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Building micro-capsules using water-in-water emulsion droplets as templates

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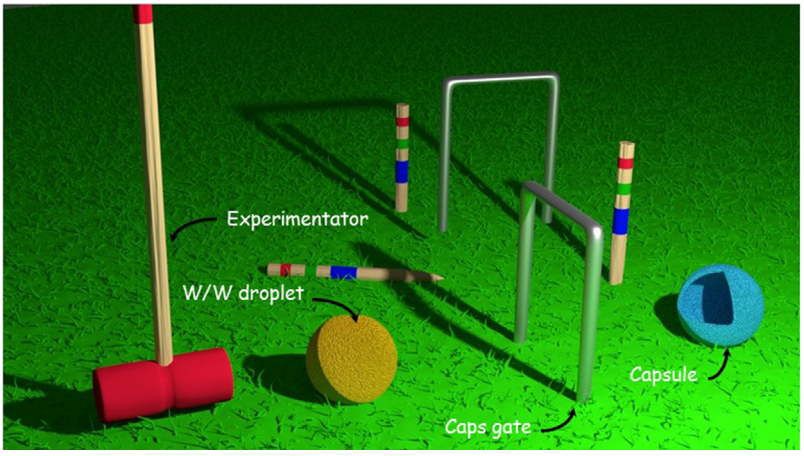
GRAPHICAL ABSTRACT



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ABSTRACT

The use of templates in materials chemistry is a well-established approach for producing membrane-bounded hollow spheres used for microencapsulation applications, but also in synthetic biology to assemble artificial cell-like compartments. Sacrificial solid or gel micro-particles, but also liquid-like oil-in-water or water-in-oil emulsion droplets are routinely used as templates to produce capsules. Yet, disruption of the core sacrificial material often requires harsh experimental conditions, such as organic solvents, which limits the use of such approach to encapsulate fragile solutes, including biomolecules. Recently, water-in-water emulsion droplets have emerged as promising alternative templates to produce capsules in solvent-free conditions. These water-in-water droplets result from liquid-liquid phase separation in dilute aqueous polymer or surfactants solutions. Their ease of preparation, the large palette of components they can be assembled from and the lack of harsh solvent or oil used for their production make water-in-water emulsions of practical importance in materials chemistry. Water-in-water droplets can also spontaneously sequester solutes by equilibrium partitioning, which provides a simple strategy to locally accumulate molecules of interest and encapsulate them in capsules after interfacial shell formation. Here, we review recent works that employ water-in-water emulsion droplets to prepare capsules and suggest possible additional applications in materials chemistry.

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1. Introduction

In materials chemistry, sacrificial templates are solid particles, emulsion droplets, self-assembled systems or any other object that can be coated with chemicals or particles then disassembled to produce a material in the shape of the initial template (Fig. 1). Templates have been used to prepare porous materials [1–5], Janus particles [6–11] or nano- or micro-meter sized hollow capsules [12–20]. The latter represent attractive materials for both technological and fundamental aspects since they have been exploited to encapsulate various solutes, protect them from the external medium, and release them on demand [21–26]. The encapsulation properties of hollow spheres depend on the chemical composition and structure of the shell used to cover the core, but also on the nature of the template initially used. While hollow spheres with sizes spanning several orders of magnitudes (from tens of nanometer to hundreds of micrometers) can be produced using such a templating approach, our discussion will focus on microscale capsules (typically 1–100 μm in diameter).

Conventional methods for the preparation of micro-capsules have been broadly reviewed recently [6–8,10,12,16,17,19–21,23,24,26–42]. Several methods are template-free as nanoprecipitation or use solvent shifting [40–46]. Solid or gel particles but also water-in-oil or oil-in-water emulsion droplets are routinely used as sacrificial templates to synthesize capsules. One advantage of using solid templates such as silica or latex beads is that they can be highly monodisperse, which provides simple access to monodisperse hollow spheres [16,47,48]. However, removing a solid template often involves harsh conditions, such as high temperatures [49], organic solvents [50] or very low pH environments [51]. In addition, encapsulating solutes within the resulting hollow spheres has to be performed after the capsules have been prepared (which often turns out tedious) or by initially loading the template beads, but the solutes can then be damaged during template removal. Gelled beads assembled from low molecular weight organogelators [52,53] have also been used as templates to form hollow capsules by the interfacial assembly of a shell. Gelled templates can be removed more easily, and do not necessarily need to be sacrificed as they can be loaded with the molecules of interest before the assembly of a shell. The use of water-in-oil (W/O) or oil-in-water (O/W) emulsion droplets as templates has also become popular in recent years [4,5,33,54,55], mainly because such dispersions can be produced using a broad range of methods and components (oils and emulsifiers) [3,36,56], and can be used to encapsulate either water-soluble or lipophilic solutes. Here again, destruction of the template is not necessary but the final capsules may eventually be transferred into the desired continuous phase, e.g. using gradual changes in solvent.

Different strategies and components have been used to produce shells onto the above-mentioned templates. Colloidal particles can stabilize W/O or O/W droplets to produce Pickering emulsions [12,17,31,37,57], and can then be cross-linked or locked-in to form robust capsules that have been referred to as colloidosomes [18,19,58]. Polymers and polyelectrolytes [59,60], including proteins, have also been used to form a shell in lieu of particles [27,30,33,61–63]. These macromolecules either stabilize the emulsion itself by spreading at the oil/water interface or are deposited at the surface of pre-formed emulsion droplets via electrostatic interactions, for instance using the layer-by-layer technique [16,33,47,61,64–66]. Interfacial polymerization, cross-linking or complexation are also powerful strategies for producing hollow spheres in emulsion dispersions [67–71]. For instance, molecular precursors solubilized in one phase (water or oil) may polymerize at the droplets' interface upon addition of a reagent in the other phase [72]. Similarly, a polymer bearing reactive groups can be solubilized in one phase and cross-linked at the interface by using a cross-linker dispersed in the other phase [73]. Two polymers can also be dispersed in one or the other phase, respectively, and meet at the droplet interface via attractive electrostatic interactions where they can further be cross-linked to form a robust shell [74,75]. Interestingly, droplet-based microfluidics has emerged as a promising approach to produce monodisperse emulsions droplets as templates to prepare uniformly-sized micro-capsules [67,76–78].

The above-mentioned templates offer powerful approaches for the construction of micro-capsules, but may be limited for handling fragile solutes, such as biomolecules. In addition, in the context of a greener chemistry, alternative strategies to build capsules with a lower environmental impact have recently emerged based on the use of water-in-water (W/W) emulsion droplets as solvent-free, biomolecule-friendly templates. The term 'water-in-water emulsions' is a generic term used to designate the formation of all-aqueous (oil-free) droplets in a continuous aqueous solution [79]. W/W emulsions encompass aqueous two-phase systems (ATPS) produced by segregative liquid–liquid phase separation of incompatible polymers (such as poly(ethylene glycol) and dextran), but also coacervates [80] resulting from an associative liquid–liquid phase separation process, e.g., between oppositely charged polyelectrolytes (complex coacervation) or between amphiphilic molecules [81] (e.g., in the case of the surfactant

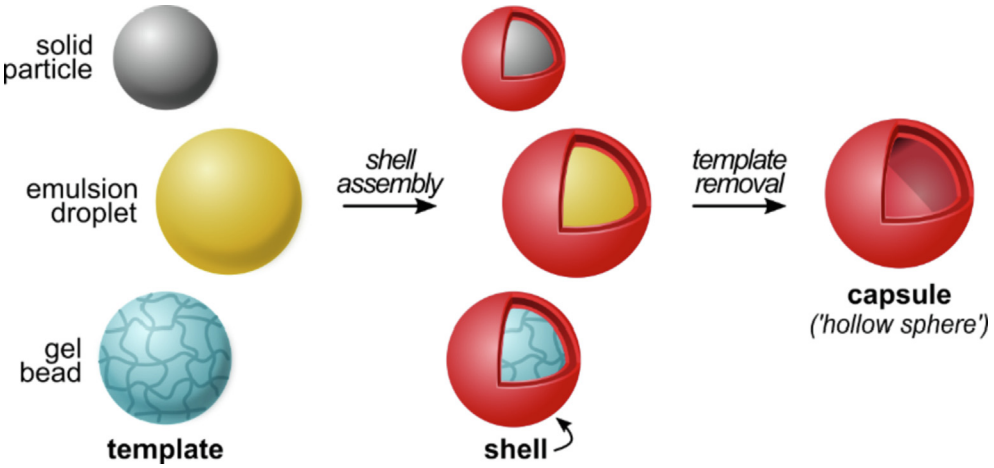

Fig. 1. Schematic representation of the templating method used to prepare capsules as hollow spheres. The template is first covered by a shell, and then eventually removed to produce a hollow sphere.

clouding phenomenon). Such W/W emulsions have been first reported in the late 19th/early 20th centuries, and have been applied to various fields, including food science, healthcare industry or underwater adhesives. In the mid-20th century, complex coacervate droplets have further been suggested as plausible prebiotic protocells [82], the first primitive cells that appeared on the early Earth. These water-in-water emulsions have recently regained strong interest with their use as synthetic or artificial cells in a bottom-up approach [83–87].

Key feature of these droplets is their ability to spontaneously uptake and accumulate a range of solutes, including biomolecules, via equilibrium partitioning, making them interesting compartments to localize species. However, droplets produced by LLPS are intrinsically unstable and undergo macroscopic phase separation with time. To avoid loss of droplet integrity, several approaches have been developed recently to stabilize all-aqueous droplets against coalescence. These approaches pave the way to the use of W/W emulsion droplets as templates to produce hollow spheres, using similar shell construction strategies as with conventional templates (Fig. 2). For instance, Pickering-like water-in-water emulsions can be produced; interfacial complexation and polymerization at the water/water droplet interface, but also polyelectrolyte layer-by-layer adsorption can be performed: in other words, all the chemistry that has been developed to produce capsules using conventional templates can be applied to water-in-water emulsions. Of course, this requires the chemicals and components used to build the shell to be adapted to be all soluble in water, and the experimental conditions (polymerization, cross-linking. . .) to be optimized to be performed in all-aqueous environment. The best example being that of proteinosomes, which have been initially produced from water-in-oil emulsions [62] and were further produced using complex coacervates [88]. Importantly, since these all-aqueous droplets spontaneously sequester solutes, it becomes possible to develop simple encapsulation strategies without the need of organic solvent or harsh conditions (as depicted in Fig. 2).

Here, we review recent studies on the production of robust capsules from all-aqueous emulsions, starting with the experimental conditions of liquid–liquid phase separation in water, followed by studies devoted to the stabilization of droplets, the sequestration of solutes, and the formation of hollow capsules in a context of materials chemistry.

2. All-aqueous emulsions produced by liquid–liquid phase separation

2.1. Polymer liquid–liquid phase separation

Liquid-liquid phase separation (LLPS) is a well-established phenomenon in aqueous polymer solutions that produces water-rich micro-droplets in thermodynamic equilibrium with a continuous aqueous phase [79,86,89–92]. Two main classes of LLPS can be distinguished depending on whether the process is associative or segregative (Fig. 3). Associative LLPS is typically referred to as "coacervation", which originates from the Latin word '*coacervare*' meaning 'coming together' or 'gather' [93]. Complex coacervation involves the association between two species (at least), such as oppositely charged polyelectrolytes, to produce polymer-rich droplets in equilibrium with a continuous dilute aqueous phase, while simple coacervation refers to LLPS of a single polymer species, such as a polyampholyte, that self-phase separate [79,85,91]. Beyond electrostatic attraction, other non-covalent interactions can also result in associative LLPS, including biomolecular recognition, hydrophobic polyampholytes, π -π or π -cation stacking, as recently reviewed [94,95].

In comparison, segregative phase separation is driven by the incompatibility between two polymer species (generally uncharged, such as PEG and dextran) to produce polymer-rich droplets suspended in a continuous phase enriched in the other polymer. This process has been referred to as an "aqueous two-phase system" [91].

2.2. surfactant liquid–liquid phase separation

Surfactants can also undergo liquid–liquid phase separation to produce surfactant-rich droplets – a process known as the clouding phenomenon [81]. This process can be compared to simple coacervation when a single surfactant species is involved, but can also occur in mixtures of positively and negatively charged surfactants [96,97] or uncharged pairs of surfactants [98]. In the latter case, the term catanionic coacervates, from the contraction of cationic and anionic, can be used, by analogy with catanionic vesicles or other assemblies produced by two oppositely charged surfactants [99–103].

Interestingly, the clouding phenomenon has recently been extended to long- and short-chain fatty acids. Solubilizing long-chain fatty acids in water is usually not trivial due to the existence of a critical temperature (known as the Krafft point) below which fatty acids crystallize. For instance, the Krafft temperature for the sodium salt of myristic acid, a 14-carbon chain length fatty acid, is around 40 °C [104,105]. Studies have shown that replacing sodium counter-ions with bulkier tetrabutyl-ammonium cations prevented the crystallization of myristate, allowing the formation of stable myristate micelles at room temperature [106,107]. Similarly, addition of guanidine hydrochloride (GuHCl) to sodium myristate (at equimolar ratio) successfully prevented crystallization, and favored fatty acid self-assembly into elongated micelles or stacked bilayers above or below 20 °C, respectively [108]. Strikingly, myristate coacervate droplets were produced by adding a ~ 2-fold molar excess of guanidinium counter-ions (Fig. 4), which was attributed to the ability of guanidinium to form hydrogen bonding networks with the carboxylate headgroup of fatty acids [109]. In comparison, flat bilayers formed upon cooling, yielding faceted gelled droplets (Fig. 4).

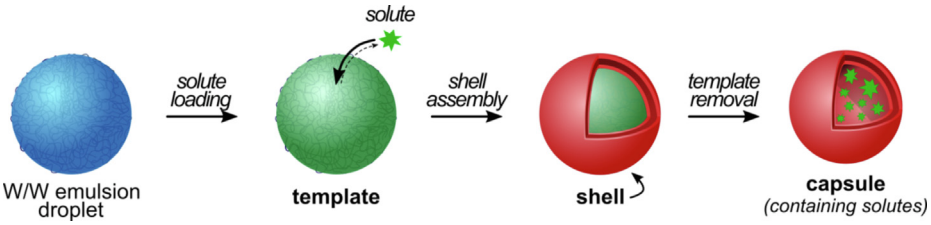

Fig. 2. Schematic representation of the formation of capsules and encapsulation of a desired solute starting from water-in-water emulsion droplets as templates. The initial emulsion droplet (blue) spontaneously sequesters a solute (green) before being covered by an additional chemical to form the shell (red), which is consolidated to form a capsule containing the solute (green). The initial template may eventually remain encapsulated within the capsule, together with the solute.

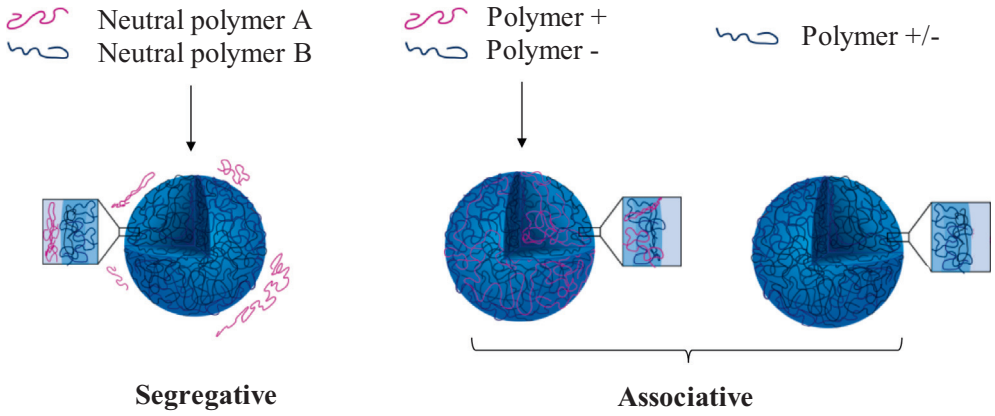

Fig. 3. Schematic representation of segregative (Aqueous Two Phase System, ATPS) and associative (Complex and simple coacervates) Liquid-Liquid Phase Separation (LLPS).

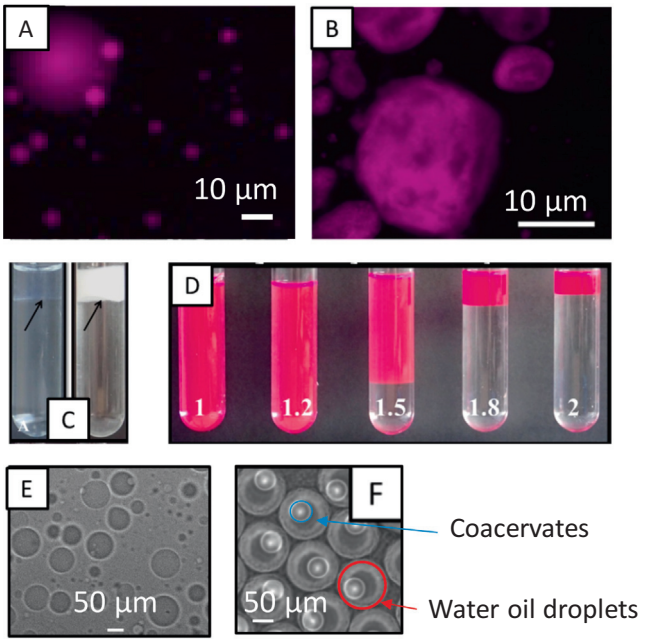

Fig. 4. (adapted with permission from ref [96,109]). Fatty acid based coacervate droplets made of sodium myristate and a 2-fold excess guanidine hydrochloride observed by epifluorescence (using Nile red) at 25 °C (A) and 15 °C (B). C) Phase separation occurring upon resting showing a fatty acid upper rich phase (see arrows) made of transparent elongated micelles at 25 °C (left) and stacked bilayers at 15 °C (right). D) Photos of samples tubes prepared at different GuHCl molar ratio (indicated below) showing the occurrence of the phase separation phenomenon at a given value (1.5). Catanionic coacervates prepared from sodium decanoate and CTAB in bulk E) and using microfluidics (F).

Using decanoic acid, a shorter fatty acid bearing 10 carbon atoms, we were also able to produce catanionic coacervates upon addition of either cetyltrimethylammonium bromide or cetylpyridinium chloride by adjusting the pH at an adequate value [96]. Highly monodisperse catanionic coacervates were also formed using microfluidics by first producing cationic surfactant-rich water-in-oil droplets followed by pico-injection of the sodium salt of the fatty acid (Fig. 4).

2.3. Water-in-water emulsions from liquid-liquid phase separation

Liquid-liquid phase separation processes in water therefore produce micrometer-sized, chemically-enriched, droplets suspended in an aqueous continuous phase. The droplets are highly hydrated, with a water weight fraction that is typically comprised between 50 and 90 wt%. Since the common solvent of the two coexisting, immiscible phases is water, we here refer to these droplet suspensions as "water-in-water", "all-in-water" or "all-aqueous" emulsions, regardless of the driving force or constituents of the droplets and continuous phase [79].

3. sequestration phenomenon in all-aqueous emulsions

As for conventional oil/water phase separation, any solute added to W/W emulsions may phase partition in one or the other phase (or both) based on chemical potentials. When the solute mostly partitions within droplets, the term 'sequestration' is used rather than 'encapsulation' since the solute can still freely exchange between the droplets and the continuous phase (there is no physical barrier that restrict solute diffusion). This sequestration phenomenon has led Oparin to suggest that coacervate droplets may have played a role as protocells, the first primitive cell-like compartments that appeared on the early Earth [82]. According to this hypothesis, prebiotic ingredients would have been sequestered within droplets, and their local accumulation could have favored reactions to produce more and more complex or evolved cells. Aqueous two-phase systems and coacervates have also gained strong interest in recent years to build artificial or synthetic cells [83,84]. Here again, the sequestration phenomenon provides a simple means to concentrate ingredients of interest within droplets. Depending on the chemical composition of the droplets, uptake of small molecular dyes, proteins, enzymes, DNA but also cell-free expression systems has been demonstrated [82,83,86,87,109–111]. The sequestration phenomenon is easily observed when using fluorescent dyes or fluorescently labelled solutes by visual inspection, epifluorescence or confocal microscopy. Direct visualization of fluorescence within droplets allows determining whether the solute is sequestered or not (Fig. 5). Taking advantage that W/W emulsions are not stable since droplets coalesce with time, macroscopic phase separation can also be exploited to separate and recover the two phases and quantitatively analyze solute partitioning using UV/vis of fluorescence spectroscopy.

While the uptake or exclusion of a component from droplets is still a largely empirical phenomenon, several parameters such as the net charge of the solutes but also the solution pH can affect partitioning and result in predictable trends for solute sequestration. For instance, studies showed that uncharged and positively charged dyes were sequestered within fatty acid (negatively charged) based droplets, whereas negatively charged dyes were excluded (Fig. 5) [109], in other studies, proteins were shown to

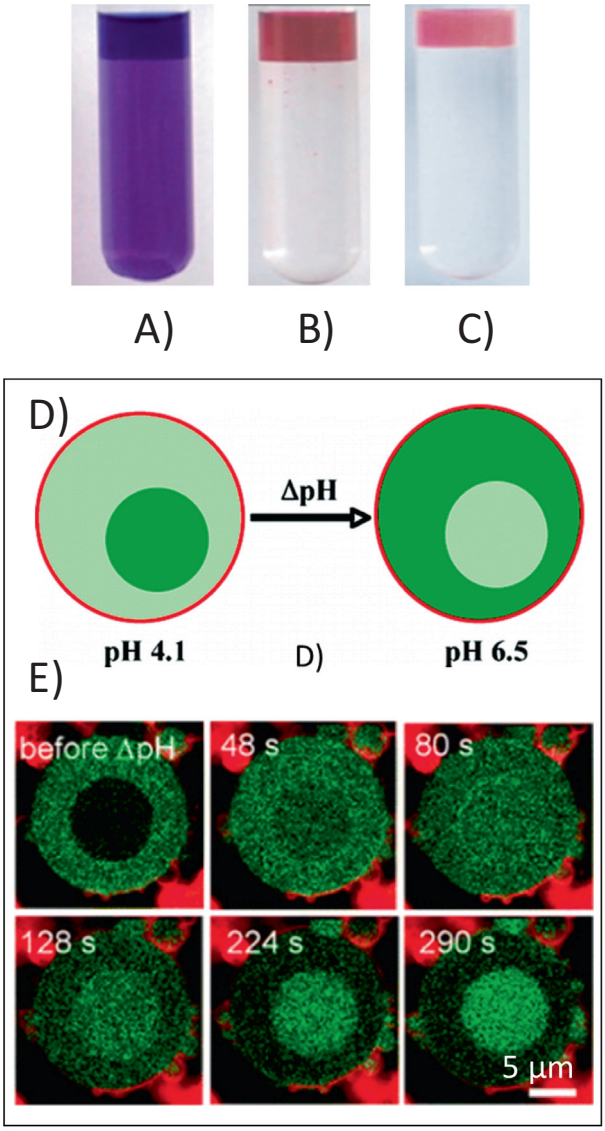

Fig. 5. (adapted with permission from ref [109,112]). Sample tubes photos of fatty acid based coacervates mixed with A) anionic dye (calcein), B) cationic dye (basic fuchsin) and C) uncharged dye (rhodamin B) after phase separation occurred. D) Schematic representation and E) experimental evidence via confocal microscopy of the change of protein sequestration upon varying the pH in giant lipid vesicles encapsulating PEG/dextran aqueous two phase system.

move from one phase to the other upon varying the pH [112] (Fig. 5). We also suggest that the sequestration phenomenon could be 'tuned' by using additives, similar to what has been done in solvent/water systems: for instance, hydrophobically modified hyperbranched polyethylene imine (HbPEI) dispersed in chloroform allowed uptake of hydrophilic dyes from water.[113] Using such polymers to form complex coacervates, or at least, doping coacervates with HbPEI could allow the uptake of chemicals that were not initially sequestered within droplets.

4. stabilization of W/W emulsions

All-in-water emulsions prepared by liquid–liquid phase separation typically contain highly polydisperse droplets with sizes ranging between 1 and 100 μm, as observed by optical microscopy [79]. In general, droplets can be seen to coalesce under the microscope, which ultimately results – in a few minutes up to a few hours – in macroscopic phase separation clearly visible in bulk with the formation of two distinct phases upon resting. The construction of capsules using all-aqueous droplets as templates therefore requires that coalescence is prevented. Yet, and unlike conventional O/W or W/O emulsions, the stabilization of W/W emulsion droplets is far from trivial for two main reasons: (i) the surface tension of water/water interfaces is significantly lower (few μN/m) than that of water/oil interfaces (∼mN/m) [114], and (ii) water/water interfaces are very diffuse, with typical thicknesses of a few tens of nanometers [115]. Conventional surfactants used to stabilize O/W emulsions therefore turn out inefficient in adsorbing at water/water interfaces, so that they do not allow stabilization of all-aqueous emulsions. Different alternative strategies have thus been developed in recent years to stabilize W/W droplets, mostly via the use of large objects – typically with a size similar to that of the diffuse interface – to produce Pickering-like emulsions, but also via the rational design of polymers able to adsorb at such interfaces. We detail below examples of stabilization approaches.

4.1. Pickering-like all-aqueous emulsions

The first work reporting the stabilization of W/W emulsions used protein, fat or quartz particles to stabilize dextran and methyl cellulose and/or maltodextrin-based W/W emulsions. Particles were shown to adsorb at the W/W interface and could be aggregated by different ways to form Pickering W/W emulsions [116]. Recently, in the same way, amine-modified commercial latex beads were shown to adsorb on dextran-rich droplets suspended in a PEG-rich continuous phase [117], an observation that was also extended to polydopamine-based particles [118] or other particles (Fig. 6) [119]. Small unilamellar vesicles [120–122], lipid corpuscle [123], together with cellulose nanocristals [124,125], protein particles and nanofibrils [126,127], yeast cellular wall fragments [128] and spiky particles [129] were also shown to adsorb at all-aqueous droplets' interface to produce Pickering-like W/W emulsions (Fig. 6).

The underlying mechanism that account for the adsorption of such objects at water/water interfaces remains yet elusive, so that this phenomenon is still largely empirical. As commented above, the low surface tension in such systems does not allow supposing that the presence of particles at the interface affords a gain of free energy by decreasing the interfacial energy, as in the case of W and O emulsions. The rational design of particles could help deciphering general rules for their adsorption at all-aqueous interfaces, maybe affording a unified description of this phenomenon. For instance, the construction of Janus particles with the two sides showing a differential affinity for the droplets and continuous phase would be excellent candidates for stabilizing such emulsions, but this has not yet been reported to date. By analogy with Janus particles synthesized from O/W emulsions after they have been immobilized and chemically modified [130,131], we also believe that such Janus particles could be synthesized using W/W emulsions by using particles that spontaneously adsorb on it, and adding a chemical partitioned in one or the other phase able to react with these particles.

An important contribution to the stabilization (and the understanding) of W/W emulsions upon addition of particles has been made by the group of Nicolaï, Benyahia and collaborators who developed various experimental and theoretical evidences of this phenomenon [115,124,132–140]. For instance, pH-responsive polymeric microgels were shown to adsorb at PEG/dextran interface and stabilize the dextran-rich droplets depending on the pH (Fig. 7) [115]. These results highlight that the surface properties of the particles play a crucial role in the stabilization of W/W emulsion. Moreover, emulsion stability could be controlled on demand by using the same particles. A similar feature has been observed

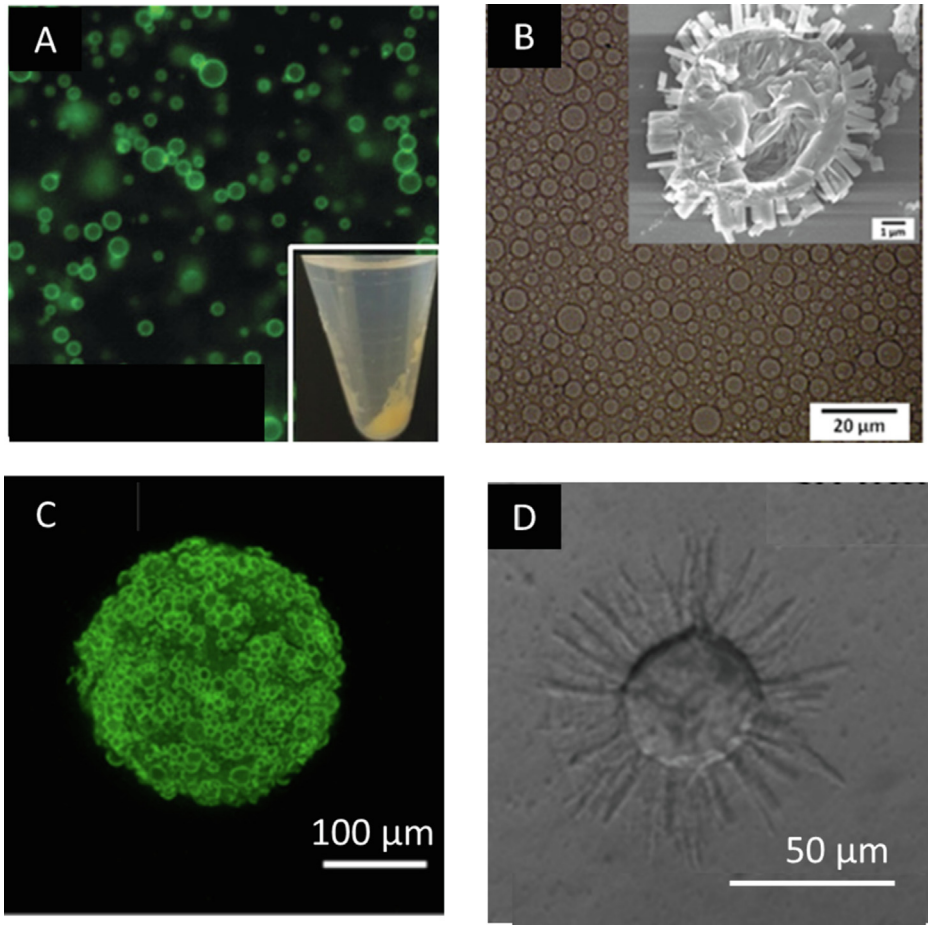

Fig. 6. (adapted with permission from ref [117–119,129]) A) Confocal images of Pickering W/W emulsions stabilized by latex beads (insert: sample tube photo of the Pickering emulsion after sedimentation); B) Optical microscopic image of dextran-in-PEG emulsion (insert: cryo-SEM image of Polydopamine Particles at the W/W interface); C) Projection image characterizing the surface of the blastosomes stabilized by fluorescent PS-COOH particles, D) All aqueous droplet stabilized by spiky particles.

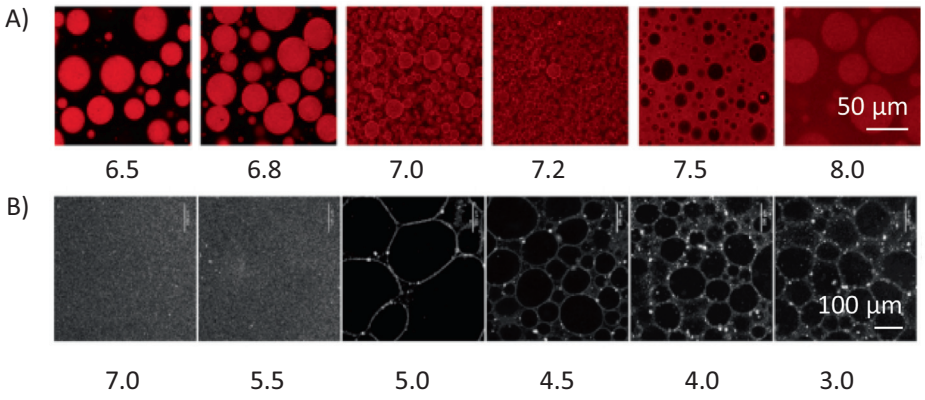

Fig. 7. (adapted with permission from ref [115,141]) A) Images PEO-in-dextran emulsions stabilized by microgels at different pH values. B) Images of W/W emulsion (xyloglucan / amylopectin) at different pH in the presence of fluorescently labeled β-lactoglobulin microgels after 24 h.

more recently with diblock copolymer-based microgels, affording temperature dependent stabilization of W/W emulsions (Fig. 7) [141].

4.2. polymer adsorption at water/water interfaces

Beyond nano- and micro-particles, single polymer chains have been reported to be able to adsorb and self-assemble at water/water interfaces, and in turn stabilize W/W emulsions. An elegant approach to drive the adsorption of a polymer at water/water interfaces is to rationally design it so that a part of it interacts with the inner droplet components whereas another part interacts with the outer continuous phase. This has been nicely achieved using a triblock copolymer that spontaneously self-assembles at the interface of dextran-rich droplets suspended in PEG [142]. The triblock copolymer was made of a central hydrophobic region flanked by two different hydrophilic moieties that exhibited affinity for either dextran or PEG so that they self-assembled at the PEG/dextran

interface, forming a polymer layer, i.e., a polymersome-like structure having a dextran-rich lumen embedded in a PEG-rich continuous phase. Such a strategy was also applied to stabilize complex coacervates, still using triblock copolymers [143].

Another approach is to use polymers that can interact electrostatically with the inner core of complex coacervate: in this case, the interaction should be not too strong so that the polymer remains at the droplet interface without destructing the coacervate droplet. We observed such a phenomenon when adding DNA to catanionic coacervates (Fig. 8) [96].

4.3. Lipid-based stabilization of W/W emulsions

Recent studies have also explored the use of self-assembling molecular amphiphiles, and in particular lipids, to adsorb at water/water interfaces. We do not include here all works reporting on aqueous phase separation occurring within lipid vesicles [146,147] but indeed, lipid membranes that really cover aqueous droplets. In a seminal example, a fatty acid, sodium oleate, was shown to self-assemble into bilayers at the surface of complex coacervate microdroplets, forming a lipid membrane [148]. Using lipids allows mimicking the membrane-bounded structure of living cells, and is attracting growing attention. Recent studies have indeed demonstrated the self-assembly of phospholipids at the surface of complex coacervates [144,145,149] (Fig. 8). The interactions driving the formation of lipid membranes at water/water interfaces remains yet elusive – it is unclear in particular how such small molecules, i.e., with a size much smaller than the interfacial thickness [115], can adsorb at these all-aqueous interfaces – so that further investigations are needed to better understand this phenomenon.

4.4. Diffusion ***across shells and membranes assembled on W/W droplets***

While all the systems discussed above appear very efficient to stabilize W/W emulsions, to date, the shells or membranes formed on such all-aqueous droplets are all still permeable to small –and in some cases large – molecules, so that passive diffusion of solutes through the membrane is observed. This diffusion limits the use of

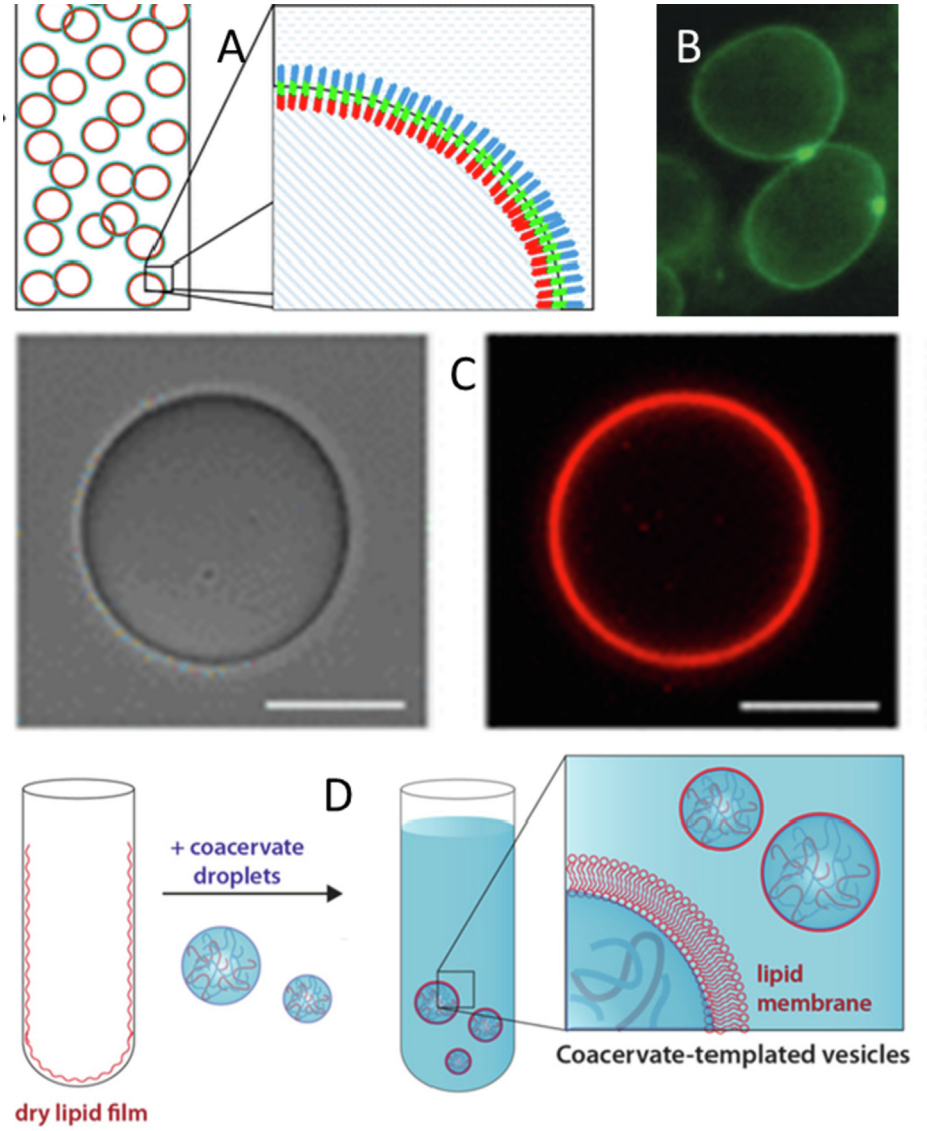

Fig. 8. (adapted with permission from ref [96,142,144,145]) A) Schematic representation of W/W emuslions stabilized by triblock copolymers; B) Capsules observed by epifluorescence and prepared from the addition of DNA (labelled with Sybr green) to catanionic coacervates. C) Giant vesicles formed by complex coacervates surrounded by a phospholipid membrane (left: optical image, right: confocal image) and D) hydration of a dry lipid film using a complex coacervates dispersion to form giant vesicles.

such systems as cell-like micro-compartments since it precludes a fine control over chemical localization and exchange with the environment. Stronger efforts should be made to restrict diffusion of solutes across the membrane and better control its permeability.

5. building capsules from W/W emulsions

The stabilization of W/W emulsions against macroscopic phase separation via adsorption of particles or molecules (polymers and lipids) does not prevent droplet dissolution (for instance upon dilution), so that droplet disassembly drives the destruction of the shell and no hollow sphere is retained. The formation of robust capsules requires the adsorbed objects to be cross-linked to maintain the shell integrity when the droplets are disassembled. Similarly to W/O and O/W emulsions, the term 'demulsification' has been used for aqueous two-phase systems, e.g., PEG/dextran solutions, to refer to the disassembly of W/W emulsions due to dilution below the critical polymer concentration required to have phase separation [118]. For complex coacervates, held together by electrostatic interactions, changes in pH or ionic strength, in addition to dilution, can also induce demulsification. Several strategies have been developed to produce micro-capsules before demulsification of W/W emulsions.

5.1. colloidosomes templated by W/W emulsions

Colloidosomes offer new opportunities for applications such as microencapsulation. For conventional W/O or O/W Pickering emulsions, a large set of methods has been developed to produce colloidosomes from Pickering emulsions [19], e.g. via particles cross-linking. However, the encapsulation of small chemicals is usually limited to short timescales because the particles shell is very permeable [19], due to the large interstitial pores produced between the adsorbed particles. Although a second shell or impermeable polymer layer can be deposited at the surface of such capsules to prevent fast release of small chemicals, the use of phase-separated all-aqueous droplets is emerging as an exciting alternative to produce colloidosomes able to retain such small chemicals by sequestration, and prevent their fast release.

Interestingly, the approaches developed to build colloidosomes from W/O or O/W systems can be adapted to W/W Pickering emulsions. This was first shown in an ATPS using fat-based particles to stabilize the emulsion, where particles were aggregated at the droplet interface to form microcapsules [116]. Other kinds of particles have also been used to produce Pickering water-in-water emulsions but were not converted into robust colloidosomes (see above) [115,124,132,135,136,139,150–152]. Amine-modified latex beads were shown to spontaneously adsorb at the interface of dextran-rich droplets suspended in a PEG continuous phase [117]. The authors attempted to cross-link the beads via amide bonds by using carbodiimide chemistry in the presence of polyacrylic acid (PAA), but observed instead that carboxylate groups on PAA were esterified with hydroxyl groups of sugars on dextran. Esterification reaction is usually unfavored in water due to hydrolysis [153], but it was suggested that the high concentrations of both polymers (PAA and dextran) within droplets favored the reaction in this case. This reaction eventually produced a dextran/PAA microgel, on which latex beads were also cross-linked (via amide bonds) concomitantly to the esterification of PAA and dextran. The resulting colloidosomes exhibited reversible swelling behavior upon dilution (Fig. 9) and were able to uptake and release macromolecules.

Other colloidosomes have been obtained using PEG/dextran ATPS as templates and dopamine-based or $CaCO_3$ particles to produce a Pickering water-in-water emulsion. The dopamine-based particles were successfully cross-linked by using both PAA and carbodiimide chemistry to produce colloidosomes (Fig. 9) [118], without forming a gel inside the droplets (in contrast to the above-mentioned study). $CaCO_3$ particles were formed *in situ* at the surface of dextran-in-PEG droplets and locked-in through enzyme-driven biomineralization to form hard-shell colloidosomes [154]. In the same way, $CaCO_3$ particles also formed Pickering emulsions when combined with a thermoresponsive and degradable polymer-forming coacervate, which were further transformed into colloidosomes upon calcium carbonate crystallization [155].

5.2. Coacervate-to-capsule transition

Other approaches have also emerged to produce capsules from all-in-water emulsions. Instead of covering the aqueous droplets with particles or other shell-forming components, a morphological transition from droplets to capsules can be induced by varying a physicochemical parameter or adding a chemical. Such a structural reorganization was first reported by the group of Mann [156] starting from complex coacervates assembled from poly(diallyldimethylammonium chloride (PDDA) and adenosine triphosphate (ATP) (Fig. 10). Addition of phosphotungstate (PTA) clusters to such coacervate microdroplets induced a structural change to capsules consisting of a double shell of PDDA/PTA and PDDA/ATP delimiting a polymer-free, dilute aqueous lumen. The stronger interaction of PTA with PDDA compared to ATP induced the formation of a semi-permeable shell, resulting in the osmotically-driven diffusion of water molecules in the coacervate droplet, and formation of a water-rich lumen. This transition from a molecularly crowded environment (coacervates) to a yolk-shell structure was further exploited to produce protocells with catalase-like activity by combining PTA and ruthenium-based polyoxometalate [157]. We anticipate that this type of structural transformation could also be induced using other chemicals, such as negatively charged polymers, but further studies are required.

A transition from coacervates to vesicles has also been observed in fatty acid (FA)-based systems upon decreasing the pH. This feature may be relevant in the context of prebiotic chemistry since fatty acids are the simplest amphiphiles capable of self-assembling into vesicles [158–161]. As discussed above, sodium myristate can form coacervates using an excess of guanidinium cations. In another paper [162], it has been shown that such a FA can self-assemble into vesicles at lower pH (7.5–9) so that transitions from coacervates to vesicles could be performed upon varying the pH. This has been achieved recently [163], which allowed the encapsulation of proteins within the so-formed FA vesicles (Fig. 10). A fluorescent protein and an enzyme were shown to be spontaneously sequestered within FA-enriched coacervates. Upon decreasing the pH, the FA-filled droplets turned to membranous hollow vesicles encapsulating the pre-concentrated proteins. In the same way, vesicles initially lacking any cargos in their lumen were transformed into coacervates that sequester proteins, which were further transformed into vesicles that now encapsulated proteins in their lumen.

We also observed such a transition from droplets to capsule-like structures in an ATPS based on gelatin and PEG [164]. This binary system can form gelatin-enriched droplets within a PEG-enriched continuous phase above the melting transition of gelatin (~45 °C) [165–168]. The droplets were shown to self-aggregate upon cooling. However, when alginate was added to the initial hot mixture, aggregation no longer occurred when the temperature was decreased [164]. Rather, isolated gelatin-rich droplets were observed and were shown to entrap smaller PEG droplets in their core, forming a PEG-in-gelatin-in-PEG double emulsion. When the sample was maintained at a temperature above the melting transition of the protein (before cooling), the smaller inner PEG

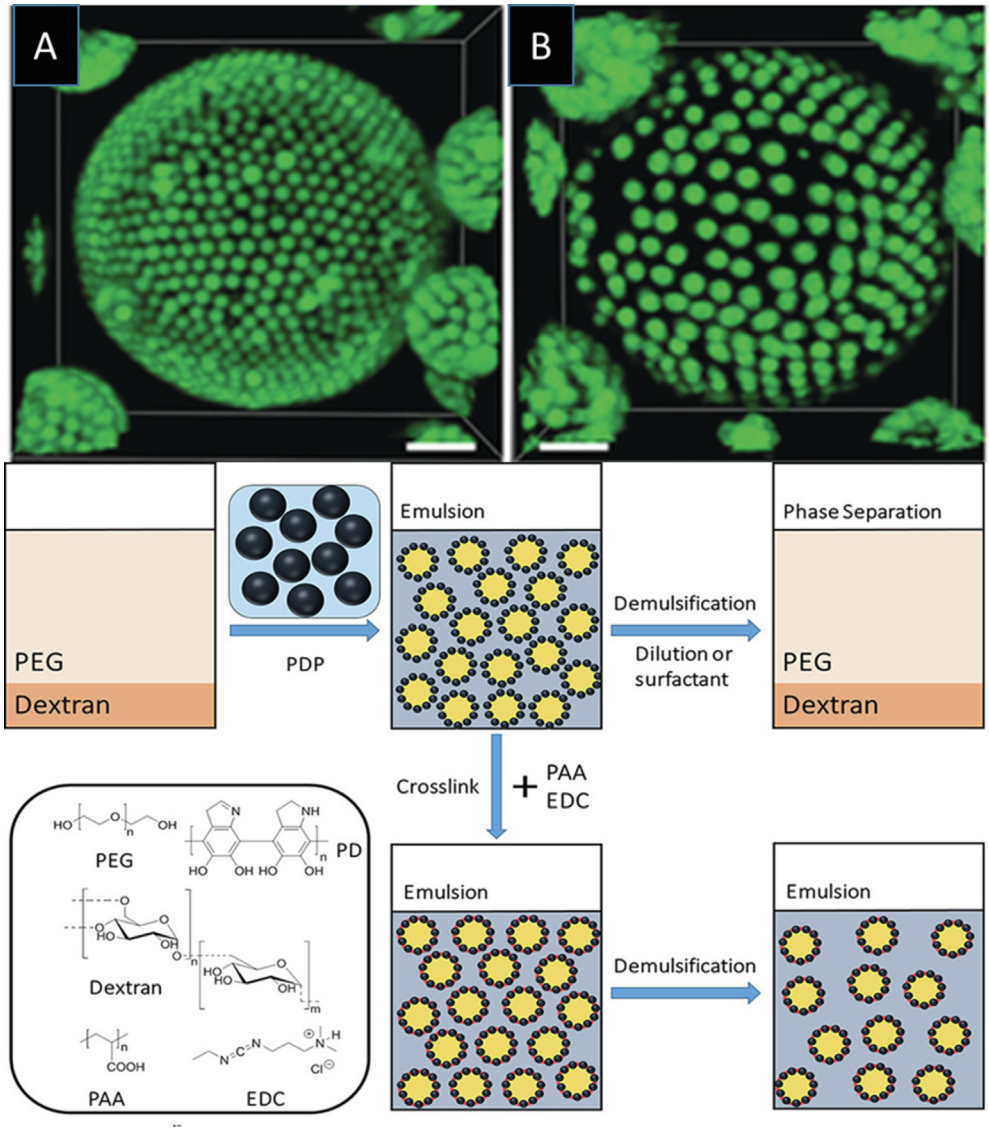

Fig. 9. (adapted with permission from ref [117,118]) Confocal images of hybrid hydrogel-colloidosomes in the pristine (A) and swollen (B) forms. Lower panel: process used for the production of colloidosomes using polydopamine-based microparticles (PDP).

droplets were shown to coalesce within the main gelatin-rich droplets, forming a lumen so that hollow spheres having a gelatin shell that gelled upon cooling were produced (Fig. 10).

6. emerging directions for the production of polyelectrolyte capsules templated by W/W emulsions.

6.1. W/W droplets and capsules using microfluidics

The W/W emulsion-templated construction of capsules discussed above typically produces polydisperse compartments that replicate the polydispersity of the template emulsion. Approaches to prepare uniformly-sized capsules are also being investigated, mostly using droplet-based microfluidics. Microfluidics allows the high-throughput production of monodisperse microdroplets suspended in a continuous phase, and has been initially developed for preparing water-in-oil droplets [169–171]. The so-formed emulsion droplets have been used to produce capsules using similar chemical strategies as those developed in bulk [76,172].

Excitingly, droplet-based microfluidics has recently started being used to produce water-in-water emulsions without the need of oil. For instance, for a PEG/dextran ATPS, monodisperse dextran-rich droplets can be produced by using a PEG solution as the continuous phase. Yet, due to the very low surface tension of W/W emulsions, the different settings, such as the flow rates, and the set-up have to be adapted to produce droplets. For example, a piezoelectric field or pulsating inlet pressures (Fig. 11) have been used to force the production of droplets [173,174], while other approaches have relied on the use of passive droplet generation, choppers or glass microcapillaries [169,173,175–181]. Coacervates were also formed exploiting microfluidic flow-focusing system. Droplets resulting from the interaction of poly(diallyldimethylammonium chloride) with either adenosine triphosphate or carboxymethyl-dextran exhibit a narrower size distributions and a higher stability compared to the conventional fabrication techniques [177].

Once monodisperse W/W droplets are produced, capsules can be easily prepared, e.g. by interfacial polymer complexation, particular cross-linking or other methods. In a seminal study, polyelectrolyte microcapsules were produced by dispersing a positively charged polymer in dextran and a negatively charged polymer in the continuous PEG phase [182]. When producing dex-

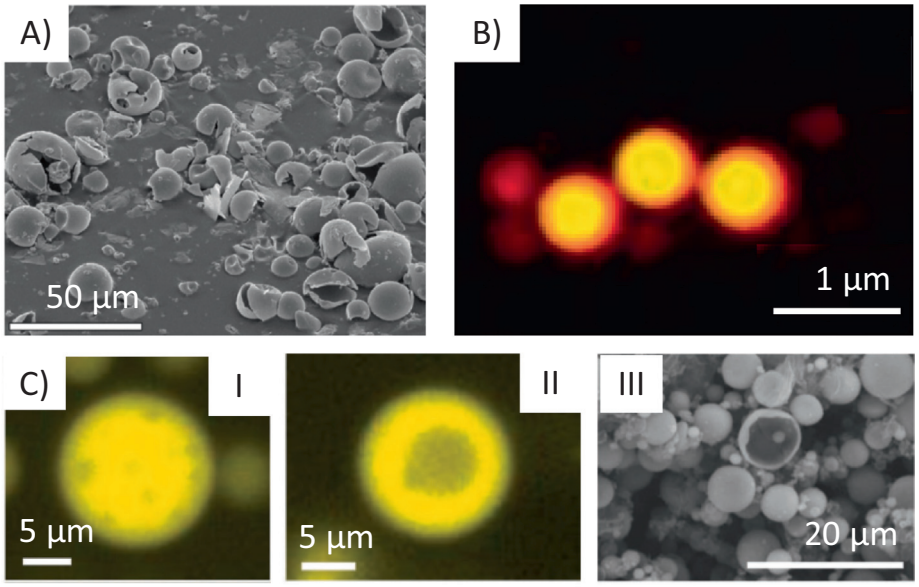

Fig. 10. (Adapted with permission from [156,163,164]) A) SEM image of dried PCVs spherical polyoxometalate coacervate vesicles; B) Epifluorescence images showing vesicles obtained from fatty acid-based coacervates upon decreasing the pH. Yellow patch stands for encapsulated YFP and Nile Red allows delineating the fatty acid bilayer; C) Epifluorescence images (I/II) of PEG/gelatin dispersion (labeled with acridine orange) containing 0.5 and 0.8 mg mL^{-1} alginate, respectively. III) SEM images of yolk–shell particles after deposition of a silica layer.

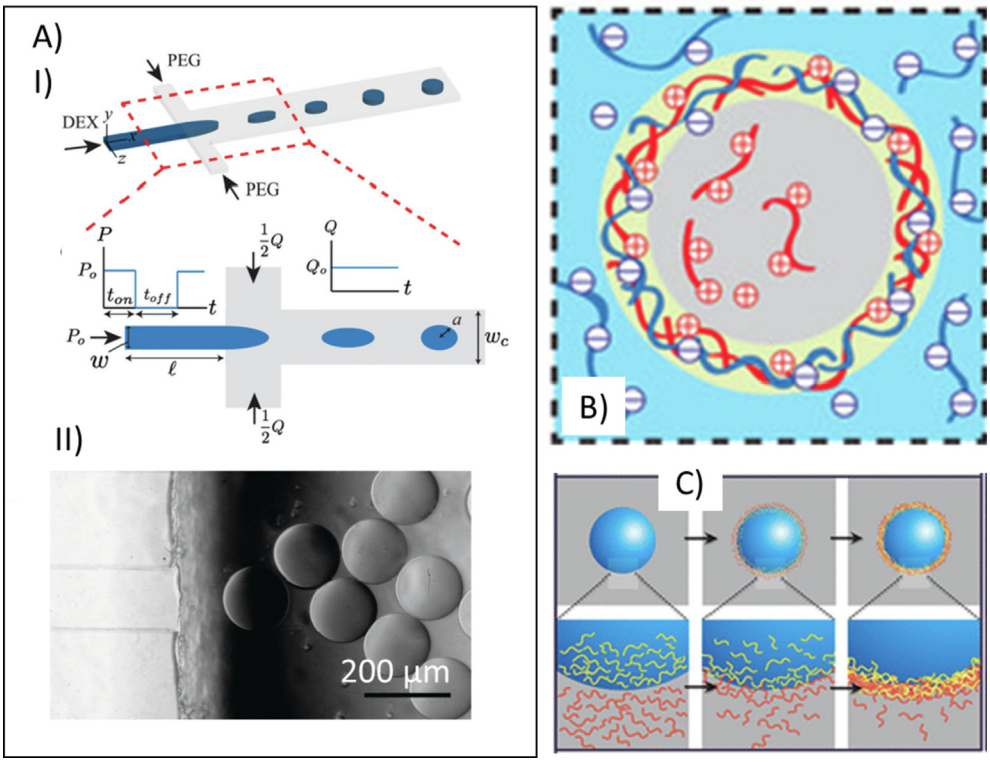

Fig. 11. (Adapted with permission from ref [173,182,186]) A) W/W droplets formed with perturbations generated by a solenoid valve, which acts on the inlet pressure. I) Schematic representation of the process, II) Optical images of the W/W droplets. B) polyelectrolyte capsules obtained by microfluidic formation of Dex-in-PEG-in-PEG double emulsion. The positively charged polymer present in the dextran phase and the negatively charged polymer present in the PEG outer phase meet in the intermediate PEG phase where they strongly interact to form a robust shell. C) polyelectrolyte capsules obtained by a similar approach using the electrospray method. Here, no intermediate PEG phase is required but charged polymers also meet at the interface to form a robust shell.

tran droplets, clogging occurred in the microfluidic channel because of the fast complexation between charged polymers at the droplets' interface. This problem was elegantly circumvented by adding an additional PEG channel, free of any charged polymers, to coat the dextran-rich droplets. Therefore, dextran-in-PEG-in-PEG double emulsion droplets were formed, where the intermediate PEG layer ensured slow mixing of oppositely charged polymers and their complexation to form a polyelectrolyte shell (Fig. 11). The

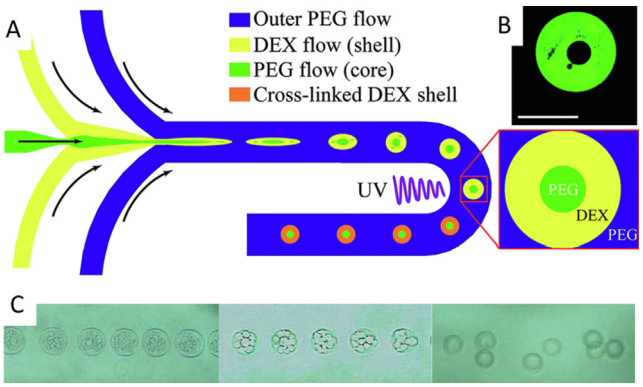

Fig. 12. (Adapted with permission from ref [190,191]) A) Schematic representation of the microfluidic device used to produce PEG-in-Dex-in-PEG double emulsion droplets. The use of acrylate-dextran derivatives allows cross-linking the intermediate dextran shell upon UV exposure. B) Confocal image of a capsule obtained by this way. C) Osmose-induced formation of multiple emulsion droplets (2 panels left) obtained by microfluidics that further evolve along the microfluidic device to yolk-shell double emulsion droplets (right panel).

need of such an intermediate layer was confirmed in another recent study [68]. Similar findings were obtained using the electrospray method [183] instead of microfluidics. In this case, monodisperse droplets and capsules have also been produced via interfacial complexation (Fig. 11) [184–189]. However, up to now, no any polyelectrolyte-based microcapsules have yet been produced in bulk using W/W emulsions.

6.2. multiphase emulsions to prepare capsules

Recent studies exploited the possibility of forming multiple all-aqueous emulsions to prepare micro-capsules. For instance, microfluidic production of PEG-in-dextran-in-PEG double emulsion droplets, coupled to the use of reactants (thiol-yne reactions) in the dextran intermediate shell, allowed preparation of microcapsules with a gelled dextran layer (Fig. 12) [190]. An interesting way to prepare all-aqueous double emulsions is to play with osmotic pressure when producing a single W/W emulsion. In an example, a low concentration of PEG was initially mixed with dextran so that no phase separation occurred. When droplets of such a mixture were produced by microfluidics using a highly concentrated PEG outer continuous phase, variation of osmotic pressure induced a phase separation (PEG-in-dextran) within the droplets to produce a PEG-in-dextran-in-PEG double emulsion (Fig. 12) [191]. Such a strategy was further extended to higher orders since multiple emulsions were produced by this way [192]. However, only double emulsions were produced but no capsules, up to now. Obviously, the formulation of W/W/W double emulsions via microfluidics opens promising perspectives and a nice way to build capsules in oil-free solutions.

Strategies for the preparation of W/W/W emulsions in bulk are also being developed. Elegant examples include multiphase complex coacervates (Fig. 13) [193–197], produced by mixing 2 polycations together with 2 polyanions in water to form all-aqueous droplets-in-droplets in an aqueous continuous phase. However, again, multiple emulsions were produced but no any capsules. This approach could pave the way to the construction of capsules where the intermediate aqueous layer would be transformed into a robust shell (e.g., upon gelation or chemical reaction).

6.3. 3D constructs from W/W emulsions

Another coming direction, although it is beyond the scope of the production of capsules, is the preparation of 3D constructs by using ATPS [198,199]. Typically, instead of building droplets, the method consists of forming tubules by using a 3D printer that can deposit a concentrated polymer within another incompatible polymer-rich continuous phase, e.g. dextran in PEG. Again, the interface needs to be stabilized by polyelectrolytes or hydrogen bonds to prevent complete dissolution of both polymers or fast deformation of tubular structures. These tubular construct could even be produced by microfluidics [200] (under flow conditions that do not form droplets) and be used for preparing non-spherical but elongated capsules or other materials, still with the capacity of sequestrating or encapsulating cargos.

6.4. adding oil to W/W emulsions

While W/W emulsions are particularly attractive because compartmentalization can be induced without using nonpolar solvents, addition of oil to such emulsions may also be of interest for microencapsulation applications, e.g., to encapsulate both hydrophilic and lipophilic cargos. This has been mainly explored by the group of P. Erni et R. De Vries [201–203] and consists of forming O/W/W droplets [17,204–206]. Oil is emulsified in complex coacervates so that oil-in-coacervate droplets can form in a continuous aqueous phase. The intermediate complex coacervate layer is further cross-linked and eventually biomineralized using silica precursors to produce hybrid capsules. There are other open doors in this direction since double emulsions are of practical interest

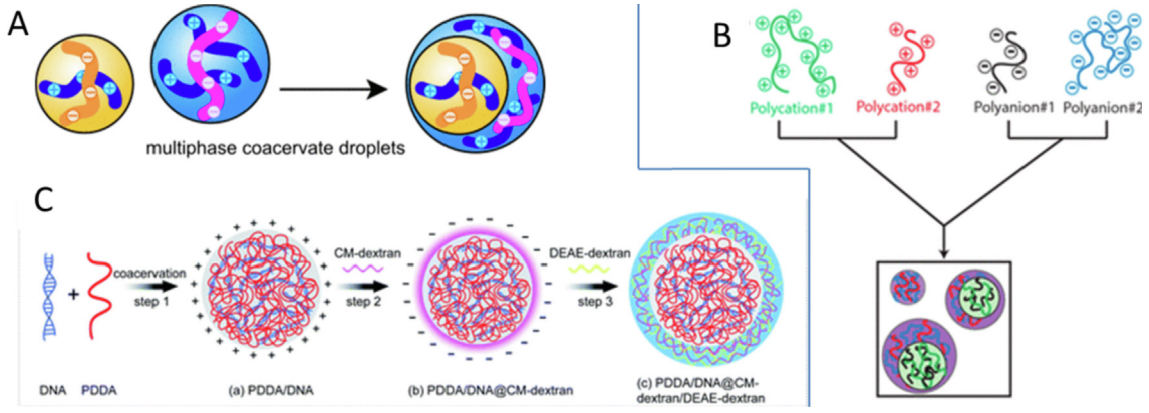

Fig. 13. (Adapted with permission from ref [193–195]) Examples of the production of multiphase complex coacervates yielding double emulsion droplets, A) mixing 2 preformed complex coacervates prepared with 2 different charged polymers, B) mixing 4 different charged polymers and C) step-by-step formation upon sequential addition of charged polymers.

for various applications [207–209] and have been widely used recently for the production of liposomes and polymersomes or other kind of capsules [75,210,211]. Out of this context but of interest, production of W/W/O droplets has been largely investigated [167,168,212–216] and can afford capsules of various forms, thanks to the aqueous phase separation occurring within droplets embedded in oil [217].

7. conclusions and outlooks

Interest in W/W emulsion droplets has been reignited a decade ago [83] in the field of protocells, artificial and synthetic cells. Yet, beyond these bio-inspired systems, these emulsions are also very attractive as templates in materials chemistry for the preparation of microcapsules. The key advantage of such all-aqueous emulsions is their ability to spontaneously sequester cargos, which provides a simple mechanism to accumulate species before the assembly of a robust shell. Interestingly, it is also possible to first form a permeable shell at the surface of droplets, to prevent their coarsening, then upload cargos via spontaneous partitioning and, ultimately, make the initial shell impermeable.

As discussed above, the sequestration phenomenon is still largely empirical and should be better investigated to predict whether a cargo will be sequestered or not in aqueous droplets. Similarly, the stabilization of such droplets to prevent their coalescence should be studied more in detail to predict whether a chemical or a particle will adsorb at the droplet interface or not. In addition, while many studies have developed strategies to prepare W/W emulsions-based microcapsules, the formation of submicrometric capsules using W/W emulsions is still unexplored to our knowledge. This domain of lower sizes may be of higher interest for pharmaceutical, cosmetic and food applications. As for the preparation of O/W emulsions, high shearing or sonication methods could be employed for forming small W/W emulsions to be transformed into few tens or hundreds nanometers capsules.

Still copying what has been done in the domain of O/W or W/O emulsions, the preparation of high internal phase W/W emulsions (HIPEs) should also be possible. In O/W emulsions, HIPEs are obtained when a high amount of oil is added with respect to water. Instead of inducing a phase inversion that would yield a W/O emulsion, O/W droplets still form and are in close contact, often forming a gel [5,218,219]. In an ATPS made of PEG and dextran, varying the amounts of both polymers (and/or their molecular weight) may yield dextran-in-PEG or PEG-in-dextran droplets, depending on the phase diagram. In other words, addition of dextran with dextran-in-PEG droplets induces a transition to PEG-in-dextran droplets. However, if one add a stabilizing agent that force the initial curvature, i.e., the formation of dextran droplets, then, dextran-in-PEG HIPEs should form for high amounts of dextran.

Overall, preparing capsules from W/W emulsions has gained interest in recent years, and open exciting perspectives for the years to come. Although studies on W/W emulsions have been reignited for being used as protocells, organelles [90] or other cellular bodies [220], it is obvious that their ease of preparation, the large palette of components they can be assembled from for their production and the potential low cost of chemicals to be used make them of practical importance in materials chemistry. Many works have been done by using microfluidics but this could also be developed in bulk affording higher amounts of final materials. A lot has also been done recently for stabilizing these emulsions, preventing coalescence of droplets, but these were not always converted into robust capsules. Obviously, the phenomenon of spontaneous sequestration of cargos in such aqueous droplets should be the best motivating focus for using such emulsions as templates for building capsules that can efficiently encapsulate solutes of various interests.

CRediT authorship contribution statement

Adeline Perro: Conceptualization, Writing – original draft, Project administration. **Noëmie Coudon:** Conceptualization, Writing – review & editing. **Jean-Paul Chapel:** Conceptualization, Writing – review & editing. **Nicolas Martin:** Conceptualization, Writing – original draft, Project administration, Funding acquisition. **Laure Béven:** Conceptualization, Writing – review & editing. **Jean-Paul Douliez:** Conceptualization, Supervision, Writing – original draft, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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