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Review

Analysis of biomolecular condensates and protein phase separation with microfluidic technology☆

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ABSTRACT

An increasing body of evidence shows that membraneless organelles are key components in cellular organization. These observations open a variety of outstanding questions about the physico-chemical rules underlying their assembly, disassembly and functions. Some molecular determinants of biomolecular condensates are challenging to probe and understand in complex *in vivo* systems. Minimalistic *in vitro* reconstitution approaches can fill this gap, mimicking key biological features, while maintaining sufficient simplicity to enable the analysis of fundamental aspects of biomolecular condensates. In this context, microfluidic technologies are highly attractive tools for the analysis of biomolecular phase transitions. In addition to enabling high-throughput measurements on small sample volumes, microfluidic tools provide for exquisite control of self-assembly in both time and space, leading to accurate quantitative analysis of biomolecular phase transitions. Here, with a specific focus on droplet-based microfluidics, we describe the advantages of microfluidic technology for the analysis of several aspects of phase separation. These include phase diagrams, dynamics of assembly and disassembly, rheological and surface properties, exchange of materials with the surrounding environment and the coupling between compartmentalization and biochemical reactions. We illustrate these concepts with selected examples, ranging from simple solutions of individual proteins to more complex mixtures of proteins and RNA, which represent synthetic models of biological membraneless organelles. Finally, we discuss how this technology may impact the bottom-up fabrication of synthetic artificial cells and for the development of synthetic protein materials in biotechnology.

1. Introduction

In the last ten years it has become clear that cells can form dense biomolecular condensates that can act as membraneless compartments [1–4]. These bodies can exhibit a variety of rheological properties and could mediate diverse biological processes and functions, which are only beginning to be elucidated. Most importantly, the formation of membraneless organelles allows the reversible organization of biochemical reactions in time and space, while guaranteeing a continuous influx and outflux of reactants and products. These characteristics are useful in a multitude of highly dynamic cellular processes, including signaling clusters [5,6], gene expression by the formation of super-enhancers [7,8], RNA flux, storage and processing in stress granules or processing bodies [9–12], and innate immune defense [13]. Unsurprisingly, these recent observations have revived scientific interest in liquid-liquid phase separation of biopolymers.

Proteins and nucleic acids associated with membraneless organelles encode multiple intermolecular interactions [14–18] that have been evolved by nature to provide for outstanding spatiotemporal control not only of stimulus responsiveness but also of the properties of the resulting compartments, including dynamics, composition, rheological properties and biochemical activity.

In addition to the intermolecular interactions encoded in protein sequences, the accurate control of phase separation is mediated by a variety of modulating factors, which include post-translational modifications, as well as interactions with RNA and DNA [13,18–22], in addition to small molecules and metabolic factors like ATP [22–25]. An imbalance of these mechanisms can often result in pathological liquid-to-solid phase transitions and the formation of aberrant protein aggregates associated with neurodegenerative diseases [17,26–31].

The spatiotemporal control of biochemical functions offered by liquid-liquid phase separation has also been suggested to play a key role in the origin of life and the formation of primitive compartments [32]. In particular, complex coacervates formed by oppositely charged

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biopolymers may represent a simple form of catalytically active, open compartments [33–36]. The complex mechanisms that cells have developed to control open compartmentalization raise fascinating questions about the physical chemistry that regulates phase separation in biological mixtures. Some of these fundamental questions cannot be addressed in the complex environment of the cell and require minimalistic *in vitro* reconstitution approaches that mimic basic biological features, while maintaining sufficient simplicity. The development of such model systems is crucially required not only to understand biological phase separation but also to generate synthetic systems with applications in biotechnology, such as adaptive protein materials and biosensors [37–42].

In this context, microfluidic technologies represent valuable tools for both the analysis of biological self-assembly and for the fabrication of bottom-up synthetic compartments [43]. First, the comprehensive characterization of phase transition processes and the properties of the resulting compartments requires a large number of experiments. A clear benefit of microfluidic systems is the ease with which experiments can be performed with high-throughput by using limited amounts of material. Moreover, the manipulation of liquid samples on the micron scale allows the performance of operations that are essentially impossible on the bulk scale. In the current context, microfluidic technologies engender the superior manipulation of protein environments in both space and time, which in turn leads to the exquisite control of both the thermodynamics and kinetics of biochemical reactions. This is especially attractive in the analysis of biological phase transitions associated with cellular compartmentalization, which typically involves non-equilibrium processes.

In this review, after describing generic features of microfluidic systems that are of particular relevance to protein phase transition, we will focus on selected examples that demonstrate the power of this technology in investigating biological liquid-liquid phase separation.

2. Advantages of microfluidic technology for protein phase transition and biomolecular condensates

Advances in soft lithography techniques have greatly facilitated and simplified the development of microfluidic platforms for biological experimentation, ensuring their adoption in a wide range of chemical and biological applications [44], including protein science [45–48].

In brief, microfluidic features are first patterned within a layer of photoresist (using photolithography) to yield a master structure. This master is then used to imprint thermally curable polymers, typically silicone rubbers such as polydimethylsiloxane (PDMS), with micron-scale channels for directing fluid flow. Functional devices are simply realized by bonding the polymeric matrix to a substrate, typically glass slides or a thin PDMS layer [49,50]. Fluid flows in such microfluidic channels are subsequently imposed either by external pumps or by controlling the pressure at the inlets or outlets.

The manipulation of fluid samples within microfluidic channels offers all the advantages that are inherent to miniaturization, and enables the analysis of small amounts of sample at high analytical throughput (Fig. 1A). The ability to process miniscule amounts of sample is particularly important when biological material is limited, which is often the case when analyzing *in vitro* models of biological phase separation, which rely on recombinantly expressed proteins and *in vitro* synthesized RNAs, or material extracted from cells, tissues or biofluids. The potential of microfluidics, however, is not driven solely by these advantages, and rather builds on the fact that several physical phenomena vary as a function of scale [51]. For example, mass transport processes in microfluidic environments can be accurately controlled simply by modifying the ratio of inertial to viscous forces [52]. Although flow regimes within microfluidic systems are almost always laminar in nature [53] and ideal for measuring the physical properties of proteins and protein assemblies [54,55], mass transport can be enhanced through chaotic advection, which allows the rapid mixing of species over a timescale of sub-milliseconds [56].

In addition to continuous flow operation, microfluidic devices can be used to create segmented flows in a passive manner, using flow focusing or T-junction geometries [57] (Fig. 1B). The adoption of a segmented (rather than continuous) flow regime allows the compartmentalization of analytes within fL–nL sized droplets that reside within a continuous and immiscible carrier fluid. Such an approach allows the generation of millions of well-defined microcompartments of exceptionally narrow size distribution, whose size and payload can be accurately controlled by varying the channel geometry or input flow rates (Fig. 1B) [58]. In addition, since the carrier fluid (continuous phase) wets the channel surface, complete isolation of the assay mixture is ensured. This contrasts with continuous flow formats, in which surface-molecule interactions are unavoidable, despite the fact that a variety of surface functionalizations have been developed to minimize adsorption of biomolecules on the walls of microfluidic channels [59–61]. Finally, droplet-based microfluidic approaches allow the formation of large arrays of microcompartments within short time periods, significantly increasing the analytical throughput and allowing for a range of end-point assays.

In the context of biomolecular condensates, the aforementioned tools allow the encapsulation and mixing of homogenous protein solutions with modulators of the phase diagram (such as nucleic acids, salt, buffer components and pH), which enables the observation of the thermodynamics and kinetics of phase transitions in compartments with cell-like volumes (pL-nL) (Fig. 1B, C) [62]. Such tiny volumes also allow the observation of stochastic events, such as rare nucleation events underlying protein aggregation and phase separation [63].

Within these micro compartments, the solution is in contact with a well-defined interface, typically comprising non-ionic surfactants, such as polyethylene glycol (PEG), which exhibit limited interactions with biomolecules (Fig. 1B). These surfactants stabilize the water-based compartments within the hydrophobic carrier fluid [64]. The limited interactions between biomolecules and the interface of the container minimize issues related to the glass or plastic surfaces present in bulk assays, which can alter the phase transition or prevent droplet coalescence events due to the sticking of the condensates on surfaces. Droplet-based microfluidic systems can be further exploited to generate more complex structures, towards accurate mimics of cellular interfaces, including for instance vesicle-like structures delimited by lipid bilayers [32].

The material of the microfluidic device, typically the thermo-curable polymer (silicone rubber) polydimethylsiloxane (PDMS), is transparent and compatible with most optical detection methods. Phase transitions and biochemical reactions occurring inside the compartments can therefore be followed by monitoring, for example, the fluorescence signal of conjugated fluorophores or the intrinsic fluorescence from native samples [65] (Fig. 1D). To monitor phase transition events over long incubation timescales (minutes to hours or even days), droplets can be stored in droplet storage arrays (Fig. 1E) by introducing traps [66], storage chambers [67] or delay channels that drastically reduce the flow rates of droplets [68,69] (Fig. 1E).

Droplets can also be manipulated before, during or after their storage. For instance, droplets can be trapped in chambers and shrunk over time by removal of water [70–72]. This reduction of the compartment volume increases the protein concentration, allowing the exploration of regions of supersaturation and phase separation within phase diagrams [73–75]. This process can mimic volume losses that are sometimes observed in cells in response to external stresses [76,77]. For instance, a reduction of 15% of the cell volume has been observed in glucose-deprived *Saccharomyces cerevisiae* cells. Since the total cell mass remained constant, this reduction leads to an increase of the intracellular mass density and molecular crowding [78], potentially facilitating phase transitions synergistically with other cellular signaling factors. Shrinking droplets can also be used to mimic increases in biomolecule concentrations that seed protein phase transitions. For instance, shut-

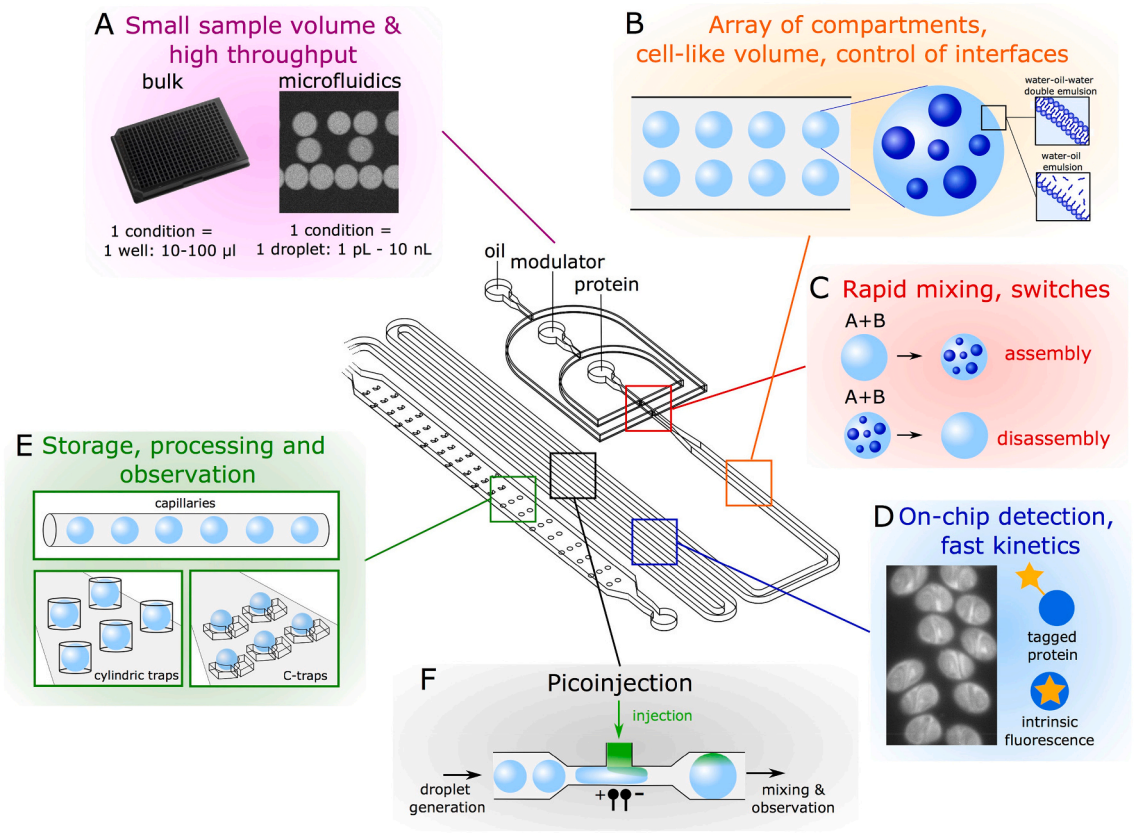

Fig. 1. Schematic illustration of some key features of microfluidic systems when analyzing biomolecular condensates. (A, B) Droplet-based microfluidic technologies allow the compartmentalization of sample into water-in-oil emulsions, generating thousands of well-defined microcompartments within a short time. Samples can be screened with high-throughput and within droplets with cell-like volumes. (C) Rapid mixing inside the droplets ensures well-defined starting conditions for kinetic assays. (D) Biochemical reactions can be observed in droplets using optical methods, such as fluorescence spectroscopy. (E) Droplets can be stored, processed and observed over long timescales by trapping droplets in traps or by transferring them into capillaries. (F) The composition of a compartment can be modified after generation by introducing additional molecules *via* pico-injection.

down of translation upon cellular stress increases the number of cytoplasmic mRNAs, which results in the formation of processing bodies in yeast by liquid-liquid phase separation [79].

Droplet-based microfluidic systems also facilitate changes in the composition of the mixture, for instance by introducing picoliter volumes of additional molecules of interest into individual water-in-oil compartments *via* pico-injection (Fig. 1F). Pico-injection is achieved by the application of an electric field (at kHz rates), which destabilizes the water-oil interface of a droplet, enabling a small volume of a flowing fluid stream to enter [80,81] (Fig. 1F). Such approaches could enable the investigation of the role of modulators not only on the assembly of biomolecular condensates but importantly also on their reversible disassembly, which is crucial for their biological functions and not yet widely studied [82]. *In vivo*, common examples that regulate the disassembly of the condensates include reversible enzyme-mediated post-translational modifications, such as (de-)phosphorylation [83] or (de-)methylation [19], as well as (de-)protonation due to pH changes [84], interactions with molecular chaperones [17,85,86] and ATP binding and hydrolysis [12]. Such reversible processes have recently been observed and the application of microfluidic technology will contribute to the understanding of the molecular mechanisms underlying the formation and dissolution of droplets.

Finally, a powerful feature of all microfluidic technologies is the ability to accurately control and modify temperature, due to the high associated surface area-to-volume ratios [87,88]. Temperature is a key physical parameter governing the kinetics and thermodynamics of phase separation and the quantification of temperature effects is a standard approach when assessing the enthalpic and entropic contributions of phase separation processes. These measurements have direct implications in the elucidation of mechanisms evolved by cells to sense temperature, in particular in response to heat stresses [89–91]. For example, it has recently been shown that polyA-binding protein 1 (Pab1) acts as temperature sensor and undergoes phase separation with increasing temperature, exhibiting LCST behavior [92]. The phase separation is likely driven by hydrophobic residues in the LCD of the protein, which promote attractive protein-protein interactions upon an increase in temperature to maximize the solvent entropy [40,92]. Notably, the extent of phase separation drastically changes over a narrow temperature range, allowing accurate sensing of the process. Replicating such biological processes on-chip is a promising approach to understanding the peculiar molecular modulators underlying control [93].

Altogether, the enabling features of microfluidic systems have enormous potential in diverse fields of biological research [45,94–99], including the characterization of phase separation and the properties of the resulting compartments (Fig. 2), as discussed in detail in the following paragraphs.

3. Valve-based and droplet microfluidics for phase diagrams of biopolymers and biomolecular condensates

Phase diagrams of macromolecules depend on a large number of molecular modulators and environmental conditions, with the most common being protein concentration, temperature, pH, ionic strength and macromolecular crowding agents [100]. For biomolecular condensates, this list includes many other specific molecules that interact

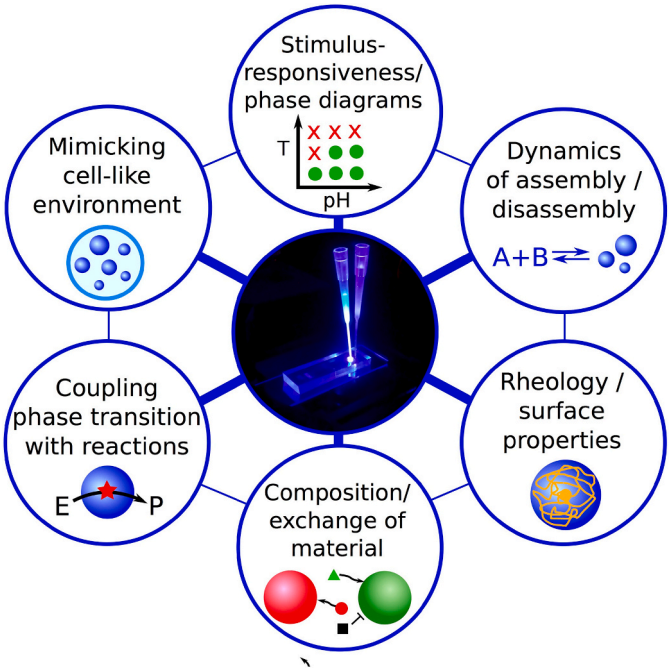

Fig. 2. Microfluidic technologies for the analysis of the process of phase separation and of the properties of the resulting compartments: (i) Phase diagrams with high throughput and using only small sample volumes; (ii) Dynamics of assembly and disassembly by accessing short timescales; (iii) Rheological and surface properties; (iv) Exchange of material with the environment and with adjacent droplets; (v) Coupling between phase transitions and biochemical reactions, including active, non-equilibrium systems; (vi) Additionally, microfluidics is an excellent tool for encapsulating phase separating systems into vesicle-like structures, creating cell-like compartmentalization.

directly with the phase separating component, such as RNA, ATP, and several other co-factors.

Phase diagrams are typically obtained through the systematic screening of these different modulators [19,26,37]. Using conventional bulk assays, however, this approach requires large amounts of sample, which are often not available when working with recombinantly expressed proteins or *in vitro* transcribed RNAs. Two main microfluidic features have been exploited to decrease the amount of sample necessary for the evaluation of phase diagrams; valve-based and droplet-based microfluidics.

The successful growth of protein crystals requires the screening of a vast chemical space to identify the best thermodynamic and kinetic conditions for crystallization. Multiple kinetic approaches, including the hanging drop, micro-batch or free interface diffusion methods have been applied to search for the optimal kinetic space for protein crystallization [101]. However, these require a significant amount of protein and yields from protein purification are often low, in particular for complex proteins which are often the hardest to crystallize as well. These issues indicate the suitability of microfluidic technologies in protein crystallization studies. Indeed, the first application of microfluidics to protein crystal growth was reported by Quake and co-workers in 2002 [102–104]. Through the implementation of microfluidic crystallization platforms, the authors devised a microfluidic paradigm for systematic crystal growth, obtaining protein crystals that were inaccessible *via* traditional methods. Massive parallelization and automation capabilities provide an ideal platform for high-throughput screening efforts, such as volumetric metering to achieve high-throughput free-interface diffusion-based screening for crystal hits [102] and automated phase space screening to identify ideal precipitants for the protein of interest [103].

Pneumatic valves (commonly termed Quake valves) have been additionally used to build peristaltic micro-pumps [103,105], which when integrated in mixing ring structures, allow high titration precision and simple multiplexing. For instance, using such a configuration the phase behavior of the endo-1,4-β-xylanase protein from the *Trichoderma reesei* fungus was screened by testing 4300 different conditions obtained in 16 different buffers and 16 precipitation agents [103].

Droplet-based microfluidic devices allow the generation of nanoliter and sub-nanoliter assay chambers. Unsurprisingly, there have been many advances in technologies for droplet-based protein crystallization over recent years. For example, Ismagilov and co-workers have produced a host of passive droplet-based devices for protein crystallography. These systems combine reagents in droplets embedded within an immiscible oil, and enable the rapid screening of crystal conditions [106]. Indeed, the authors have demonstrated techniques for altering crystallization kinetics by mixing [107] and seeding [108], and have also presented platforms for *in situ* X-ray diffraction of protein crystals grown on-chip [109].

Phase transitions can be induced by changing not only the buffer composition but also the protein concentration. In this context, droplet-based microfluidic platforms have been used to encapsulate homogeneous solutions at sub-critical concentrations into water-in-oil compartments, which are subsequently trapped and shrunk by water extraction [73]. In this way, the concentration of molecules in each compartment increases until supersaturation is reached, leading to a phase transition. Water removal can be controlled by the regulation of the osmotic pressure between the trapped droplet and a second fluid layer containing a high-salt solution [70] (Fig. 3A, B), water evaporation through porous PDMS [74], flowing air through microfluidic channels after droplet trapping [110] or the extraction of water molecules into the oil phase surrounding the trapped droplets [72] (Fig. 3C). All these strategies can be used to increase the concentration of biomolecules inside compartments until supersaturation and phase separation are observed. [70,72–75] (Fig. 3D).

While liquid-solid phase transitions have been extensively studied using microfluidic systems [111,112], liquid-liquid phase separation has been much less explored [70,75]. However, these platforms have the potential to analyze liquid-liquid phase separation of biologically relevant systems. In this regard, we recently showed the monitoring of the phase transition of a biologically relevant *in vitro* model system of processing bodies (represented by the DEAD-box ATPase protein Dhh1 in the presence of ATP and polyU) by increasing the concentration of the constituents in a microfluidic droplet concentrator [72] (Fig. 3E). The concentrator is designed to leverage buoyancy and ensure that the droplets remain trapped in the chambers even upon severe shrinkage, thereby enabling content assessment over extended periods of time using a conventional microscope. Here, severe shrinkage provides access to a broad concentration factor of approximately 100,000 fold, which in turn allows promotion of phase transitions at high biomolecular concentrations, while using only nanograms of sample. Moreover, coalescence events of the protein-rich condensates into one single droplet confirmed the liquid-like nature of the dispersed phase [72].

Recently, an elegant approach based on capillary valving has been demonstrated for the accurate and precise determination of phase diagrams [113]. This approach avoids the need of microfluidic flow control elements such as actuated valves or pumps, facilitating the implementation of the technique by non-expert users [113].

4. Droplet microfluidics to analyze the dynamics of protein phase transition and biomolecular condensates

As a result of the ability to rapidly mix reagents on chip (Fig. 1C), droplet-based microfluidic systems have been applied to investigate not only the thermodynamics but also the kinetics of protein phase transitions. Fast (bio)chemical reactions on timescales of μs-min can be followed in real-time [114,115] by monitoring changes in the droplet content. In analogy to thermodynamics, the first applications of

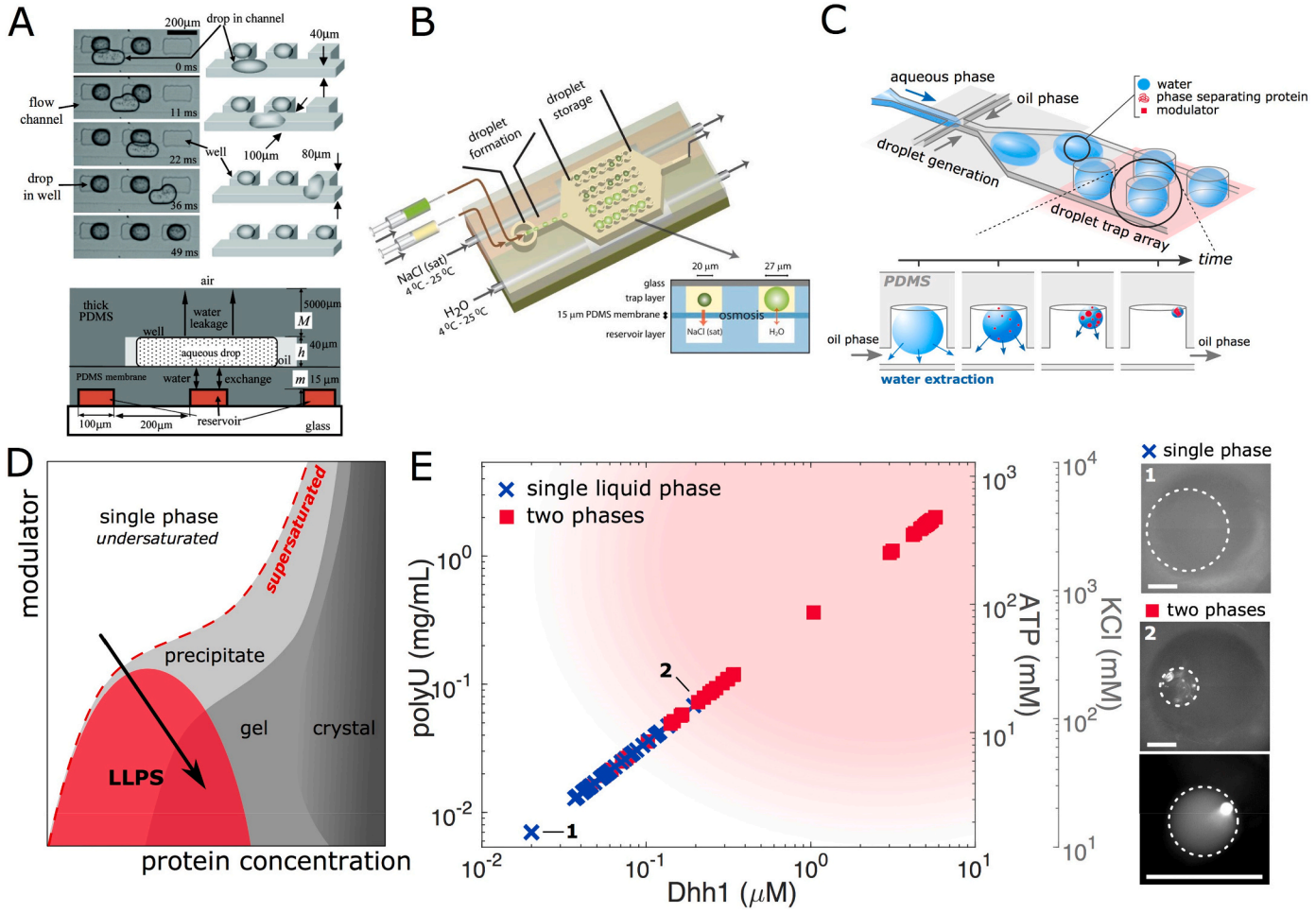

Fig. 3. Phase diagram of protein solutions on chip. (A, B, C) A droplet-based microfluidic system is used to screen phase diagrams at high throughput, while consuming small amounts of sample. Droplets filled with solutions at sub-critical concentrations are trapped inside a microfluidic chip and concentrated by removing water either by osmosis (A, B) or by water extraction into the surrounding oil phase (C). (A) Reprinted (adapted) with permission from [73]. Copyright (2007) American Chemical Society. (B) Copyright (2013) National Academy of Sciences. (D) Schematic phase diagram of a protein solution. The boundaries and the accessed physical states depend on the considered protein and solution conditions. The arrow indicates a possible trajectory during droplet shrinkage in a microfluidic device as shown in (A, B, C). (E) Phase separation of a model system of processing bodies. The DEAD-box protein Dhh1 shifts from a homogeneous solution (blue cross) to a phase separated dispersion (red square) upon increase of the concentration of the protein and of three modulators: ATP, polyuridylic acid (polyU, RNA mimic) and KCl, obtained in the microfluidic device shown in (C). The liquid-like properties of Dhh1 condensates are confirmed by the merging of all protein-rich droplets into a single condensed phase, as shown in the fluorescence images on the right-hand side. Scale bars are 50 µm. Reprinted (adapted) with permission from [72] Copyright (2020) American Chemical Society.

microfluidics in the analysis of the kinetics of phase separation were for liquid-to-solid transitions, such as the formation of protein crystals [73] and amyloid fibrils [69]. Kinetic control is particularly important for protein crystallization, since the number and morphology of the resulting crystals is highly affected by the balance of nucleation and growth events, which in turn depends on the level of saturation. Specifically, large single crystals, which are required for structure determination by X-ray crystallography, can be obtained by promoting the growth of existing nuclei over the nucleation of new ones. Droplet-based microfluidic approaches enable not only the screening of multiple conditions with low sample volumes, but also the precise control of the level of supersaturation (Fig. 4A) [73]. Importantly, supersaturation can be dynamically controlled by changing the droplet volume over time. For instance, large crystals can be generated in a two-step process performed in trapped water-in-oil emulsions. First, nucleation is induced by osmotically shrinking the droplet volume by flowing a high-salt solution through a second fluid layer to increase supersaturation. After nucleation, the droplet volume is osmotically increased (by flowing a solution of low salt concentration), therefore decreasing the level of supersaturation and disfavoring further nucleation with respect to crystal growth by Ostwald ripening [73] (Fig. 4A).

Protein aggregation represents another example of a liquid-to-solid transition. As before, the balance of nucleation and growth events in this process controls the size and morphology of the resulting aggregates [116]. A particularly interesting class of protein aggregates is represented by amyloid fibrils, which are associated with a variety of neurodegenerative disorders [117]. Understanding the nucleation and growth of amyloids is crucial for the development of therapeutic strategies against protein misfolding diseases [118]. These fibrils are initially formed by rare nucleation events, a process which is difficult to monitor in conventional bulk assays. Droplet-based microfluidic tools can reproduce nucleation and growth events in cell-like volumes, enabling the analysis of the stochastic formation of the first nuclei as well as the propagation of aggregation in space and time (Fig. 4B) [69]. These studies demonstrate that stochasticity occurs only in tiny volumes (pL-nL) and cannot be responsible for the variability of aggregation profiles which is typically observed in *in vitro* kinetic assays [119,120].

Kinetics is also crucial in the context of biomolecular condensates, since living systems are intrinsically out-of-equilibrium and ensure a

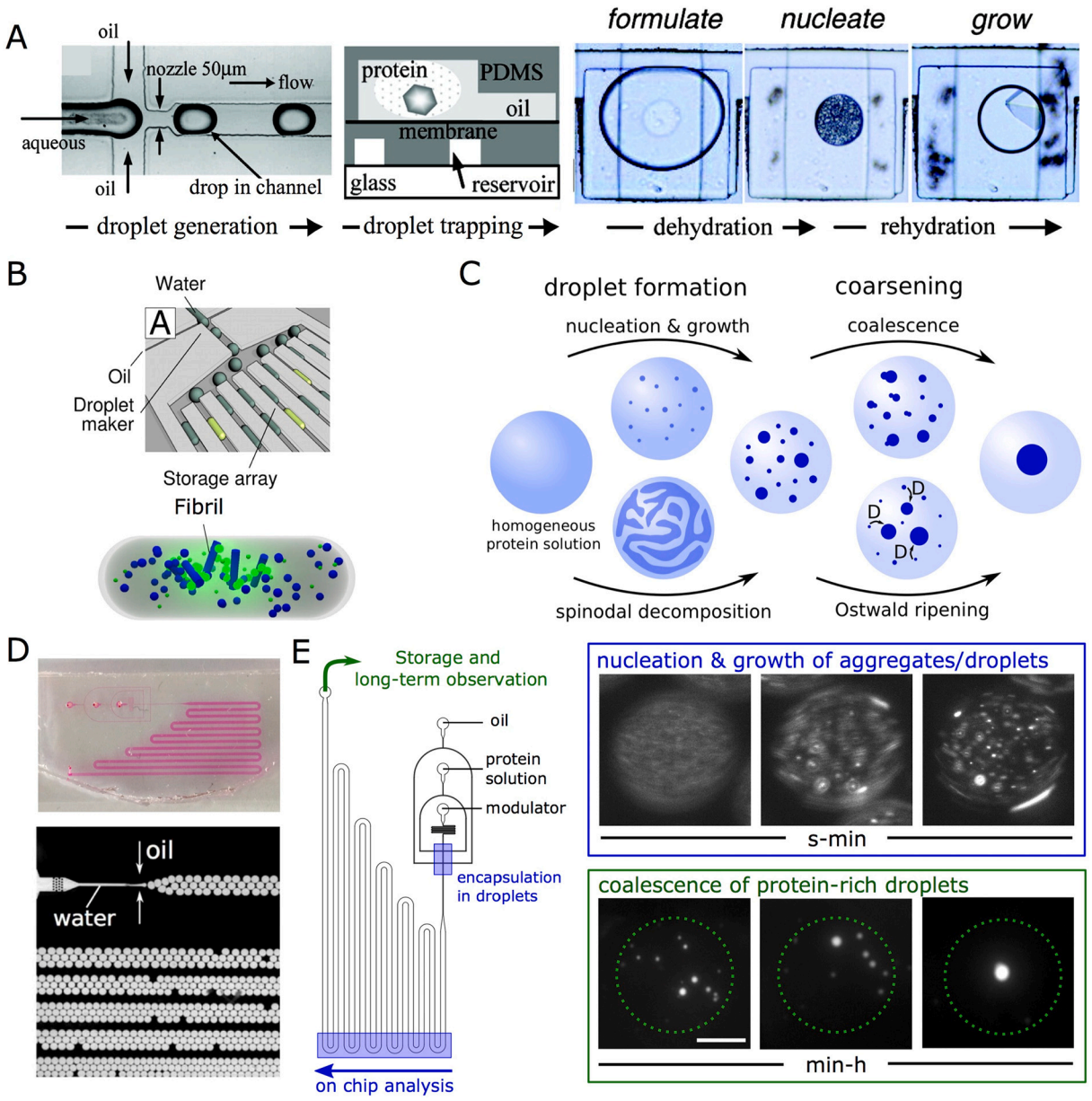

Fig. 4. Dynamics of phase transitions in droplet microfluidics. (A) Formation of protein crystals in microfluidic droplets *via* the control of supersaturation. Nucleation of crystals was induced by reducing the volume of the compartments that encapsulate the protein solution. Subsequently, crystal growth was promoted by increasing the droplet volume and by decreasing the level of supersaturation, thereby disfavoring nucleation and promoting growth by Ostwald ripening. Reprinted (adapted) with permission from [73] Copyright (2007) American Chemical Society. (B) Nucleation and growth of amyloids observed in microfluidic droplets, which allow one to investigate volume confinement effect and stochasticity of primary nucleation events. Copyright (2011) National Academy of Sciences. (C) The dynamics of formation of condensates involves several possible microscopic mechanisms such as spinodal decomposition, nucleation and growth, droplet growth by Ostwald ripening or droplet coalescence. (D) Dynamics of liquid-liquid phase separation in microfluidic droplets, representing an *in vitro* model system of processing bodies. The phase separating protein was encapsulated into water-in-oil droplets and the phase separation induced by rapid mixing with molecular triggers. (E) Condensate formation could be tracked on short timescales, with nucleation and growth being monitored on chip. Furthermore, droplet coarsening can be observed over longer timescales by collecting the compartments in capillaries. By extracting size distributions over time, droplet coarsening mechanisms like Ostwald ripening and coalescence were distinguished.

constant turn-over of biochemically active agents [121,122]. Biomolecular condensates are open reactors and exhibit rapid internal mixing, fast exchange with the surrounding environment and can rapidly assemble and disassemble through a constant flux of molecules [123–126]. Moreover, over longer timescales, biomolecular condensates can undergo “aging”, in which the physical properties of the protein-dense phase changes and the liquid, protein-rich phase forms hydrogels and/or solid aggregates [127–129]. The investigation of such transitions is particularly important since the formation of liquid phases at high protein concentrations may lead to the formation of solid structures associated with a variety of devastating pathological conditions, including Amyotrophic Lateral Sclerosis, Alzheimer's and Parkinson's Disease [17,26,27,29–31]. Understanding the molecular mechanisms underlying such “aging” processes is therefore crucial in fighting against aberrant behaviors.

Despite the essential role of dynamics in living organisms, mechanisms have remained largely unexplored. This is in part due to the lack of suitable tools able to monitor dynamics in an accurate and quantitative way on both short and long timescales. In this regard, droplet-based microfluidics is useful in monitoring events such as

spinodal decomposition, nucleation and growth of condensates and coarsening mechanisms *via* Ostwald ripening or droplet coalescence (Fig. 4C).

In addition to the construction of phase diagrams (Fig. 3D, E), we recently applied droplet-based microfluidics to the analysis of the dynamics of the liquid-liquid phase transition of an *in vitro* model system of processing bodies [12,79,130]. Here, the processing body-associated protein DEAD-box ATPase Dhh1 was rapidly mixed with molecular triggers (ATP and polyU) and encapsulated into water-in-oil compartments using a flow focusing geometry (Fig. 4D). On timescales ranging from milliseconds to seconds, the nucleation and growth of phase-separated droplets was then monitored, as well as coarsening events that occur over longer timescales (minutes to hours). This analysis highlighted an increase of the rate of formation of the condensates as a function of the compartment volume. Moreover, the results indicated coalescence as the dominating mechanism for droplet growth [62] (Fig. 4E). Another attractive feature of droplet-based microfluidic systems is the ability to form more biologically relevant structures. For instance, hydrogel matrices can be assembled inside droplet compartments to mimic a cytoskeleton-like structures, with the formed hydrogels arresting droplet coalescence [62,131].

Recently, a combinatorial droplet microfluidic platform has been developed for the rapid and high-resolution acquisition of protein phase diagrams, which have been demonstrated with condensates of the protein FUS [132].

5. Microfluidic tools for the analysis of rheological properties and composition of biomolecular condensates

In addition to the analysis of the thermodynamics and kinetics of phase separation, microfluidic technology has been recently applied to probe the material properties of the resulting biomolecular condensates as well as the recruitment of client molecules (Fig. 5). In this situation, the advantages of microfluidics rest on the accurate control of mass transport processes on the micron scale.

Particle tracking methods are commonly used to analyze rheological properties and measure diffusion coefficients and viscosities [21]. However, in many cases the size of the phase-separated droplets is limited to a few microns and thus incompatible with this technique. To overcome this limitation, a microfluidic device integrating micron-sized pillars was used to merge pre-formed droplets into a single phase-dispersed phase. Subsequently, tracer particles could be recruited into this now large phase and successfully tracked [133] (Fig. 5A). In addition to passive microrheology, the same authors developed a microfluidic configuration to analyze viscosity under flow, which is highly relevant for out-of-equilibrium systems [133]. Here, a protein-rich, phase-separated fluid stream was co-flowed with a protein-lean phase, with both phases being supplied with tracer beads to measure flow profiles. The viscosities of the individual phases could then be extracted by the measured velocity profiles [133] (Fig. 5B). Such microfluidic approaches have been applied to the analysis of the viscosity of condensates of Laf1, Whi3 and GAR-1ΔN proteins, which are respectively associated with P granules, nucleoli and Whi3 assemblies [133]. Since biological condensates can exhibit a broad range of rheological properties, ranging from liquid to gel-like, these microfluidic tools are crucial in correlating biomolecular interactions with viscoelastic properties and biochemical functions.

Moreover, such material properties can change over time. For instance, it has been recently shown that application of shear forces can promote liquid-to-solid transition in protein-based condensates [134]. A key advantage of microfluidic platforms is the possibility to apply highly controlled shear stresses and elongational strains [134,135].

In addition to bulk features, microfluidics can also be applied to measure surface properties, as recently demonstrated by the measurement of the surface electrostatic potential of biomolecular condensates [136].

Composition and the exchange of material between condensates and their surrounding are another important aspect of biomolecular condensates. Such rapid mass transport processes can be conveniently monitored within microfluidic environments. Indeed, a recent study analyzed the recruitment of client proteins into protein-rich droplets in a microfluidic device containing a flow focusing geometry. Here, phase separation was triggered on-chip and the resulting droplets brought into contact with a homogenous solution of a fluorescence-tagged cargo protein flowing parallel on both sides of the droplet stream. In this way, cargo proteins were able to diffuse inside the droplets, with the kinetics of uptake being observed on timescales of milliseconds by monitoring the increase in protein fluorescence over time [137,138]. This approach was used to investigate the ability of nuclear pore complexes to selectively transport cargo biomolecules across the nuclear membrane [137]. The model *in vitro* protein-rich droplets consisted of phenylalanine/glycine-rich nucleoporins (FG-Nups), which are highly abundant in those complexes. Client proteins were injected in the absence and presence of importins, which mediate trafficking through the nuclear pores by forming complexes with the cargo proteins. The results showed that only the complexes formed by importins and the cargo proteins could be recruited, while in the absence of importins the phase-separated FG-Nups were impermeable to client proteins. In addition to proving the key role of importins in mediating transport across the nuclear membrane, the microfluidic method also allowed the measurement of the kinetics of this process on very short timescales.

Recently, a droplet microfluidic device was applied to quantitatively characterize with high-throughput the partition coefficient of different molecules within individual coacervates of different chemistry [139] (Fig. 5C, D). The technique finds several applications, for instance in the study of enzymatic reactions in phase separated condensates.

6. Droplet microfluidics to analyze biomolecular phase separation in artificial cell-like systems

Until now, we have focused our discussion on microfluidic devices for the study of *in vitro* models of biological condensates associated with different membraneless organelles in cells. In addition to the analysis of biological compartments, microfluidic technologies have been widely exploited in recent years to generate synthetic compartments for the bottom-up synthesis of artificial cells. Membrane-bound compartments as well as simple and complex coacervates have been assembled on chip to address fundamental questions on how life originated from simple chemical systems. This topic has been comprehensively summarized in some excellent recent reviews [140–142]. Accordingly, we focus now on a few selected examples that are particularly relevant for the study of biomolecular condensates, the assembly of biological interfaces and the coupling between compartmentalization and biochemical reactions (Fig. 2).

A central characteristic and property of a cell is the ability to compartmentalize and organize biochemical reactions in both space and time [32]. In artificial systems, such microenvironments can be established by encapsulating membrane-bound and membraneless compartments into liposomes or giant unilamellar vesicles (GUVs). These vesicles consist of a phospholipid bilayer, which represents a mimic of the plasma membrane of eukaryotic cells [143]. Microfluidic approaches are excellent tools for the assembly of such constructs, since they provide for the precise control of size and composition of the generated vesicles [144–147]. Lipid bilayers can be generated using simple PDMS-based flow focusing devices to produce water-in-oil-in-water double emulsions. Here, phospholipids are introduced in the oil phase and assembled by solvent removal [148] (Fig. 6A). In addition to flow focusing devices, double emulsions can also be generated by capillary microfluidics [93] (Fig. 6B) or by droplet transfer methods [149,150]. In the latter approach, a droplet surrounded by a phospholipid monolayer is transferred off-chip from a hydrophobic oil phase into an aqueous phase [149,150]. Droplet-based microfluidics enables

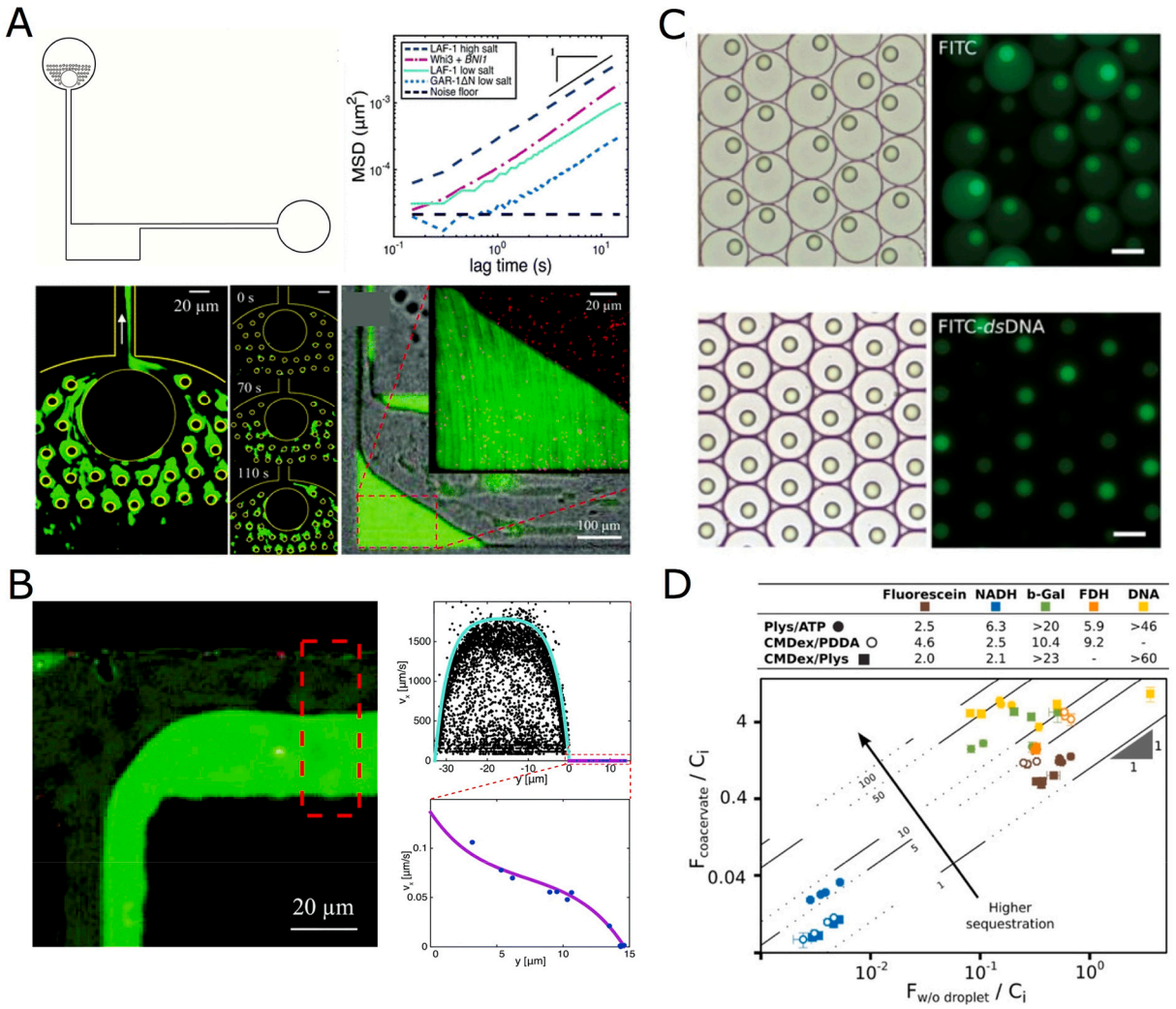

Fig. 5. Microfluidic devices can probe rheological properties of phase separated condensates and the recruitment of molecules. (A) Pre-formed droplets are flown through a network of pillars to generate a large dispersed phase, which can be supplied with nanoparticles, therefore allowing particle tracking analysis. (B) Measurement of viscosities of the disperse and continuous phase by flow-based active microrheology. Tracer particles are used to measure flow profiles in both phases, from which viscosities are extracted. (A,B) Reproduced from Ref. [133] with permission from the Royal Society of Chemistry. (C) High-throughput measurement of partition coefficients to characterize the recruitment of fluorescein (top) and FITC-dsDNA molecules (bottom) into pLys/ATP coacervates formed in microfluidic droplets. (D) Quantitative assessment of the partition coefficients of various molecules (fluorescein, NADH, b-Gal, FDH, DNA) into different types of coacervates (pLys/ATP, CMDex/PDDA, CMDex/pLys). (C,D) was reprinted from [139] under the terms of the Creative Commons Attribution-NonCommercial License for non-commercial purposes.

the formation of progressively more complex structures that closely mimic biological membranes. For example, it is possible to add proteinaceous pores into the lipid bilayer, enabling active or passive transport of molecules across the membrane [36] (Fig. 6C).

Artificial cells can be generated by assembling membraneless compartments within containers enclosed by these cell-like interfaces. For such an application, microfluidic technology is of great benefit, since membraneless compartments can be encapsulated inside cell-like compartments "on-chip" in a robust fashion. Currently, synthetic open organelles have been formed inside artificial cells mainly by complex coacervation, for instance by using peptide-nucleotide complexes [33], polycations (*e.g.* polyarginine, polylysine, spermine) and polyanions (*e.g.* polyU, ATP, CoA) [93,143] or elastin-like peptides [151]. The phase transition can also be regulated by modulating the pH inside the cell-like compartments [152,153], mimicking the changes in the pH value that are often observed in cells under stress [154–156] (Fig. 6D). These important platforms set the basis for the encapsulation of more complex *in vitro* models of biomolecular condensates, moving towards a closer representation of cells.

Besides the encapsulation and observation of simple phase separating systems, microfluidic technologies also allow for an analysis of the coupling between compartmentalization and biochemical reactions (Fig. 2), which is becoming increasingly important in light of the emerging findings in the field of non-equilibrium active compartmentalization [122,157,158]. In microfluidic environments, precise measurements of biochemical reaction rates are enabled by the accurate control of the composition of the mixture and the rapid mixing reagent, which ensures well-defined initial conditions.

The interplay between biochemical reactions and biomolecular condensates can occur in various ways [159,160]. In some cases, biochemical reactions modify the building blocks of biomolecular condensates, either by increasing or inhibiting their tendency to phase separate, which in turn promotes the assembly or the disassembly of the compartments [152,161–163] (Fig. 6E, i). For example, in one study complex coacervation between cations and polyuridylic acid sequences was induced by progressively increasing the length of initially short sequences of the RNA-mimicking molecules *via* an enzymatic reaction, until a critical length was reached [36]. The required enzymes and

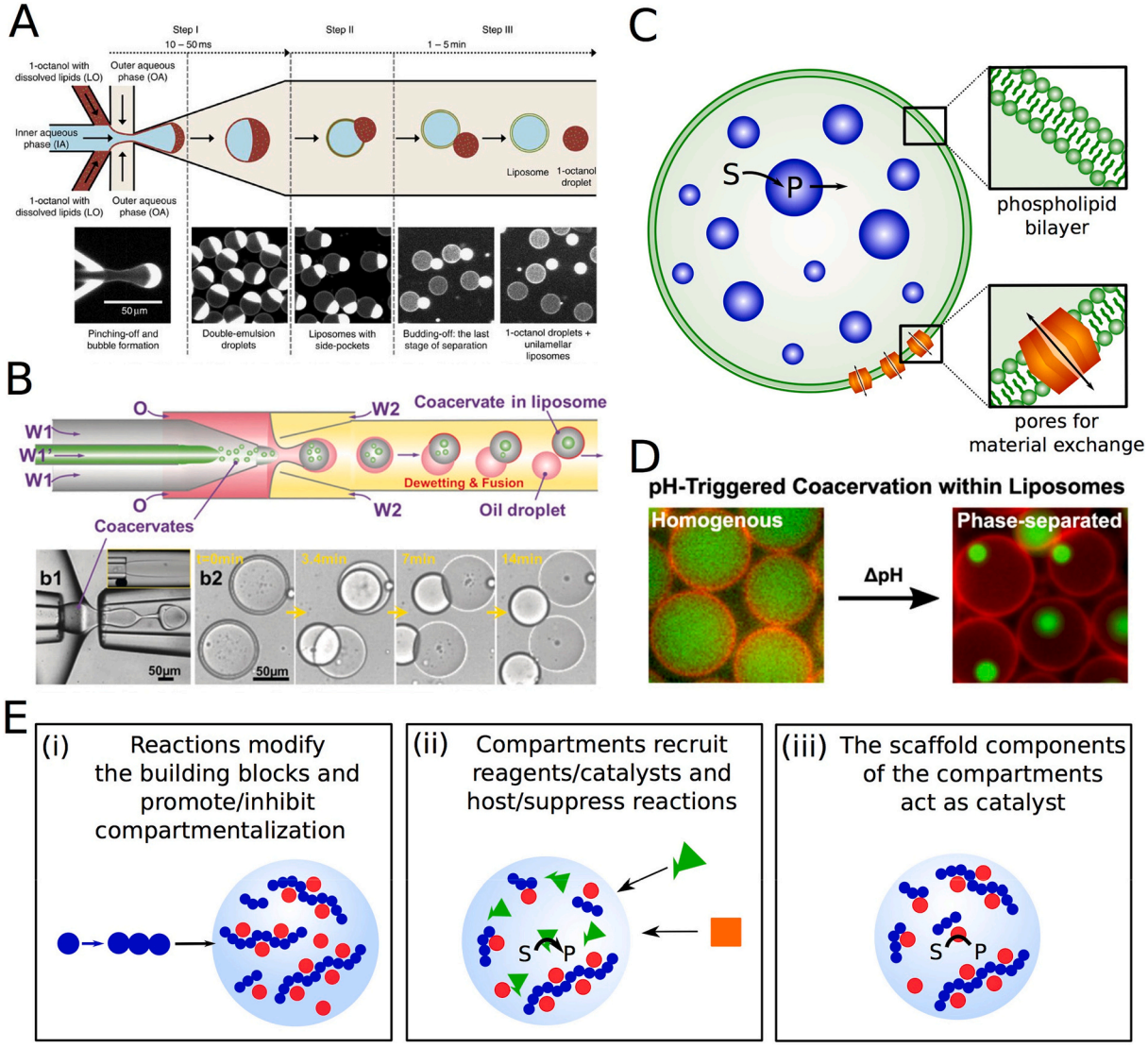

Fig. 6. Examples of microfluidic approaches that mimic compartmentalization in artificial cells. (A, B) Droplet microfluidics is used to form cell-like compartments surrounded by a phospholipid bilayer that mimics the cell membrane. This can be achieved by using a PDMS- (A) or a capillary-based (B) device to generate double emulsions, where the phospholipids are introduced in the oil phase. (A) Material reused from [148]. (B) Material reused from [93]. (C) In such compartments it is possible to encapsulate synthetic organelles and add pores into the phospholipid bilayer to allow transport across membranes. (D) The assembly and disassembly of coacervates can be regulated by changes of the pH value, modulated by proton diffusion across lipid membranes. Material reused from [152] https://pubs.acs.org/doi/10.1021/acsnano.9b10167. Further permissions related to the material excerpted should be directed to the ACS. (E) Coupling between compartmentalization and biochemical reactions: (i) reactions can modify the building block of the condensates, either promoting assembly or disassembly; (ii) condensates can recruit reagents, inhibitors and catalysts, hosting reactions in their interior; (iii) the building-blocks of the condensates can act as catalysts themselves.

reactants were encapsulated together with the counterions in lipid vesicles into the droplets. In other cases, biomolecular condensates recruit client molecules that act as reagents or catalysts of biochemical reactions hosted inside pre-formed compartments (Fig. 6E, ii) [42,93,164,165]. For instance, the formation of a model cytoskeleton system was reproduced by partitioning of the bacterial cytoskeleton protein FtsZ into the dextran phase of a PEG/dextran aqueous two-phase system, followed by polymerization in the presence of GTP [150]. In another study, *in vitro* transcription and translation leading to the expression of GFP was realized in coacervates encapsulated into water-in-oil compartments as well as in liposomes (Fig. 6E, ii) [70].

Under different conditions, the building blocks of membraneless organelles can exhibit enzymatic activity themselves and can act as catalysts for biochemical reactions [37,41,42] (Fig. 6E, iii). In this regard, studies have recently demonstrated that compartmentalization of biochemical reactions affects their rate, and often the increase in the local concentration of substrates and enzymes enhances kinetics with respect to the bulk phase, in agreement with observations *in vivo* [41,70,93]. However, the high viscosity and the different environment of the condensates could also have an opposite inhibitory effect, and the coupling between open compartments and biochemical reactions is thus an area of much current activity [159]. In this context, microfluidics can play a key role in allowing a quantitative analysis of biochemical process and reproducing cell-like environments.

7. Conclusions and outlook

Microfluidic systems are particularly attractive tools for the analysis and synthesis of supramolecular structures of biomolecules. Key advantages of such systems are related to the benefits inherent to miniaturization and to the superior control of the reactive environment on the micron scale, which in turn leads to the accurate modulation of self-assembly in time and space. Prompted by these advantages, microfluidic tools have been widely applied in the last decade to the bottom-

up synthesis of artificial cells and the field has experienced the development of several attractive platforms [81,93,166,167] for studies on cell-like systems. These studies already provided important insights, such as the possible acceleration of biochemical reactions in separated compartments [41,70,93].

Simultaneously, a rapidly growing number of studies in biology have generated important knowledge on the role of membraneless organelles in cell compartmentalization, shedding light on key molecules and molecular triggers that regulate this process [2,4,168,169]. It is therefore timely to reproduce *in vitro* model systems of these biological compartments in microfluidic platforms, moving towards the replication of cellular compartmentalization on the bench. In this review we have illustrated the power of microfluidics in gaining insights into the process of phase separation and on the properties of the resulting compartments. This knowledge is crucial for understanding the complex coupling between compartmentalization and biochemical functions (Fig. 2). This technology will be particularly important when analyzing phase transitions in biology and their role on cellular metabolism, as well as unearthing new therapeutic strategies against a variety of diseases associated with phase separation. In addition to increasing our understanding of biological condensates [62], the reproduction of synthetic compartments in microfluidic platforms will have clear implications for the bottom-up development of artificial cells and for the generation of bio-inspired protein materials in biotechnology [37,41,42].

Declaration of competing interest

The authors have no conflicts of interest.

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