Coacervate-Based Protocells: Integration of Life-Like Properties in a Droplet

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ABSTRACT

The formation of compartments with life-like properties from abiotic origins is one of the most drastic, yet least-understood transitions in the emergence of cellular life. Similarities between the protoplasm of modern cells and liquid droplets formed by condensation of oppositely charged macromolecules led Oparin to postulate in the 1930s that primitive protocells might have formed by coacervation. However, in order to proliferate, protocells must be stable, preserve their identity, and be capable of fuelled growth and division. At the time, it was not clear how passive coacervate droplets made from large polymers could fulfil these requirements. Recent advances in systems chemistry and physics of active droplets have revived the idea that various life-like properties could be integrated in a single droplet. The challenges on the way for coacervates to become viable protocells are being addressed one by one, and coacervate systems with increasing complexity and emergent properties are being developed. Here, we review the recent developments aimed at providing coacervates with the characteristics required to become self-sustained. Our discussion includes the incorporation of genetic material in coacervates, their stabilization by interfacial membrane assembly, and ways to achieve growth and division by fuelled reactions. From a distance, coacervates may be far from the complexity of modern cells, but they represent a promising systems chemistry approach to create protocells with properties beyond the sum of their parts [1].

Keywords: coacervates, protocells, self-organization, active systems, droplets.

Introduction

Protocell research aims to design and build supramolecular assemblies comprising elements that are central to living cells [2–8]. Ultimately, a complete and self-sustained protocell should combine all the essential ingredients for a cell to proliferate, and could resemble the first generation of cells on Earth. These ingredients include a stable compartment, a way to transduce energy to fuel metabolic reactions, processing of information, growth and division, and adaptation to changing conditions (*Figure 1*) [7]. Many different protocell models are currently being investigated, examples of which include lipid and polymer vesicles [9], polyelectrolyte microcapsules [10], water-in-oil emulsions [11], colloidosomes [12], proteinosomes [13] and coacervates [14] (*Figure 2*). All of these models mimic the compartmentalization of reactions and molecules we see in cells today, albeit in different ways. Lipid and polymer vesicles, microcapsules and proteinosomes form

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compartments that are separated from their environment by a self-assembled layer of molecules, whereas water-in-oil emulsions, most colloidosomes and coacervates are phase separated from their respective solutions when they are first formed. This phase separation allows for spontaneous concentration of relevant chemical building blocks *via* a partitioning equilibrium.

The advantage of coacervates over other phase-separated systems, is that coacervates contain the same solvent (usually water) both inside and outside the compartment. Coacervates are droplets enriched in one or more solute species, such as polymers, proteins or nucleic acids, that are formed by liquid-liquid phase separation (LLPS) [15]. The phase separation is often driven by associative interactions between molecules of a single type (simple coacervates), or between multiple interacting species that form a complex together (complex coacervates). Upon complexation, condensed droplets are formed that are highly concentrated in the associated molecules. These droplets are in equilibrium with the surrounding medium and they usually coalesce readily for lack of a surrounding membrane. A wide variety of molecules have been used to create coacervates *in vitro*, including synthetic polyelectrolytes, polysaccharides, peptides, proteins and (poly)nucleotides [16, 17]. The best-known example is complex coacervation of two oppositely charged macromolecules, such as polylysine and RNA.

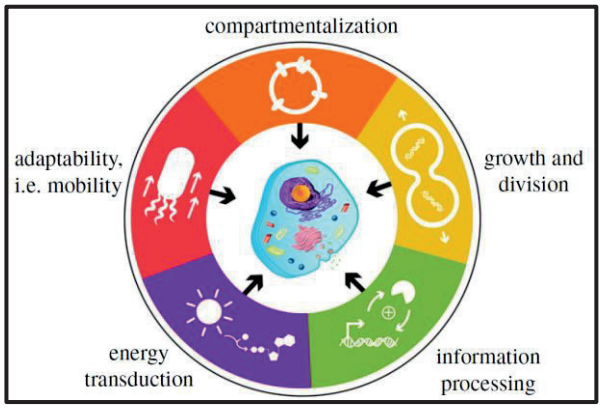

Figure 1. Hallmarks of living systems. Reprinted from [7].

Coacervation was first observed in the early 20th century [18–20], and the term was coined when the underlying mechanism was described several years later by *Bungenberg-de Jong* and *Kruyt* [21]. *Oparin* was inspired by this phenomenon and linked the physicochemical properties of coacervates to the protoplasm of modern cells, and reasoned that primitive cells could have formed by coacervation [14]. In a primordial soup of molecules, small droplets enriched in some of those molecules could have spontaneously formed and sequestered other essential compounds without the need of an enclosing membrane. The elevated concentrations and crowded interior of coacervates could lead to stronger binding and enhanced rates of chemical reactions [22], ultimately resulting in the evolution of a self-sustained protocell. *Oparin*'s theory did not go without criticism. Coacervates generally lack specificity and take up most molecules in their proximity. This is the reason why they have been referred to as molecular 'garbage bags', capturing their most notable weakness, namely a lack of identity [23]. Additionally, the coacervates studied at the time consisted of long polyelectrolytes,

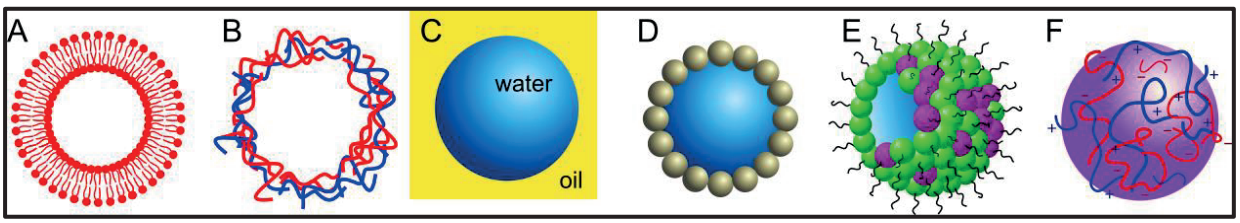

Figure 2. Schematic representation of different protocell models. *A*) Lipid vesicles, *B*) polyelectrolyte microcapsules, *C*) water-in-oil emulsions, *D*) water-dispersible colloidosomes based on silica nanoparticles (*grey*), *E*) proteinosomes made of BSA-NH_2/PNIPAAm (*green*) and GOx-NH_2/PNIPAAm (*purple*), and *F*) complex coacervate droplets.

which are considered too complex for prebiotic conditions. Finally, a clear mechanism by which coacervates could persist and reproduce has remained elusive, leaving them far from living systems.

Other protocell designs addressed some of these concerns and gained attention in the late 20th century. Particular focus was given to protocells based on amphiphilic vesicles encapsulating autocatalytic RNA molecules, as they resemble modern cells in important ways and provide a route to self-replication [9, 24, 25]. However, these and other protocell designs all have limitations [26], and the fact that coacervates can form spontaneously and concentrate solutes remains appealing. Recent findings of liquid-liquid phase separation of disordered proteins in cell biology, resulting in the formation of intracellular droplets that are highly similar to coacervates, have also sparked renewed interest in coacervates as protocells [27 – 31].

In the new generation of coacervate systems, several of the previous limitations have been addressed. Here, we discuss how these recent developments have improved coacervate systems as plausible protocells in which several life-like properties can be integrated [32]. We centre our discussion on four key aspects that are also part of the hallmarks of living systems (*Figure 1*). First of all, coacervate protocells should consist of simple, small molecules, and the compartments should remain stable. We will discuss how encapsulation in vesicles and interfacial assembly of membranes can help to achieve this. Secondly, coacervate protocells should contain a type of genetic material to provide them with a (genetic) identity and allow them to process information. We showcase recent work on RNA-based coacervates and ribozyme activity in coacervates, as an important step in this direction. Thirdly, coacervate protocells should have a way to transduce energy to fuel metabolic reactions. We present progress on integrating metabolic reactions in coacervates and creating out-of-equilibrium coacervate systems, in which coacervate formation or dissolution is fuelled by light or chemical reactions. The final requirement is that coacervate protocells must grow and divide in order to proliferate, and we discuss recent developments in this direction.

Simplicity and Stability

Coacervates have been studied extensively in colloid science, making use of long, often synthetic polyelectrolytes [16,17]. For a long time, their potential as protocells has not been investigated, because model systems based on simple and biologically relevant building blocks were missing. *Koga et al.* were the first to reintroduce coacervates as protocells, when they reported that complex coacervates could be formed from simple cationic peptides like polylysine (pLys) in combination with mononucleotides, such as adenosine tri-, di-, and monophosphate (ATP, ADP, and AMP, respectively) [33] These coacervate droplets were stable at different pH values ranging from 4 to 10 and temperatures up to 90°C, which shows their resistance to changes in the environment.

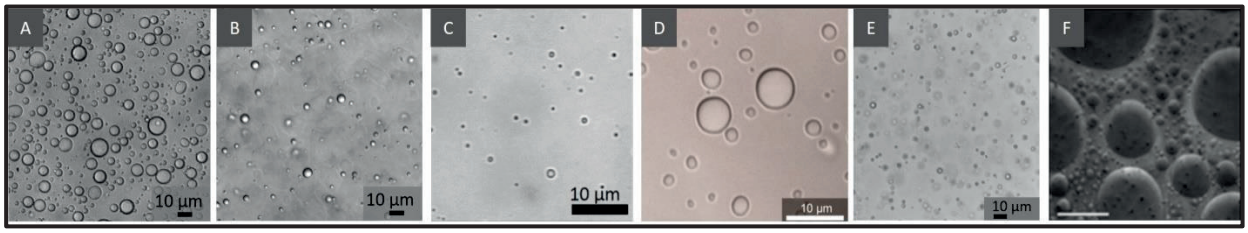

Figure 3. Different complex coacervates composed of one or more simple molecules have very similar appearance and properties. *A*) Peptide-nucleotide (pLys/ATP). *B*) Two oppositely charged peptides (poly-L-Lys/poly-D,L-Glu). *C*) Single peptide inspired by mussel foot protein-3S. (Reprinted with permission from [39]. Copyright (2016) *John Wiley and Sons*. *D*) Spermine/poly-U nucleic acid. (Reprinted with permission from [40]. Copyright (2016) American Chemical Society. *E*) Peptide/ferricyanide (pLys/$Fe(CN)_6^{3-}$). *F*) Catecholic zwitterionic gemini surfactants. (Reprinted from [42].)

Since then, this system has been used by many other groups, both as protocell model and as artificial organelle inside lipid vesicles [34, 35] In addition, several other coacervates with one or more simple constituents have been developed, including coacervates formed by two oppositely charged short peptides [36 – 38], coacervates formed by a single peptide derived from mussel foot protein [39], coacervates formed by spermine and oligonucleotides [40], coacervates formed by gemini surfactants [41,42], and coacervates formed by divalent ions, such as calcium and magnesium, and inorganic polyphosphates [43] (*Figure 3*).

Despite the large difference in size between the polyelectrolytes used originally and the smaller and less complex molecules used in recent coacervate protocell models, and despite the chemical and structural differences between each of the building blocks, the physicochemical properties of the resulting coacervates are surprisingly similar. Most complex coacervates contain a large amount of solvation water (usually between 50-90 wt%) [44, 45], take up a wide range of both hydrophobic and charged solutes by partitioning [33, 46], and are viscous liquids with a low surface tension [47 – 49]. Complex coacervates formed by small molecules are usually less stable at high salt concentration, but the effect of charge density is far greater than length: coacervates formed by Asp_{10} and Arg_{10} remain stable up to 1.5 M [38], while coacervates formed by a cationic elastin-like polypeptide and kilobase RNA readily dissolves at 0.2 M [50]. Although the precise reason for this is not fully understood, it is relevant for the potential use of coacervates as protocell models. Coacervates formed by small molecules can be stable at elevated temperatures, salt concentrations and over a wide pH range, and could thus have been formed under the broad range of conditions that could be expected on early Earth.

Apart from being compatible with different environmental conditions, coacervate protocells should also remain stably dispersed in a given environment. Several approaches to coacervate stabilization have been explored recently (*Figure 4*). Many strategies involve a type of surrounding membrane layer, with the setback of potentially detracting from the free exchange of solutes with the surrounding solution. However, some stabilizing layers hold real promise: they have high permeability and can assemble spontaneously around coacervate droplets.

Love et al. and *Last et al.* independently showed that coacervates can be formed inside existing lipid vesicles [51, 52]. Both groups incorporated coacervate ingredients, such as pLys and ATP, in

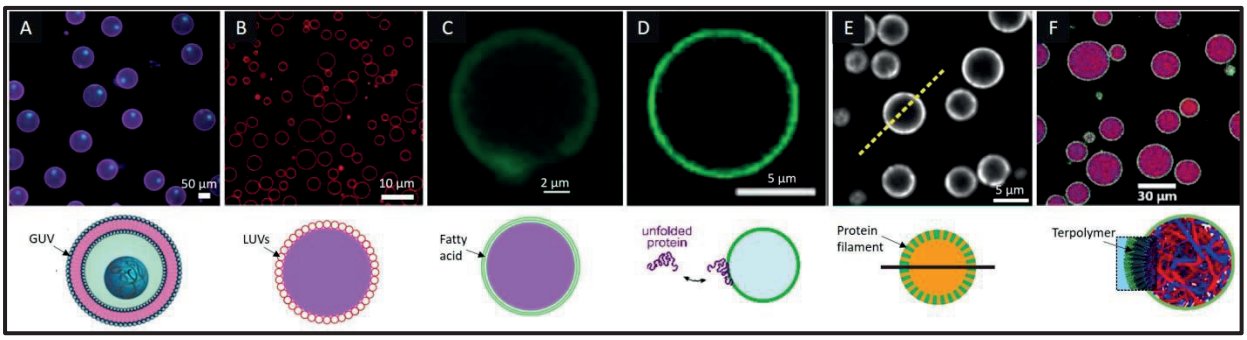

Figure 4. Observations of stabilizing layers assembled around coacervates. *A*) pLys/ATP coacervates in giant unilamellar phospholipid vesicles. (Reprinted from [51].) *B*) LUVs assembled around PDDA/PAA coacervates. (Reprinted with permission from [53]. Copyright (2019) American Chemical Society. *C*) Oleate/oleic acid multilamellar layers assembled around pLys/ATP coacervates. (Reprinted with permission from [54]. Copyright (2014) Nature Publishing Group. *D*) Unfolded BSA adsorbed at coacervates PDDA/PAA coacervates. (Reprinted with permission from [55]. Copyright (2016) American Chemical Society. *E*) Actin filaments assembled at the interface of poly-L-Lys/poly-D-Glu coacervates. (Reprinted with permission from [56]. Copyright (2018) *Elsevier*. *F*) Terpolymer assembled around cationic/anionic modified dextran coacervates. (Reprinted with permission from [57]. Copyright (2017) American Chemical Society.

DOPC or POPC vesicles at a pH where coacervation normally does not occur, and subsequently changed the pH of the outside solution, which resulted in a correlated change of pH inside the vesicles and nucleation of coacervates. These coacervates remained stable for days inside the vesicles (*Figure 4, A*), and could interact with the inner leaflet of the membrane *via* charged lipids [52]. Interaction with the membrane resulted in significant deformation of the coacervates, but the integrity of the lipid membrane seemed hardly affected.

A similar conclusion was reached by *Pir Cakmak et al.* when they investigated the interaction of existing coacervate droplets with lipid vesicles [53] They found that negatively charged phospholipid vesicles assembled at the interface of positively charged coacervate droplets (*Figure 4, B*), but remained intact. The resulting corona of lipid vesicles around the coacervates did prevent coalescence and thus enhanced coacervate stability. The uptake of RNA was reduced by 20-50%, but, interestingly, the RNA inside the vesicle-coated PDDA/PAA coacervates was better able to exchange with the outside solution than in naked coacervates. These results indicate that the vesicles are not a simple barrier between the coacervate interface and dilute phase.

Dora Tang et al. used fatty acids instead of phospholipids, and added these below the CMC to existing coacervate droplets [54]. Like in the case of phospholipid vesicles, they found spontaneous electrostatic assembly of the fatty acids at the interface of the coacervate droplets, but fatty acids formed a multilamellar membrane instead of a corona of intact vesicles (*Figure 4, C*). The multilamellar membrane resulted in exclusion of negatively charged solutes, while positive and neutral solutes were still taken up in the coacervate interior. It is not clear if the oleate/oleic acid membrane enhances coacervate stability. At higher concentrations of salt, vesicles were found to fuse into densely packed multilamellar onion vesicles with release of the coacervate components into solution. The resulting architecture depends on the surface potential of the coacervate and vesicles, and the CMC of the amphiphiles. Nevertheless, it seems reasonable that amphiphiles like these could have assembled spontaneously at the interface of coacervate protocells.

Besides a fatty acid or phospholipid membrane, the assembly of proteins around a coacervate droplet could also function as a *protomembrane*. The eukaryotic cell membrane consists of approximately 50% protein by weight, and proteins have a significant effect on membrane rigidity and permeability. *Martin et al.* showed that proteins unfolded at high urea concentrations adsorb preferentially at the interface of polyelectrolyte complex coacervates (*Figure 4, D*) [55]. As the protein was gradually refolded by diluting the urea, it got taken up more inside the coacervates.

Another example of proteins accumulating at the interface of coacervates is the assembly of actin filaments at the surface of polypeptide coacervates (*Figure 4, E*). *McCall et al.* showed high partitioning of globular actin in pLys/pGlu coacervate droplets [56]. The localization of actin filaments at the surface of the droplet was attributed to surface tension and electrostatic interactions. When bundles of F-actin grow longer than the diameter of the coacervate, they do not protrude the droplet but instead bend. Both examples show how proteins can assemble or accumulate at the interface of coacervate droplets, thereby forming a membranous layer. It suggests that other (partially) unfolded or disordered proteins or peptides could do the same, but it remains unclear if such a layer would show any emergent properties, like an increased stability against coalescence or pose a limit to coacervate size.

An effective synthetic membrane-like layer to stabilize coacervate droplets was developed by *Mason et al.* [57]. They synthesized a biodegradable terpolymer containing blocks of poly(ethylene glycol), poly(caprolactone-*gradient*-trimethylene carbonate) and poly(glutamic acid) (PEG-PCL*g*TMC-pGlu) that adsorbs *via* the negatively charged glutamic acid block at the interface of polyelectrolyte complex coacervates and self-assembles into an enclosing membrane (*Figure 4, F*). These membranes effectively prevent droplet coalescence and keep coacervate

dispersions stable for hours. The membranes are permeable to small molecules, such as glucose and resorufin, but not to macromolecules, such as enzymes. While the terpolymer is not a prebiotic molecule, it confirms the potential for molecules to self-assemble at the interface of coacervates and establish a stabilizing layer that could regulate the uptake of solutes. From a systems-chemistry point of view, such a spontaneous and hierarchical assembly process would offer a credible path to enhanced stability of coacervate protocells. A broader exploration of the types of molecules capable of assembling at the surface of coacervates would help to ascertain the rules governing this process.

Genetic Identity and Programmability

The lack of a clear mechanism by which coacervate protocells could reproduce has always been perceived as an important weakness. This is commonly linked to the absence of molecules that carry (genetic) information, such as RNA, and the fact that any sequestered molecules are free to escape into solution and exchange with nearby coacervates. Although the prospect of a stabilizing layer, as discussed in the previous section, addresses the unwanted release of sequestered molecules, the classical coacervates studied by chemists still lack in genetic identity and programmability, which are essential for protocells to process information [7]. The concept of a set of self-replicating and catalytic RNA molecules in an RNA world is an attractive idea, but also faces certain challenges, such as the higher stability of the complementary replicate strands compared to short primers, which could hamper effective replication [26]. Recent advances in coacervate research have shown that RNA can be both a building block of coacervates and be subject to polymerization and catalytic cleavage. Surprisingly, coacervates might offer a chance solution to the problem of product inhibition by stable duplexes, as they have been reported to melt long nucleic acid duplexes, preventing irreversible annealing [58].

Since coacervates of peptides and simple nucleotides have been reported by *Koga et al.*, various groups showed that different types of nucleic acids can also be used to form complex coacervates. Coacervates can be formed by complexation of polycations with low sequence complexity RNA, like poly-U or poly-A [59], total RNA [50], and single-stranded DNA [60, 61] (*Figure 5*). Interestingly, long RNA has been found to form coacervates at elevated concentrations of divalent ions, such as Mg^{2+} and Ca^{2+}, similar to polyphosphates discussed above [62 – 64]. This observation raises the question if phosphate-rich molecules with attached bases, resembling modern RNA, could have become compartmentalized in pools with high local concentrations of divalent metal ions.

From the point of view of protocell identity and programmability, a relevant question is whether

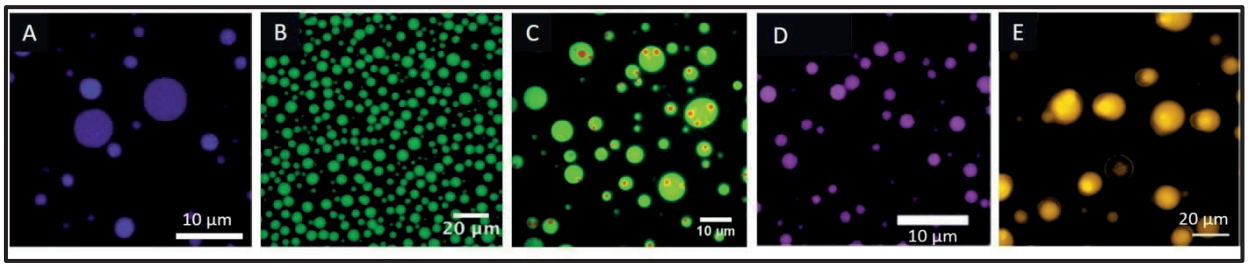

Figure 5. Uptake and activity of RNA and DNA in coacervates. *A*) Uptake of rA_{15} by spermine/poly-U coacervates. (Reprinted with permission from [40]. Copyright (2016) American Chemical Society. *B*) Coacervates of poly-U in the presence of 75 mM Ca^{2+}. (Reprinted from [64].) *C*) Hierarchical organization of RNA in multiphase coacervates with pLys and ELP K72. (Reprinted with permission from. [46]. Copyright (2020) American Chemical Society. *D*) Coacervates of dsDNA with cationic trans-azobenzene. (Reprinted with permission from [65]. Copyright (2019) *John Wiley and Sons*. *E*) Uptake of active hammerhead ribozyme in pLys/CM-Dex coacervates. (Reprinted from [67].)

nucleic acids are able to form base pairs when condensed in a coacervate droplet. Recent studies indicate that the answer is not straightforward and may depend on the precise coacervate chemistry. Several groups have reported the formation of coacervates with double-stranded DNA. *Martin et al.* recently made photoswitchable coacervates with double-stranded DNA and cationic azobenzenes [65]. Both *Vieregg et al.* [62], and *Shakya* and *King* [61] reported liquid coacervates of double-stranded DNA with simple cationic oligopeptides at high monovalent salt concentrations. The presence of duplexes was confirmed by UV melting [62]. *Aumiller* and *Keating* further demonstrated that hybridization is also possible, if one of the strands is already present in a complex coacervate [40]. They showed that the uptake of oligo-rA into poly-U/spermine coacervates is by orders of magnitude higher than the uptake of oligo-U or a random oligoribonucleotide of the same length, which was attributed to favourable base-pairing interactions.

On the other hand, *Nott et al.* showed that DNA duplexes of 12 base pairs are melted upon uptake in coacervates of a disordered protein Ddx4, which is capable of self-coacervation due to its charge patchiness [58]. In the study by *Vieregg et al.*, no change in melting temperature of a 10 bp DNA duplex was observed upon complexation with various polycations [62]. It is possible that this effect is only evident for sufficiently long duplexes, which do not fit in the coacervate mesh, as suggested for Ddx4 [58]. Another possibility is that the presence of other amino-acid residues, which are present in the case of Ddx4, but absent in the complex coacervates of dsDNA with pLys, influence duplex stability [22]. It is clear that if partial duplex destabilization is possible in certain coacervate environments, this offers new possibilities for coacervates as protocells. One attractive option is that a coacervate environment prevents trapping nucleic-acid replicators in a hybridized state by partially destabilizing long duplexes after each completed replication cycle.

Apart from the ability to form base pairs, especially RNA has attracted significant attention for its ability to catalyse reactions, including phosphodiester hydrolysis, ligation, and polymerization [66]. These functions rely on a well-defined tertiary structure of the respective ribozymes. *Drobot et al.* [67] and *Poudyal et al.* [37] showed that hammerhead and hairpin ribozymes can be taken up by different types of complex coacervates and remain functional, which is a strong indication that the required tertiary structure is intact. Coacervates formed by pLys and carboxymethyl-dextran supported ribozyme-catalysed cleavage of a substrate, although compartmentalization of the ribozyme in the coacervate phase reduced the cleavage rate significantly in comparison to buffer conditions [67]. Interestingly, the ribozyme has a high partitioning coefficient and low diffusion coefficient compared to other RNA substrates, which suggests that the coacervate droplets can be used as RNA-cleaving microreactors. Short RNAs are able to diffuse out of a protocell after being cleaved and are simultaneously replaced by new RNA substrates.

Poudyal et al. found slightly enhanced cleavage rates by hammerhead and hairpin ribozymes and DNAzyme at low enzyme and reduced magnesium concentrations in both synthetic polyelectrolyte coacervates and oligopeptide complex coacervates [37]. Moreover, they found that the same coacervates supported template-directed RNA polymerization using guanosine 5'-phospho-2-methylimidazolide, and the coacervates formed by quaternary amine-containing PDDA showed enhanced activities at strongly reduced magnesium concentrations. This enhanced rate is a result of the concentration of magnesium by the coacervates to levels required by ribozymes [59]. However, coacervates with other polycations, including lysine and arginine, showed strongly reduced RNA elongation.

The results discussed here show how coacervates can sequester various nucleic acids, which remain accessible for hybridization. From a systems-chemistry point of view, the combination of spontaneous compartmentalization and selective interactions may give rise to emergent properties of coacervate protocells. Although initially all types, lengths, and sequences of nucleic acids are

sequestered by most coacervates, random variations between protocells could become enhanced as a result of increased partitioning of complementary bases [40], boosted local elongation at optimal magnesium levels [37, 59], and a possible metabolic process to push the protocells away from thermodynamic equilibrium, which we will discuss next.

Fuel and Metabolism

Living systems exist out of equilibrium and dissipate energy to sustain themselves. One of the key ingredients of self-sustained protocells is, therefore, the incorporation of metabolic processes in which energy is transduced to fuel reactions, process information and drive growth. Coacervate-based protocells offer an advantage over other protocell designs in this respect, as they lack a membrane barrier and in principle allow free exchange of solute species across the phase boundary. Because the coacervate environment is chemically different from the surrounding solution, local concentrations of solutes can be increased by orders of magnitude and the energy landscape of reactions altered, resulting in fundamentally different behavior of chemical reaction networks in the presence of coacervates. Going a step further, coacervates, whose own formation or existence are controlled by a fuelled process, bring us closer to living systems, and recent work in this direction has provided ideas to create energy-transducing coacervate protocells.

There has been an increased interest in how reactions and reaction networks behave when incorporated in coacervate droplets, in particular with reference to intracellular membraneless compartments [22]. A general principle in these studies is that concentration of reactants or catalysts (usually enzymes) leads to a rate enhancement. *Koga et al.* found a twofold kinetic enhancement of NADPH production in a coupled enzymatic network incorporated in pLys/ATP coacervates. In a recent further development, *Love et al.* showed that, at reduced concentrations of both enzymes and substrates, reactions that are prohibitively slow in solution, can progress after localization and concentration in a coacervate droplet [54]. This provides a perspective for coacervate protocells to enable also small molecule reactions to take place within a reasonable time frame, and thereby direct the outcome of a complex network of sometimes competing reactions.

At the same time, efforts have been made to link the formation or existence of coacervates to a dissipative process, analogous to the achievements in dissipative self-assembly. The central idea is to develop a coacervate protocell that could sustain itself by actively converting energy-rich molecular building blocks into coacervate material, which in turn is slowly converted back to a soluble species, either spontaneously or by a second reaction. *Nakashima et al.* used two enzymes to control the formation of pLys/ATP coacervates (*Figure 6*, *A*) [68]. The first enzyme, pyruvate kinase, converts ADP to ATP, which undergoes phase separation under the experimental conditions, while the second enzyme, hexokinase, converts ATP back to ADP, resulting in dissolution of the coacervates. Overall, the cycle is driven by the conversion of energy-rich phosphoenolpyruvate (PEP) and glucose, the co-substrates of both enzymes, into pyruvate and glucose-6-phosphate, the by-products. By tuning the concentrations of the enzymes, coacervates could be made to appear and disappear at timescales set by the dissipation rate. The system could be refuelled by addition of PEP for another five cycles, until the accumulation of waste products prevented further coacervation.

This approach of fuelled coacervation is not limited to the conversion between ADP and ATP. *Deshphande et al.* [35] and *Spoelstra et al.* [69] showed that UDP can be polymerized into poly-U, which undergoes phase separation with small polyamines, such as spermine, beyond a critical length. They used the enzyme PNPase as a catalyst, and observed that the coacervates not only grow, but also display non-spherical shapes in the initial stages of the dissipative condensation. The polymerization is reversed by adding a large excess of phosphate, which resulted in gradual dissolution of the coacervates [69]. Although this is not a complete dissipative cycle in the sense that the cycle is driven in one direction by the conversion of

a fuel, the forward path from UDP to poly-U is an effective way to nucleate and grow coacervates by feeding with UDP. Moreover, an interesting feature of this system is that the polymerization reaction underlying coacervation can keep going, and does not necessarily stop after the phase transition point is reached. In principle, RNA oligomers can continue to be elongated, and it is even possible that elongation proceeds at a different rate inside the coacervates, as discussed above. One could imagine how this can give rise to further evolution of coacervate protocells as long as the building blocks are provided.

Tena-Solsona et al. used oil droplets instead of coacervates and showed how a chemical reaction network can be used to generate phase-separated compartments which protect the reaction products from hydrolysis [70]. A carbodiimide fuel, EDC, was used to create an anhydride of two aliphatic carboxylic acids. The anhydride has a significantly lower solubility than the free acids, and phase separates under the experimental conditions. Normally, such anhydrides are quickly hydrolysed in water, but phase separation into an oil droplet protects them from hydrolysis (*Figure 6*, *B*). The droplets are metastable, as slow hydrolysis does convert the anhydrides back to carboxylic acids. However, as long as EDC fuel is supplied, the anhydrides can be formed again, and droplets can be maintained. Interestingly, in a mixture of carboxylic acids, droplets composed of anhydrides with the strongest propensity for phase separation are selectively formed.

More recently, authors from the same laboratory developed a coacervate analogue of these dissipative droplets, in which the coacervates are made of RNA and an arginine-rich peptide with a C-terminal aspartic acid [71]. Using the same fuel, the aspartic acid of the peptide is converted into an anhydride, which increases the net positive charge

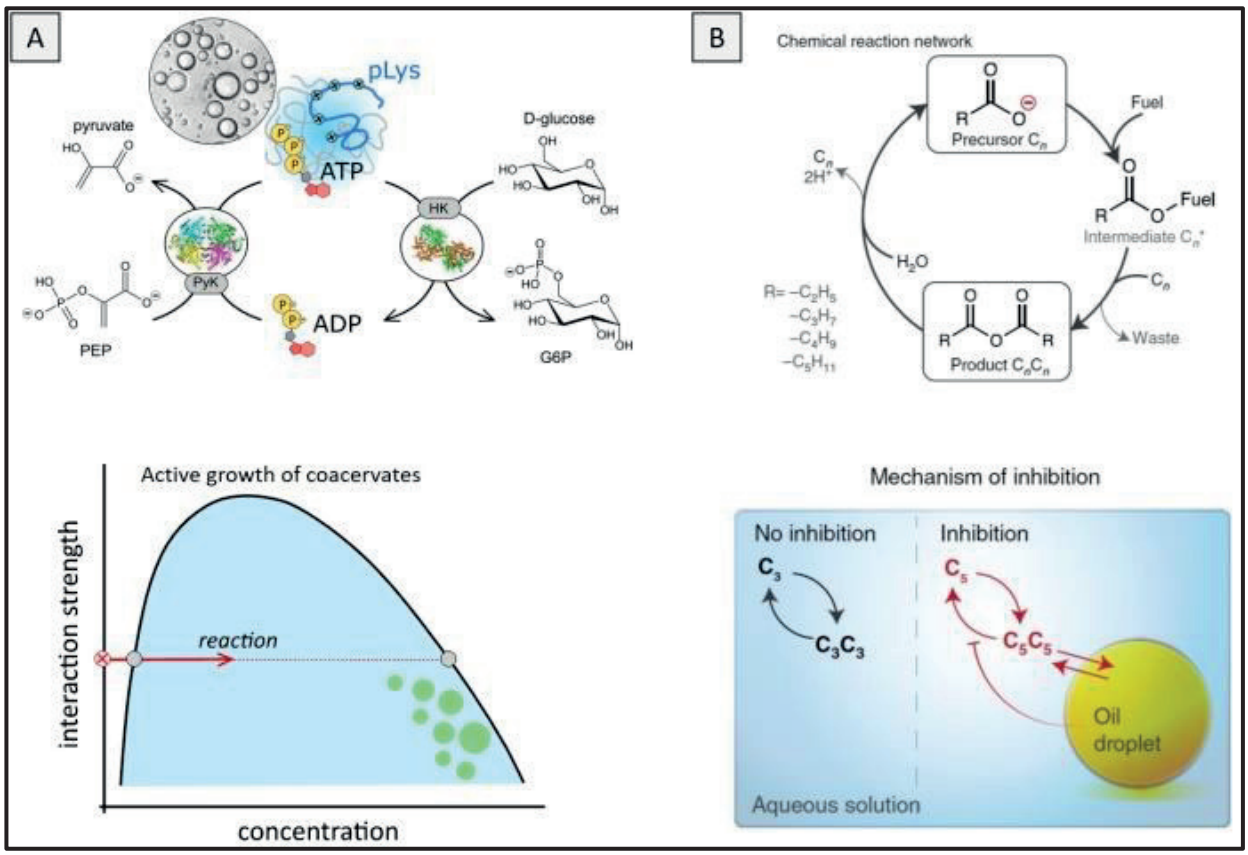

Figure 6. *A*) **Enzymatic and** *B*) **chemical reaction networks to control droplet nucleation and growth**. Adapted from [22, 70].

of the peptide and resulted in its coacervation with RNA. Similar to the previous system, these coacervates can transiently encapsulate solutes, such as RNA, and facilitate reactions.

Finally, a fundamentally different approach to couple energy transduction to coacervate protocells was explored by *Martin et al.* [65]. They used a photoswitchable cationic azobenzene to form coacervates with DNA. In thermal equilibrium, most of the azobenzene is in the *trans*-conformation, which forms stable coacervates with DNA. Upon irradiation with UV light, the azobenzene undergoes *trans*- to *cis*-isomerization, followed by dissolution of the coacervates. Thermal relaxation leads to coacervate formation again, which can also be accelerated and locally induced by blue light.

These systems show that active coacervates, which sustain themselves by energy dissipation, can be made in a variety of ways, and that these coacervates have emergent properties linked to their out-of-equilibrium nature. Currently, most experimental systems still rely on enzymes or relatively unstable and reactive molecules, such as PEP and EDC, which would not have been available on early Earth to sustain coacervate protocells. Ongoing research will show whether dissipative coacervation is also possible under prebiotic conditions.

Growth and Division

A protocell should ultimately be able to proliferate by growth and some form of division. Replication of protocells is one of the requirements for evolution [72] and arguably one of the most distinctive hallmarks of living systems. In the case of coacervates, the number of droplets normally decreases over time, as a result of ripening and coalescence, both driven by the interfacial tension. Replication of coacervate protocells means countering this natural tendency, which requires an input of energy as discussed in the previous section.

It has been hypothesized that a dissipative chemical reaction network could not only lead to controlled formation and growth of coacervates, but also to spontaneous division. A theory developed by *Zwicker et al.* shows that chemical reactions can suppress *Ostwald* ripening in dispersions of phase-separated droplets, if the rate of formation of droplet material is large enough [73, 74]. This means that coacervate protocells could retain a stable size, provided coalescence does not occur, even without a stabilizing layer. For faster production of droplet material, the droplets grow in size, and thermal fluctuations about the equilibrium spherical shape may lead to an instability that eventually results in division (*Figure 7*) [73]. This work shows how a proto-metabolism consisting of two simple reactions is theoretically enough to make a droplet divide and proliferate. However, such behaviour has not been observed yet in any of the experimental dissipative reaction networks. In most cases, the typical reaction rates seem to be too low, and the supersaturation that can be reached not high enough.

Besides a spontaneous shape instability resulting

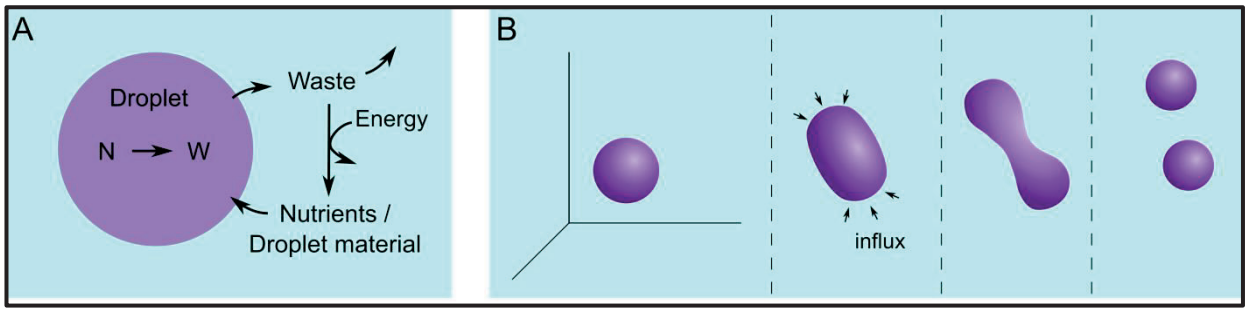

Figure 7. Theoretically predicted shape instability [73] **leads to division of a phase separated droplet in the presence of a fuelled reaction.** *A*) Schematic representation of an active droplet. *B*) Snapshots of the droplet as the reaction progresses, showing initial growth, shape instability, and ultimately division.

in droplet division, others have explored more forced disruption of coacervates to induce division in experiments. *Te Brinke et al.* used a bacterial cell division protein FtsZ, which polymerizes into long filaments when supplied with energy in the form of GTP, to stretch coacervate droplets to the point of breaking up in the middle (*Figure 8*) [50]. They used coacervates of a cationic elastin-like polypeptide and RNA. The membrane-free nature of these coacervates meant that the FtsZ monomers were able to partition freely into the coacervates, resulting in a net accumulation of FtsZ and filaments in the coacervates. In the presence of GTP, the coacervates were stretched and split into two within several minutes. This process could repeat itself multiple times until the GTP was depleted. In a similar vein, *Weirich et al.* used myosin molecular motors to deform and split droplets of actin condensed with filamin [75].

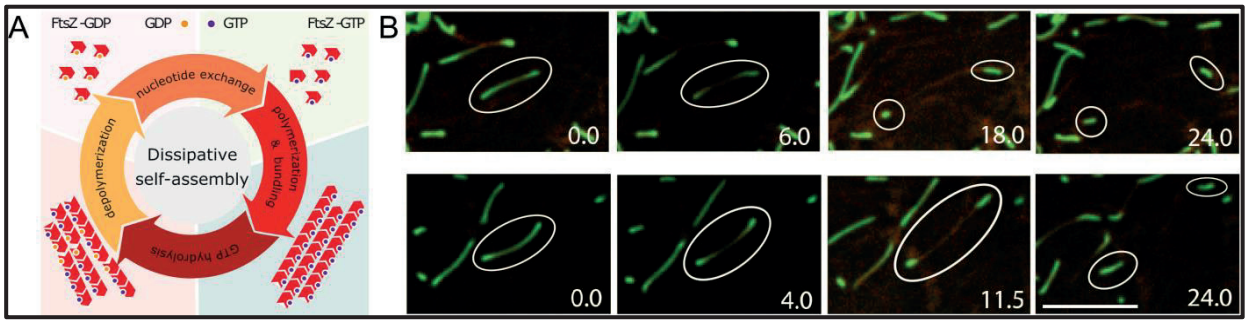

Figure 8. Dissipative self-assembly and compartmentalization of FtsZ in coacervate droplets leads to self-dividing coacervates. *A*) Schematic illustration of the GTP-driven assembly and disassembly cycle of FtsZ. *B*) Snapshots of splitting fibrils. The numbers indicate time in minutes and the scale bar represents 10 μm. Reprinted with permission from [50].

Forced droplet splitting can also be achieved without making use of highly evolved biological motor proteins, as was shown by *Song et al.* [76]. They made droplets of dextran containing a gel network of polymerized lysozyme nanofibrils, by spraying them in an external PEG solution. Although not true coacervates, these aqueous two-phase systems share many characteristics with coacervates, including the membrane-free nature of the droplet compartments. Because the PEG solution had a higher osmolarity, the fibril-containing droplet underwent dehydration during which the whole droplet started budding and ended up fragmenting into a finite number of daughter droplets. A distinct difference with previous observations of budding in PEG/dextran aqueous two-phase systems [77] is that the daughter droplets in this case contain the same phase, which is important for self-replication.

Similar budding was already observed by *Oparin* in his work on coacervates, but induced in a different way [78]. *Oparin* added polyphosphate or pLys to coacervates made of protamine/poly-A and histone/gum arabic, respectively, and observed irregularities appearing at the surface of coacervate droplets, which increased into buds and ultimately led to fragmentation. Although the mechanism behind this fragmentation is not evident, it highlights the fact that coacervates are quite susceptible to perturbations, as solutes and solvent can move in and out of the coacervates in an attempt to re-equilibrate the system. Carefully dosed perturbations may result in fragmentation, rather than complete disintegration.

Finally, instead of a distinct division process, coacervate protocells could also be regenerated and amplified through repeated disassembly and reassembly. Work by *Aumiller* and *Keating* demonstrated that phosphorylation and dephosphorylation of a short peptide like RRASLRRASL can induce coacervate dissolution and formation in the presence of RNA [79]. Similarly, the reversible reaction networks discussed in the previous section that form and dissolve coacervates could all be employed to

regenerate a population of coacervate protocells. Such an approach could be used to counter the effects of coarsening, and lead to an overall evolution of the system if the transient compartmentalization activates certain reaction pathways. However, an obvious downside of this approach is that the genetic identity of individual protocells is lost every time they are regenerated.

Recent contributions to the field of active coacervates have shown that a basic form of droplet replication may be feasible without complex division machinery. By making use of the inherent characteristics of coacervates, self-assembling units that have the potential to deform and split the droplets can be concentrated, and perturbations in the environment can induce fragmentation through the nucleation of vacuoles [71] or budding [78]. To understand and predict which conditions can give rise to division, a systems perspective seems essential.

Outlook

We started this perspective by describing how a complete and self-sustained protocell would need to combine all the essential ingredients for a cell to proliferate, including a stable compartment, a way to store and process specific information, the ability to use energy, and a mechanism to divide. All of these seemed unrealistic for coacervates when they were first described. However, recent advances have offered prospects for realizing each of these ingredients separately in "simple" coacervate droplets. In most cases, the trick is to make use of self-organization and adopt a systems-chemistry approach: coacervates can acquire a stabilizing membrane-like layer by interfacial self-assembly; coacervates accumulate (functional) nucleic acids and relevant cofactors, such as magnesium, giving rise to locally enhanced reactivity; coacervates may weaken long nucleic-acid duplexes to allow for replication; and active coacervates can exist in dissipative reaction networks to enable growth and division. Coacervates are clearly still far from being self-sustained protocells, but further integration and new connections with other areas of systems chemistry, including molecular replicators, dissipative self-assembly, and complex reaction networks [80] will lead the way to transform dull droplets into promising protocells.

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Competing interests

The authors declare that they have no competing interests.

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