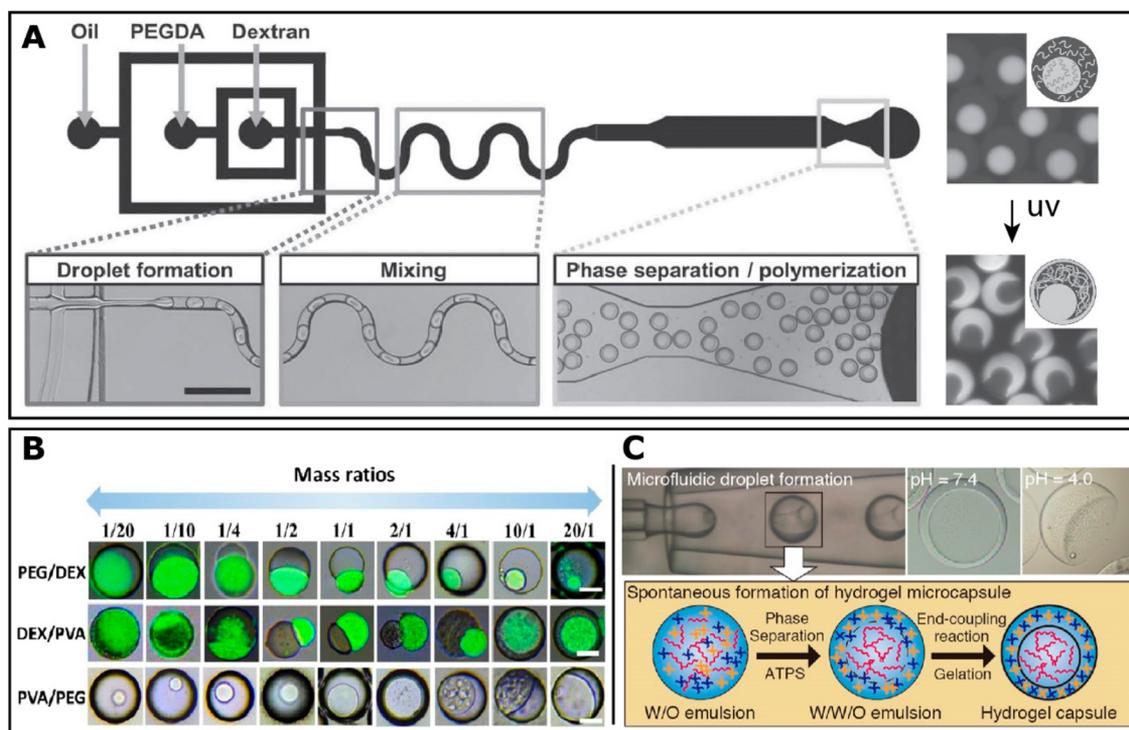


Figure 5. (A) Schematic representation of a flow focusing microfluidic chip to generate double emulsions of which one phase can be UV-polymerized resulting in asymmetrical gels. Adapted with permission from ref 73. Copyright 2012 John Wiley & Sons, Inc. (B) Examples of double emulsions consisting of different compositions. Adapted with permission from ref 69. Copyright 2017 American Chemical Society. (C) A glass capillary microfluidic device generating double emulsions; depending on pH, they are either core-shell or Janus-type. Adapted with permission from ref 72. Copyright 2019 American Chemical Society.

frequency of the shaker and could generate droplets reproducibly. Many other possible external forces can be used, either on- or off-chip, such as electric or magnetic forces, these were reviewed before and are beyond the scope of this perspective.⁵⁷

The main disadvantage of w/w emulsions is that they are highly unstable and upon coalescence will fuse together usually shortly after their formation inside the microfluidic chip. Due to the low interfacial tension and relative thickness of the interface, small molecules do not adsorb onto the interface and can thus not be used for stabilization of the droplet. However, particles do adsorb at the interface and result in stable Pickering emulsions.⁶² Other options are the generation of a thin shell or a hydrogel in situ by either a precipitation or gelation reaction at the emulsions interface.^{61,63,64} This way, stable droplets can be generated, capable of containing their intended cargo. Although all-aqueous microfluidics is simple in its setup, it can be increasingly complex to obtain and maintain stable flow rates and frequent breakup of the jet into monodisperse droplets. Higher order emulsions are difficult to generate using w/w microfluidics; only a few examples are known of higher-order emulsions.^{65–68} Combining ATPS with classical w/o microfluidics makes it possible to increase the stability and complexity of the emulsions and obtain more flexibility in choice of materials.

ATPS Double Emulsions. In the past decade, more and more scientists rediscovered the use of ATPS in combination with traditional oil–water microfluidics to generate complex multiphase droplets. While stability is a problem for w/w emulsions, water/oil (w/o) emulsions can be stabilized with surfactants and thus they will not fuse together and can be easily produced, stored and even manipulated. Simple flow

focusing microfluidic chips produce single water droplets in oil. Introduction of an extra inlet for an immiscible aqueous phase will produce an ATPS jet, forming droplet-in-droplet morphologies upon emulsification by the oil phase (Figure 5A). Tuning the phase separation and flow rates allow for the generation of droplets with different ratios of phases and compound compositions, which can either be concentric or asymmetric depending on the interfacial tension of the system (Figure 5B).^{69,70} These double emulsions can be gelated using different methods, such as chemical cross-linking^{71,72} or photoinitiated polymerization,^{73,74} which enables long-term storage in aqueous solutions. Any chemical reaction linking two polymer strands together can, in principle, be used. The different reactants are separated in different solutions so that, upon formation of the emulsion, the reaction occurs, and the droplets are solidified (Figure 5C). By incorporating a polymerizable phase, such as diacrylate functionalized polymers, droplets can be cross-linked upon UV-exposure (Figure 5A). In the case of an asymmetric PEG diacrylate–dextran double emulsion, PEG diacrylate can be selectively UV-polymerized, forming a hydrogel, while dextran templates an asymmetric shape. This has been utilized for various applications, such as cell growth chambers,⁷³ cargo buckets,⁷⁵ and as a base for micromotors.⁷⁴ By manipulating the aqueous phases and flow rates and by tuning the gelation reaction, different shapes and types of microgels can be generated.

■ INDUCED PHASE SEPARATION

Increasing the number of phases inside a droplet will result in an increased complexity of the microfluidic setup. While simply expanding the microfluidic chip with more inlets is possible,

some practical limitations will arise, such as increasing complexity in the fabrication of the master chip and increasing the amount of pumps for the microfluidic setup. Furthermore, the formation of more complex morphologies, such as those known for traditional emulsions, including three-phase or so-called Cerberus droplets and onion-shaped droplets consisting of all aqueous emulsion droplets proved difficult. Induced phase separation can overcome these limitations. Phase separation only occurs at certain conditions and at high enough concentrations. If these requirements are not met, a homogeneous mixture is obtained. Taking advantage of this knowledge, a simple w/w or w/o single emulsions can give rise to complex multiphase droplets upon changing the conditions to favor APS. There are two methods to induce phase separation, either mass-transfer induced or stimulus-induced phase separation, MTIPS or SIPS, respectively.

Mass-Transfer Induced Phase Separation. MTIPS is based on the extraction of a solvent from a homogeneous, multicomponent, single emulsion system. In most cases, the extracted solvent is water. APS within this system can only occur when all polymeric components exceed their critical concentration, above which phase-separation occurs. Below the critical concentration, all components are miscible and will form one homogeneous solution. A single emulsion generated with this solution will contain all components necessary for APS, only in dilute concentrations. By actively extracting water from the homogeneous multicomponent emulsion droplets, the internal concentrations of all components will inevitably exceed their respective critical concentrations, upon which aqueous multiphase systems (AMPSs) are achieved. This can be done either by the addition of water-attractants,⁶⁹ by evaporation,^{76–79} or by osmosis.^{67,80} The former two can be used in w/o systems, while the latter is used in w/w microfluidics.

The addition of water-attractants allows for the generation of droplet-in-droplet morphologies via controlled phase separation, as Cui et al. reported.⁶⁹ Here, aqueous homogeneous multiphase beads, containing poly(vinyl alcohol) (PVA), PEG, and dextran, are generated using a simple coaxial microfluidic setup. The continuous phase used here is an oil solution, containing the water attractant dimethyl carbonate (DMC). As water is more easily dissolved in DMC compared to the oil, water is slowly extracted from the droplets, resulting in decreased droplet size and increased compound concentrations. As concentrations keep increasing, the critical concentrations of the polymeric components within the droplet are exceeded, leading to phase separation and ultimately resulting in multiphased emulsion droplets (Figure 6A). The degree of phase separation is tunable, as prolonged DMC incubation times yield more distinct phase separation and smaller droplet sizes in comparison to shorter DMC incubation times.

Evaporation can be a tool to induce phase-separation as well.⁷⁶ Here, a universal method has been proposed for the generation of Janus particles, which is based on evaporation driven liquid–liquid phase separation. Janus particles are spherical, multicomponent particles that display different polar characteristics. Traditionally, these particles are generated by using microfluidic setups, where a biphasic laminar monomer stream is broken into Janus droplets as a result of side by side emulsification. However, as similar monomer viscosities are crucial to maintain stable biphasic laminar flow, the choice of monomers that fit that criteria are limited. To circumvent this problem, other avenues needed to be pursued. Using simple

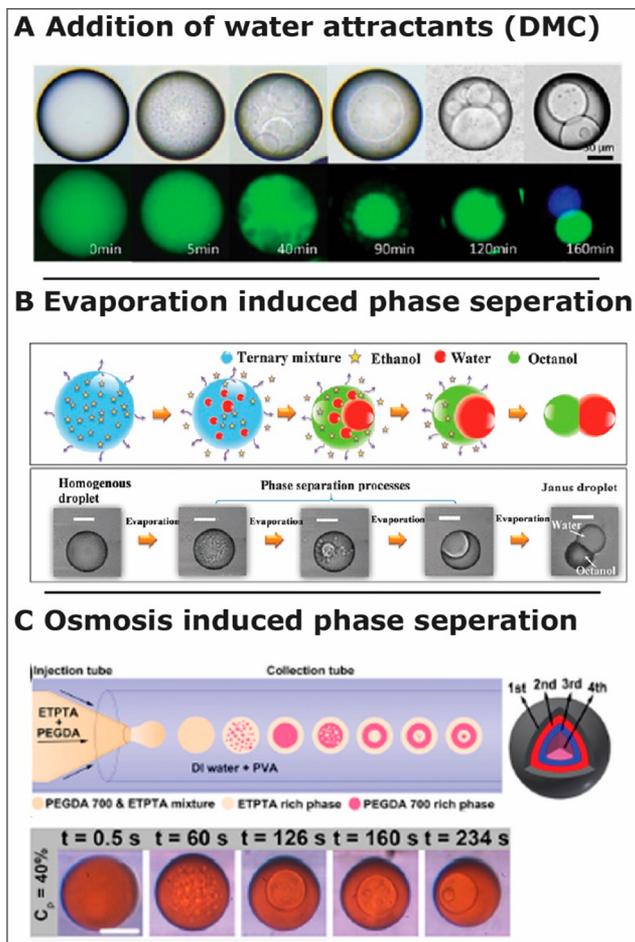


Figure 6. (A) Optical (top row) and fluorescence (bottom row) images depicting the formation of multiphase droplets out of homogeneous droplets by the addition of a water-attractant. The scale bar is 50 μm . Adapted with permission from ref 69. Copyright 2017 American Chemical Society. (B) Schematic representation of the Janus droplet formation via evaporation induced phase separation. The scale bar is 40 μm . Reprinted with permission from ref 76. Copyright 2016 Royal Society of Chemistry. (C) Schematic representation of an all-aqueous microfluidic device for multiphase droplet generation through osmosis (top). Formation of double, triple, and quadruple droplets over time with initial PEG diacrylate concentrations. The scale bar is 200 μm . Adapted with permission from ref 80. Copyright 2016 American Chemical Society.

microfluidic chips, homogeneous ternary aqueous droplets could be generated, using fluorinated oil, FC-40, as the continuous phase. As FC-40 features a high gas/vapor permeability, volatile cosolvent molecules, such as ethanol, can evaporate with ease, triggering phase-separation in the process (Figure 6B). Various morphologies can be generated via this method by simply changing the volume ratio or by adjusting the liquid composition of the ternary mixture.

Induced phase separation for w/w microfluidics can also be achieved through osmosis, as Liang et al. reported.⁸⁰ Initially, the generated particles are homogeneous in nature. Once they move down stream within the microfluidic channel, exchange of the dispersed and continuous phases takes place at the droplet interface, namely, the continuous phase enters the droplet and the dispersive phase leaches into the surrounding solution creating onion-shaped droplets (Figure 6C). Usually, the continuous phase consists of a solubilized polymer, such as

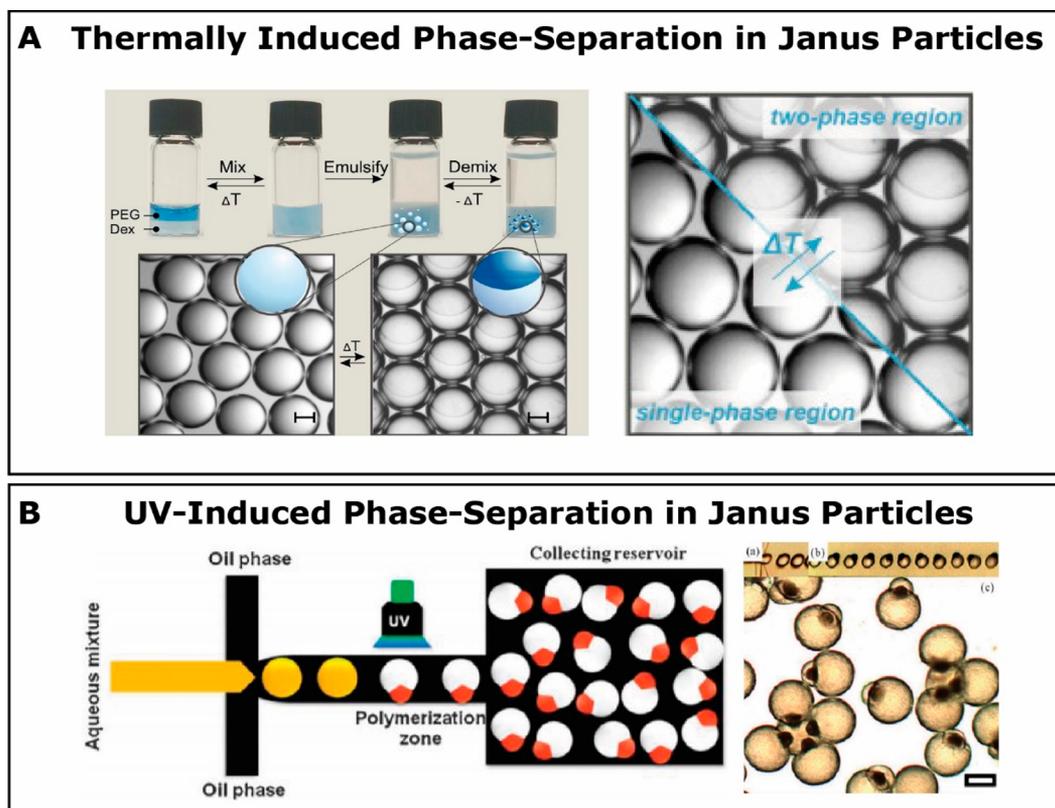


Figure 7. (A) Generation of double emulsion via temperature-controlled phase separation. Aqueous solutions of miscible hydrophilic polymers emulsified at low temperature leads to the formation of single-phase emulsion droplets. Upon warming up to room temperature, phase separation occurs, resulting in the formation of Janus droplets. Scale bar is 50 μm . Reprinted with permission from ref 87. Copyright 2020 Elsevier. (B) Schematic representation of microfluidics setup and process for the fabrication of UV-induced phase-separated Janus particles. Reproduced with permission from ref 85. Copyright 2011 Royal Society of Chemistry.

PVA, in water, while the dispersed phase can consist of ionic liquids or polymeric solutions. A wide range of structures can be obtained using this method, as reported in literature.⁶⁵

Stimulus-Induced Phase Separation. SIPS is the induction of a phase separation in homogeneous aqueous mixtures by an external stimulus, which can be a change in physical conditions or addition of a chemical effector.^{81,82} SIPS is possible when the effect of the stimulus on the system leads to changes in the composition of phases in ATPS or when the solution is made out of at least one stimuli-responsive material. Stimuli-responsive materials, such as polymers, respond to small changes in environmental stimuli with large, sometimes discontinuous, changes in their physical state or properties. The phase separation can be triggered by different physicochemical stimuli, such as temperature,^{83,84} light,⁸⁵ and pH,⁸⁶ depending on the chemical nature of the responsive polymer.

Thermally induced phase separation relies on the change in polymer solubility as a consequence of a change in temperature. This technique is based on the thermodynamic demixing of a homogeneous polymer–solvent solution into a thermosensitive polymer-rich phase and a thermosensitive polymer-poor phase in case of one polymer systems. PNIPAM is an excellent candidate for the fabrication of temperature-induced phase separating micro- and nanoparticles using microfluidics. In combination with other aqueous polymer solutions, Janus particles have been synthesized with a finely tunable internal architecture.⁸⁴ This was achieved by the thermally induced formation of PNIPAM colloidal nanoparticles that, after

formation, phase-separated. This transforms homogeneous microdroplets consisting of polyacrylamide and PNIPAM to Janus microparticles, of which one side is composed of aggregated colloidal nanoparticles, PNIPAM, and the other side of polyacrylamide hydrogel.

Recently, another technique for the one-step fabrication of double emulsions based on a thermal phase separation approach was introduced.⁸⁷ The researchers do not rely on a thermoresponsive polymer, but on the temperature-dependent phase separation. The phase diagram of PEG and dextran was found to be dependent on temperature. At low temperatures, the binodal curve shifted up to higher polymer concentrations, while for higher temperatures, the binodal curve shifted down. For this thermo-induced approach, both polymer concentrations should lie in between the two binodal curves. This ensures that for low temperatures the composition lies below the curve and thus forming a homogeneous mixture, while at higher temperatures the polymer composition lies above the curve forming a phase separated system. A single emulsion is generated at low temperatures, the aqueous mixtures of polymers exists as a single phase, but tends to return to their thermodynamically preferred phase-separated state at room temperature when allowed to warm up (Figure 7A). Once formed, the phase-separation inside the droplets can be reversibly switched between mixed and phase-separated states as controlled by the temperature. This system was extended by incorporating a third aqueous solution to form three phase Cerberus emulsions.

When light-sensitive polymers are present in the emulsion, phase separation can be induced by light as well. Lone et al. presented a simple and efficient method for the preparation of Janus particles by UV-directed phase separation of a light-sensitive polymer using a cross-junction PDMS microfluidic device (Figure 7B).⁸⁵ A homogeneous w/o emulsion is generated containing a light-sensitive random copolymer and a cross-linker. Upon UV exposure the light-sensitive polymer forms zwitterionic moieties which leads to inter- and intrachain ionic interactions. These interactions lead to water-expulsion and thus phase separation. This is a reversible reaction and over time the emulsion will form a homogeneous emulsion again. To maintain the induced asymmetry, a cross-linker was dissolved in the aqueous phase and a UV-initiator in the oil phase. Upon UV exposure phase separation of the light-sensitive polymer is induced, at the same time the initiator is activated generating radicals which react with the cross-linker to form a polymer shell at the emulsion interface. The resulting Janus microparticles consist of a smooth, hollow body and a protruded head composed of the light-sensitive polymer.

CONCLUSION AND FUTURE PROSPECTS

In this Perspective we highlighted various interesting studies to show different methods to generate increasingly complex APS emulsions. APS has evolved from a biocompatible extraction method to one of the most studied and interesting topics today. What started with the immiscibility of gelatin and agar has grown to be a phenomenon related to many different polymers, salts, and other water-soluble compounds. Its popularity lies in its biocompatibility and its versatility, something that was underestimated for years and only recently rediscovered upon increasing interest in membraneless compartmentalization. The rise of microfluidic techniques, with their main advantage being to generate monodisperse droplets, opened up new possibilities to generate emulsions of different morphologies and compositions. The spontaneous liquid–liquid phase separation together with the aqueous nature of APS makes it an interesting choice to use in combination with microfluidics to study confinement and compartmentalization, as well as utilize it as a precursor to fabricate microparticles.

Many exciting possibilities in combining APS and microfluidics still remain to be investigated, more specifically, in the field of SIPS and its applications. SIPS can boost the applicability of APS by enhancing phase separation and increasing selectivity toward the desired purpose. The design and use of new, smart polymers would allow the generation of induced, dynamic phase-separating systems. Some examples from the nonaqueous polymer field show us what might be possible in the future, such as reversible in situ SIPS upon light exposure to induce different morphologies.^{88–91} Other interesting studies are the accumulation of different cargoes in different phases of complex emulsions for drug delivery purposes or incorporation of different catalysts for reaction cascades.⁹²

Probably one of the most challenging and exciting applications is LLPS as mimic of the cell. The existence of membraneless liquid-like organelles was discovered only recently and since then attracted the interest of many scientists.⁹³ Since membraneless organelles, formed through associative liquid–liquid phase separation were found to play an important role in the cell's spatial organization, a lot of research went into this specific type of LLPS. Complex

coacervates were recognized to resemble these membraneless organelles and serve as cell model systems^{94,95} and were proposed as protocells for the origin of life.^{96,97} Since then, many interesting studies showed coacervates dynamic assembly and disassembly upon different stimuli^{98,99} and their assembly into more complex, multiphase systems.^{98,100,101} However, using complex coacervates, a polymer-rich phase is generated, as well as a polymer-poor phase. This is not an accurate representation of the cell. Even though coacervation occurs in the cell for specific molecules, many other molecules are present as well that maintain the crowded environment throughout the whole cell. APS emulsions started as simplistic models of the cells cytoplasm as a confined, crowded space, currently, it plays a more crucial role in the design of artificial cells. APS can induce localization of specific biomolecules in one or the other compartment while maintaining an overall crowded environment.¹⁰² Furthermore, combining this with induced phase separation, such as the temperature-dependent phase separation of PEG and dextran, reversible localization and delocalization of biomolecules is possible, making it more dynamic. Recently, Zhao et al. combined both LLPS phenomena to design a new protocellular system.¹⁰³ By combining both APS and coacervation a crowded, dynamic environment with spatial control over its constituents and high-order complexity was obtained. It is clear by now that nature has some remarkable phenomena in store, which are not yet completely understood or utilized to their full capacity. We believe that aqueous-phase-separated systems can help unravel some of these mysteries.

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Funding

NWO Chemische Wetenschappen VIDI Grant 723.015.001, Ministry of Education, Culture and Science (Gravitation Program 024.001.035), and Erasmus Program.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors acknowledge financial support from the NWO Chemische Wetenschappen VIDI Grant 723.015.001. M.B. acknowledges support from the Erasmus program. We also acknowledge support from the Ministry of Education, Culture and Science (Gravitation Program 024.001.035).

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