Perspective

Supramolecular fibrillation in coacervates and other confined systems towards biomimetic function

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As in natural cytoskeletons, the cooperative assembly of fibrillar networks can be hosted inside compartments to engineer biomimetic functions, such as mechanical actuation, transport, and reaction templating. Coacervates impose an optimal liquid-liquid phase separation within the aqueous continuum, functioning as membrane-less compartments that can organise such self-assembling processes as well as the exchange of information with their environment. Furthermore, biological fibrillation can often be controlled or assisted by intracellular compartments. Thus, the reconstitution of analogues of natural filaments in simplified artificial compartments, such as coacervates, offer a suitable model to unravel, mimic, and potentially exploit cellular functions. This perspective summarises the latest developments towards assembling fibrillar networks under confinement inside coacervates and related compartments, including a selection of examples ranging from biological to fully synthetic monomers. Comparative analysis between coacervates, lipid vesicles, and droplet emulsions showcases the interplay between supramolecular fibres and the boundaries of the corresponding compartment. Combining inspiration from natural systems and the custom properties of tailored synthetic fibrillators, rational monomer and compartment design will contribute towards engineering increasingly complex and more realistic artificial protocells.

The bottom-up construction of synthetic minimal cells (i.e., protocells) that reproduce some of the functions observed in living organisms is gathering great attention from the scientific community[1–4]. Cellular function encompasses sophisticated processes that are tightly coordinated to support life. This network of covalent reactions and supramolecular processes can demand a precise organisation in certain intracellular compartments[5,6]. Inside living cells, individual compartments can be found that contain a unique set of molecules and microenvironments, allowing the segregation and specialisation of funcional processes[7]. Within the intracellular space, the dynamic elongation/decay of biomolecular fibres also allows the regulation (e.g., activation-inhibition) of reversible cellular functions like molecular uptake, cell motility, and transport in response to metabolic cues. The cytoskeleton, for instance, allows spatiotemporal control in (de)poly-merisation processes, modulating cell shape, motility, and division[8–10]. The not so recent discovery of functional protofilaments in archaea shocked the scientific community by confirming that cytoskeletal fibres were not unique to eukaryotic cells[11,12]. It is now widely accepted that most, if not all, bacterial

and archaeal cells possess at least one functional protofilament system[11–13]. This confirmation that cytoskeletons were not a late-in-evolution organelle suggests that confined fibrillar networks in early protocells could have represented an evolutionary advantage during the long transition from chemistry to biology.

The plausible existence of confined protofilament networks could have been involved in different functional processes of early protocells, for example, in chemical exchange with their environment and the regulation of the certain properties and functions of the protocytosol (e.g., viscosity, cargo partitioning, etc.)[14–16]. In modern cells, specialised proteins (e.g., actin) constitute filamentous machinery essential for certain biological functions. However, the complexity of the cellular environment, together with the structural sophistication of these large proteins, hinders the development of artificial mimics using identical design motifs. In contrast, synthetic building blocks (i.e., supramolecular monomers able to self-assemble as fibres under certain conditions) could provide structurally simpler fibres by rational monomer design. Besides, the use of artificial building blocks can

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also endow with new functional capacities (e.g., redox activation, light triggered responses, etc.) compared to naturally occurring networks[17]. In this context, the dynamic permeability of coacervates, capable of freely exchanging guest molecules with the environment, offers an excellent opportunity to host synthetic monomers and study their stimuli-responsive assembly under confinement[18–20].

Coacervates are aqueous droplets formed through the spontaneous association of macromolecules, such as proteins[21], nucleic acids[22], and peptides[2]. These aqueous condensates, enriched in the fundamental biomolecular components, constitute excellent compartments to trigger and localise self-assembling and autopoietic processes[2,23]. The determinant factors that dictate their formation through liquid-liquid phase separation (LLPS) are non-covalent interactions (e.g., electrostatic and van der Waals forces) and entropic effects (e.g., hydrophobic packing and counterion release)[24,25]. This cooperative and multivalent interplay between non-covalent interactions can generate disordered domains, which can remain unaffected by changes in their external conditions (e.g., pH or ionic strength)[26]. Ranging from the nanometre to micrometre scale in size, coacervates provide different properties compared to the continuous aqueous phase. As a result, coacervates can selectively accumulate molecules from their environment based on complementary supramolecular interactions (e.g., electrostatics, polarity, etc.)[27], which combined with their high permeability, make coacervates one of the optimal model compartments to study supramolecular fibre assembly under confinement[28].

In this paper, we survey the latest developments in supramolecular fibrillation confined in coacervate systems, with a particular focus on the consequences of confinement, the chemical origin of the fibres (natural *vs.* artificial building blocks), and the emergence of functional responses. Throughout this manuscript, we will refer to 'fibres' or 'fibrils' as supramolecular polymers that propagate in a single direction, both natural (e.g., cytoskeleton, amyloids) or synthetic, regardless of their chemical composition. 'Compartments' will include a range of colloidal systems that can confine supramolecular fibrillation in their dispersed phase, focussing on coacervates, which are membrane-less droplets formed by associative LLPS[25]. Both compartmentalisation and supramolecular fibrillation are important cellular phenomena that can be mimicked by scientists in order to improve our fundamental understanding of their functional properties and for the future development of functional biomaterials[3]. Reports of coacervates are here compared to supramolecular fibrillation in analogous compartments, mainly water-in-oil droplets and phospholipid vesicles. The aim is to provide a perspective on the importance of bottom-up fibrillar self-assembly in confined aqueous systems and how, by exploiting these supramolecular architectures, the behaviour of individual aqueous compartments and their collective interactions can be designed to mimic certain cellular processes. Examples highlighted in this paper demonstrate how a fundamental understanding of confined fibrillation can shed light on the role that primitive fibres may have played in early cells, as well as on the current engineering of soft materials and synthetic prototissues[29,30].

Natural protein fibres reconstituted in coacervates

Coacervates provide a suitable model to study the confined fibrillation of natural proteins in a dense aqueous compartment like the cytosol[31–33]. Natural fibrillating proteins (e.g., actin and tubulin in eukaryotes, or FtsZ in prokaryotes) are dynamically interconverted between dormant monomeric and active self-assembling states by phosphorylation events, generating fibres with structural, transport, and motile functions[8–10]. The dynamic (de) polymerisation of natural fibres and its effects on cellular function can be mechanistically studied under controlled conditions when reconstituted in simple coacervates, outside the biochemical complexity of a cell. This section covers key advances and fundamental learnings from the study of confined natural protein fibrillators in coacervate droplets. We will address three biomimetic features of intracellular fibrillators that can be preserved in simple coacervate models: high molecular crowding, compartment

reshaping by mechanical actuation, and the hierarchical organisation of fibres in space.

Unlike dilute buffered solutions, the reconstitution of natural proteins within coacervates can mimic the high macromolecular crowding found in the cytosol that helps the nucleation and the elongation of supramolecular fibres[18]. Molecular crowding can indeed have dramatic effects on protein folding and aggregation, and it is not surprising some intracellular processes might require highly crowded environments like that of the cytosol, which can reach 400 mg·mL^{-1} in macromolecular content[34]. For example, phase-segregated condensates of VASP protein, involved in actin filament nucleation and polymerisation[35], can be formed in presence of crowding agents (i.e., polyethylene glycol 8 kDa)[36]. Despite crowding is not strictly required for actin fibrillation, this effect is responsible for the LLPS of VASP condensates and hence to confine the fibrillation of actin, which accumulates in the coacervate phase. Similarly, crowding is required for prokaryotic protein fibrillation under confinement, as shown likewise by the ability of PomY condensates, a protein part of the PomXYZ cluster that regulates bacterial cell division, to trigger FtsZ fibrillation (Fig. 1i)[37]. The authors revealed the ability of PomY to form protein condensates through intrinsically disordered regions (IDRs). Molecular crowding with polyethylene glycol also promoted the LLPS of PomY by synergistic electrostatic and hydrophobic effects, which could result from the remodelling of its IDRs in crowded environments[34]. Similarly, actin-binding LIM protein (abLIM) condensates form through specific interactions between their intrinsically disordered regions and then accumulate and polymerise actin from abLIM coacervates[38]. Overall, these examples illustrate that biomolecular fibrillation not only relies on protein structure, but also the environmental aqueous conditions (i.e., crowding) can be critical, which should be carefully considered when designing and working with synthetic biomimetic fibrillators.

Dynamic cell reshaping, for example during phagocytosis, pseudopodia motility, and cytokinesis, is governed by protein fibrillation events within the cell[39]. Analogously, coacervates can serve as minimalistic compartments to study the influence of protein fibrillation on protocellular shape and motion under controlled external settings. It should be noted that the composition of coacervates will always affect their own viscosity and deformability, which may have an impact on their reshaping in response to the mechanical stress induced by fibre polymerisation. Inspired by microtubule catastrophe and rescue events[40,41], te Brinke et al. studied the dynamic instability of FtsZ fibres within coacervates consisting of an unfolded cationic peptide with a repeated cationic sequence (VPGKG with a GFP tag) mixed with an RNA polyanion (Fig. 1ii)[42]. The resulting LLPS condensates preferentially hosted the FtsZ monomers with a partition coefficient of ~12 compared to the aqueous continuum. Upon addition of GTP and Mg^{2+}, FtsZ formed dynamic fibrillar bundles inside the coacervate phase, where GTP uptake from the aqueous solution could maintain the out-of-equilibrium polymerisation of FtsZ filaments. Once all the GTP was consumed, the fibrillar structures depolymerised and eventually dissolved into a homogeneous dispersion of disassembled FtsZ. Fibre propagation resulted in the mechanical deformation of the coacervates, inducing the elongation of the condensates and emergence of invaginations. Once all GTP was depleted, fibre depolymerisation returned fibre-deformed droplets back to their original spherical shape, confirming the fluid-like character of the coacervate. In contrast, more structurally rigid gel-like coacervates prepared from high molecular weight proteins can restrict compartment deformation, while also limiting fibre mobility and reorganisation within the compartment[38]. Indeed, dynamic fibre reconfiguration and diffusion across the cytoplasm is critical to regulate vital processes like cell division, where topological control over protein fibrillation will precisely position the required biomolecular machinery and determine cell fate. Analogously, the lenticular deformation of coacervates by the collective action of protein filaments (e.g., FtsZ)[37] resembles to the structural reorganisation of dividing cells, which may shed light into the biophysical determinants (e.g., viscosity, curvature, etc.) affecting the spatiotemporal control of cell division machinery beyond biochemical inputs.
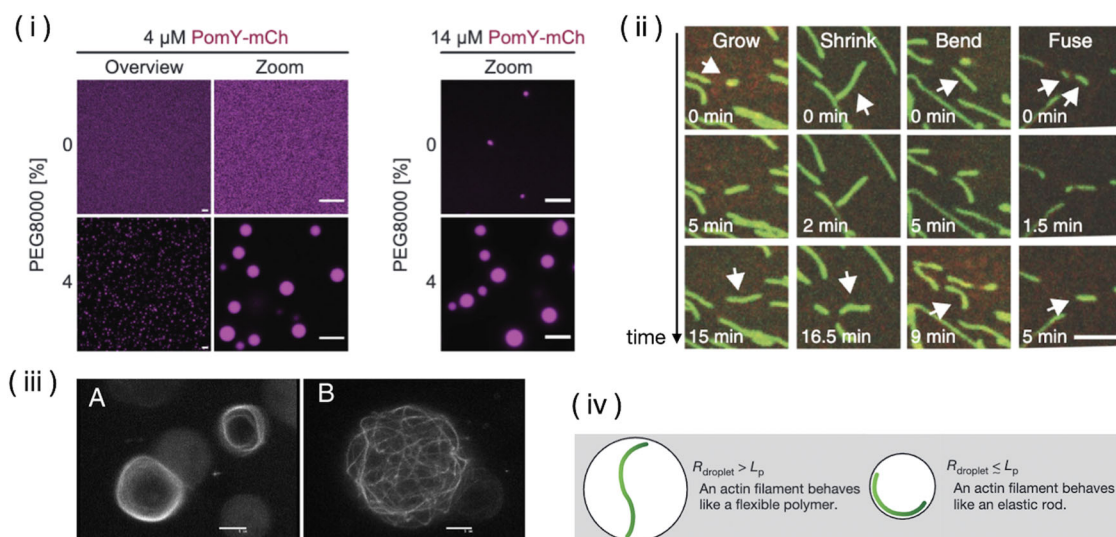
Fig. 1 | Fibrillation of biogenic motifs within coacervates. i Coacervation of PomY protein labelled with mCherry (PomY-mCh) in absence and presence of poly(-ethylene glycol) 8 kDa (PEG8000) as crowding agent. LLPS of PomY-mCh coacervates could be triggered at low protein concentration (4 μM) only in presence of PEG8000, while higher protein concentrations (14 μM) did allow the formation of some small coacervates in absence of PEG8000. Reprinted with permission from Springer Nature[37]. **ii** Dynamic reshaping of coacervates (green) induced by confined FtsZ fibrillation. Reprinted with permission from Springer Nature[42]. **iii** Dependence of actin/α-actinin network structure on vesicle size: rings obtained in vesicles of diameter d < 12 μm (**A**) and fibre mesh formed in vesicles of diameter d > 12 μm (**B**). Reprinted with permission from American Physical Society[49]. **iv** Proposed behaviour of actin fibres in response to the size of their droplet container, explaining why actin rings could only be formed in droplets with radii (R) smaller or close to the persistence length of actin filaments (Lp). Reprinted with permission from Springer Nature[51]. All scale bars = 5 μm.

Cooperative bundling and structural specialisation of protein fibres play a pivotal role in cell function[43], establishing controlled asymmetries across the cytosol like mitotic spindle polarisation and motile membrane protrusions[44]. Importantly, protein fibrillation within the cellular environment provides an elegant task-managing mechanism, as shown by actin as cytokinetic mediator when fibrillated in the cytosol or as DNA repair scaffold when elongated in the nucleus[45]. These fibrillation phenomena with highly specialised functions are all spatially controlled by intracellular coacervation events[18]. Particularly interesting is the assembly of specialised protein rings by eukaryotic and prokaryotic cells, for example, bacterial FtsZ is known to control cytokinesis via fibre coiling into a ring (i.e., Z-ring), which can attach to the cell membrane for cell division[46]. An increasing number of coacervate systems and other protocell models (e.g., vesicles) have successfully confined the hierarchical assembly of natural protein rings. VASP protein coacervates could host the ring-shaped bundling of actin fibres, resulting in the remodelling of the droplets into ellipsoidal, disk-shaped and rod-like domains[36]. abLIM protein coacervates have also shown to confine actin polymerisation into rings, where actin could continue to elongate as ring-anchored fibres that projected towards the outside of the droplets[38]. Ring-shaped actin polymerisation was also explored inside complex coacervates of poly-L-lysine (ca. 15 kDa) and poly-L,D-glutamic acid (ca. 15 kDa), leading to compartmentalised actin rings positionally restricted to the periphery of the droplets[47]. Displaying such distinct polymorphism between linear and cyclic filamentous bundles, the question arises as to what determines protein polymerisation into fibres or rings under confinement. Early studies from the 90s investigated tubulin fibrillation in solution inside micron-sized chambers, revealing an assembly pathway selection for aster-like or spiral fibre clusters based on protein concentration, as well as the size and shape of the chamber[48]. A few years later, in-vesicle studies suggested a strong dependence of ring formation based on the size of its compartment, with only vesicles smaller than 9–12 μm in diameter forming protein rings (Fig. 1iii)[49]. Elaborating on this observation, it could be argued that the smaller the size of a spherical container, the larger the curvature of its boundary. Thus, small droplets impose geometrical constraints on elongating fibres, which can either deform their container or bend into a ring or spiral if sufficiently flexible[50].

This theory was later supported by Miyazaki et al., who reconstituted ATP-dependent contractile actin rings in vesicles, observing that only small droplets could induce the formation of ring-shaped protein fibres[51]. The authors proposed a critical persistence length for actin fibres at approximately the radius of the droplet, under which the protein filaments behave as elastic rods able to curve and cluster into rings (Fig. 1iv). Actin fibres longer than the droplet radius behave as flexible polymers, leading to disperse fibrillar networks or vesicle protrusions if fibre bundles reach a certain rigidity[52].

Overall, in-coacervate fibrillation can mimic some of the topological and morphological characteristics natural protein fibres display in the cell, suggesting some parameters governing intracellular fibrillation may result from unspecific biophysical and biomechanical effects simply imposed by confinement (e.g., molecular crowding, container size, droplet rigidity, and viscosity, etc.), instead of highly specific molecular recognition (e.g., substrate-protein recognition).

Artificial molecules as biomimetic fibres within coacervates

Rationally designed synthetic building blocks can be used to replicate and understand the more sophisticated protein polymerisation processes that take place in the intracellular medium. However, the confinement of synthetic supramolecular fibrillators with hierarchically controlled organisation and function remains challenging. Several systems have been developed in recent years that use external stimuli to promote fibrillation inside coacervates and related aqueous droplets. This section is focused on the recent advances in artificial peptide fibrillators as minimalistic protein analogues, allowing the engineering of de novo confined filamentous assemblies with potential emergent behaviour.

One of the advantages of compartmentalisation is the preferential partitioning of guest molecules inside the coacervate droplets[2]. The higher local concentration of supramolecular monomers in the coacervate phase can overcome their critical aggregation concentration, eventually inducing the confined assembly of supramolecular structures even at low monomer (overall) concentrations[36–38]. Particularly interesting systems to study compartmentalised fibrillation are molecules capable of both processes, firstly undergoing LLPS for the subsequent assembly of fibres inside the

droplets. Examples can be found of naturally occurring proteins that undergo LLPS prior polymerisation into fibrillar networks, such as elastin[53]. To understand this process, Vidal Cebalos et al. studied the stepwise coacervation-assembly of an elastin-like protein consisting of alternating hydrophobic and cross-linking domains[54]. This 252-amino acid protein initially undergoes LLPS by hydrophobic packing, increasing its apparent concentration in the coacervate phase as compared to the bulk solution. Over time, the resulting coacervates grow into amorphous protein aggregates, which despite not fibrillar, demonstrate the stepwise supramolecular transition from the same monomer into coacervates and solid-like aggregates.

While the structural complexity of natural proteins may suggest that fibre formation requires a set of highly specific and directional interactions, the replication of this processes using minimal systems (e.g., peptides) could help to elucidate the underlying —and hence fundamental— molecular and biophysical mechanisms triggering and controlling supramolecular fibrillation under confinement. To rationalise in-coacervate fibre proliferation, Hsieh et al. developed a theoretical framework to predict phase transitions in a clever and rather simple synthetic system[55]. The model describes the evolution from a dispersed phase to the final thermodynamic assembly, whose structure is determined by the least soluble component (i.e., monomer or assembly). The phase transition is characterised by the formation of a metastable intermediate in the form of a condensed phase, where monomers accumulate when their solubility limit is surpassed. As a result, supramolecular fibrillation is triggered inside these condensates by the localised accumulation of building blocks (Fig. 2i). This mechanism was validated using a short amyloid peptide, Ac-KLVFFAE-NH$_2$, adjusting solubility in water:acetonitrile mixtures. This one-component molecular system confirmed the formation of a coacervate phase at low monomer solubility, transitioning into supramolecular fibres generated inside the initial metastable peptide. The appearance of this intermediate condensate parallels the compartmentalised fibrillation of natural models, where intracellular

coacervates induce fibre proliferation with impeccable spatial control[36–38]. Thus, this model could be extended, at least in terms of thermodynamic processes, to the existence of metastable condensed phases governing the formation of fibrillar networks. Indeed, the desolvation of monomers through changes in water:acetonitrile ratio presented in this paper could be compared, to some extent, to the aggregation induced by crowding effects on macromolecular nucleation in cells (Fig. 1i)[41]. Molecular crowding can perturb the hydrogen bond network of solvating water around the monomers, which in turn will affect the entropic barrier for solubilisation and assembly of condensates and fibres[56].

Transitions in one-component systems from monomer to coacervate and subsequent in-coacervate self-assembly are controlled by supramolecular interactions, which ultimately result from rational molecular design and manipulation of the external conditions (e.g., pH, temperature, etc.). Lipiński et al. explored the sequence-assembly relationships of monomers consisting of two dipeptides linked by a flexible connector[57]. As previously observed in the case of elastin[54], the non-directional solvophobic packing of hydrophobic aminoacids (e.g., alanine) could lead to the formation of coacervates, but not to the assembly of fibres. Directional π-π interactions between aromatic phenylalanine sidechains were responsible for the confined supramolecular fibrillation after LLPS. However, bulkier tryptophan side chains caused the rapid assembly of amorphous aggregates prior to coacervation, proving the importance of balanced supramolecular interactions for LLPS and compartmentalised fibrillation when using a single building block.

Moving from protein fibrillators to short peptide mimics poses the challenge of efficiently confining the monomers, as small peptides are much more weakly retained inside the coacervate phase than large multivalent proteins[58]. To tackle this issue, Jain et al. developed a three-component coacervate system based on the amyloid fragment LVFFA coupled to an oligo-arginine (LVFFAR$_9$) as an amphipathic building block[59]. Mixed with R$_9$ and ATP, the three-component system forms a coacervate phase at pH 8.
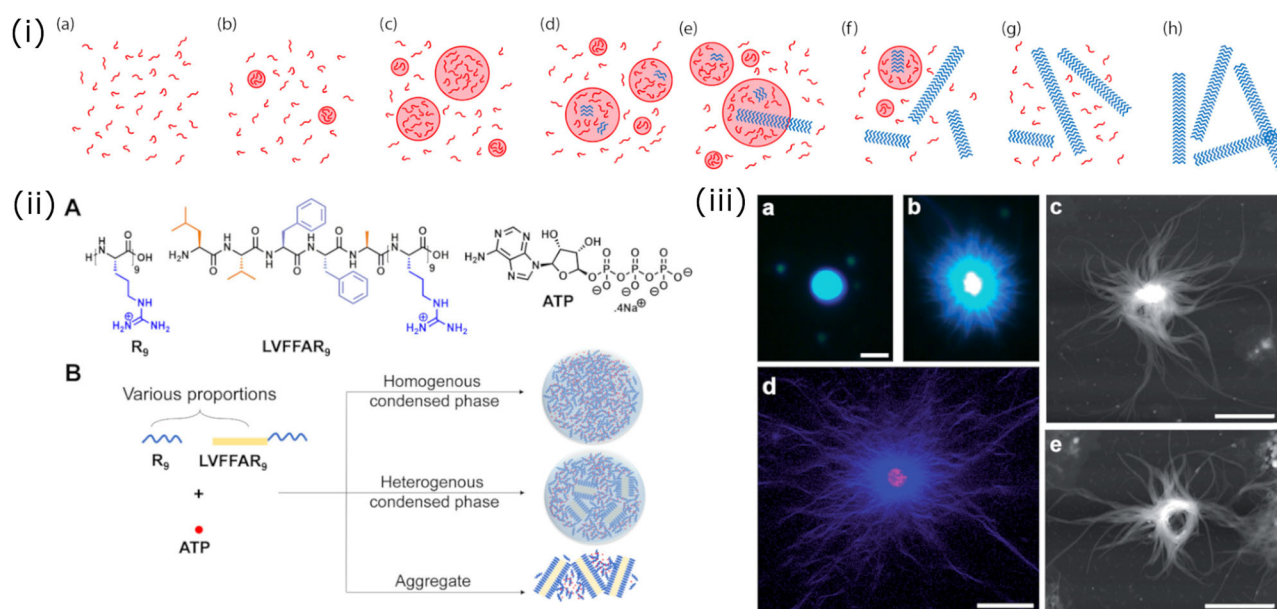

Fig. 2 | Supramolecular assembly of artificial fibrillators within coacervates.
i Mechanism of multiphase peptide fibrillation: **a** free peptides (red traces) in solution, **b, c** metastable peptide coacervates (red circles) nucleate and grow, **d** confined peptide begins to form fibres (blue), which can grow beyond the particle's boundaries (**e, f**) fibres can propagate by consuming peptide monomers from the coacervate phase, **g** particles start to dissolve at low free peptide concentration, and **h** the fibres become the only species in the system at poor solubility conditions. Reprinted with permission from American Chemical Society[55]. Copyright 2017 American Chemical Society. **ii** Artificial three-component complex coacervate with confined fibrillation: **A** molecular components and **B** compositional control of the

resulting assemblies. Reprinted with permission from American Chemical Society[59]. Copyright 2022 American Chemical Society. **iii** Structural transition from a PDDA/Fmoc-D-Ala-D-Ala droplet to an aster-like structure upon addition of GDL. Fluorescence microscopy images at t = 0 min (**a**) and t = 10 min (**b**) labelled with Hoechst 33258. Scale bar = 10 μm. AFM images of a droplet 3 h after the addition of GDL with (**c**) and without (**e**) a coacervate core. Scale bar = 2 μm. **d** Confocal fluorescence microscopy of a matured aster-like structure, where the polymer and the peptide show red and blue emission, respectively. Scale bar = 16 μm. Reproduced from with permission from the Royal Society of Chemistry[61].

The authors could induce peptide self-assembly solely inside the droplets, with structural transitions between disassembled-aggregated-fibrillar states controlled by the molecular ratio between the three components of the system (LVFFAR$_9$-R$_9$-ATP; Fig. 2ii). The formation of confined fibres was shown to affect the collective behaviour of the droplets, facilitating droplet coalescence, and also causing an increase in the mechanical integrity of the coacervate phase by the gain in solid content of the droplets. This polyelectrolyte-based design constitutes a key step towards understanding the molecular requirements of small fibrillators to preferentially accumulate in complex coacervate phases, here achieved by the strong Coulombic complementarity of polyarginine (R$_9$) and ATP[60].

A particular aspect of fibrillation in biological compartments is the active response of the monomers to external stimuli to regulate supramolecular polymerisation. In natural systems, this is often achieved by the incorporation of an energy source at the molecular level, such as GTP[42]. The engineering of synthetic monomers with similar fibrillation dynamics remains challenging but highly coveted towards adaptive materials and protocells[17]. In this context, Kumar et al. developed a reconfigurable coacervate system that undergoes fibrillation when acidified[61]. This system consisted of cationic poly(diallyldimethylammoium), PDDA, and a *N*-fluorenyl-9-methoxycarbonyl (Fmoc)-functionalised dipeptide, Fmoc-D-Ala-D-Ala. At pH 8.5, the carboxylate group at the peptide's C-term is deprotonated, hence anionic, and capable of complex coacervation with the polymer. Addition of glucono-δ-lactone (GDL) provides a slow release of protons through hydrolysis that acidified the system to pH 4.5, causing the protonation of the peptide's C-term and its assembly into nanofilaments. As a result, the spherical coacervate droplets developed aster-like structures consisting of a dense core surrounded by a shell of polymer-decorated peptide fibres (Fig. 2iii). The progressive bundling of fibres triggered hydrogelation, possibly due to entanglement of the supramolecular nanofilaments, consistent with the behaviour of Fmoc-based dipeptide fibrillators in aqueous solution[62–65]. Notably, the fibrillation process was reversible due to the electrostatic nature of the complexation, where changes in the pH could be used to transition between the coacervate phase and the fibrillar state.

The scientific community has been also focused on the study of chemically active compartments, i.e., those that respond to external chemical stimuli[2]. In particular, redox-active compounds, such as ferricyanides, constitute an excellent proxy for mediating redox-coupled supramolecular assembly[66]. In a recent study, Wang et al. presented the redox-triggered fibrillation of a molecular gelator within polylysine/[Fe(CN)$_6$]$^{4-}$ condensates[67]. Initially, the coacervates allow the compartmentalised redox-activation of the dormant *N*-benzoyl-L-cysteine (BC) to form the active monomer *N,N*-dibenzoyl-L-cysteine (DBC). In acidic conditions, the protonated form of DBC polymerised into supramolecular fibres inside the coacervate phase, which subsequently matured into rigid bundles. One interesting aspect of this design is the reversible (de)polymerisation of fibres by addition of oxidating/reducing agents or by fluctuations in pH. Thus, coacervate compartments that respond to external triggers can be exploited to control supramolecular polymerisation inside the coacervate phase. Constructing on these advances, the development of reactive dormant precursors whose fibrillation can be activated by chemical reactivity needs to be further investigated in coacervate systems, from their molecular requirements and with a special focus on the functional consequences, which could open new levels of coupled reactivity-assembly complexity.

Lessons from fibrillation in other biomimetic compartments—vesicles and droplets

The development of confined microreactors that control the assembly of different functional assembling systems and reaction networks has also been explored in compartments such as water-in-oil droplets, liposomes, and polymerosomes[68]. With increasing orders of functionality attributed to confinement, scientists have explored the regulation of enzymatic cascades[69], chemical coupling between compartments[70], and localised rheological modifications[71], amongst others. Learnings from these systems will allow to understand, extrapolate, and compare the emerging behaviour of supramolecular fibrillar networks across compartments of different nature. Pioneering experiments with protein monomers were aimed to the reconstitution of natural cytoskeletal fibres under confinement[72–77]. In this section we will highlight selected examples of fibrillation and functional responses under confinement in a collection of different compartments and, where relevant, their correlation to in-coacervate fibrillation.

The molecular machinery found in nature constitutes a perfect example of self-regulated functional networks. By mimicking this covalent and supramolecular interplay, Maeda et al. reconstituted a minimal bacterial cytoskeleton inside lipid vesicles by cell-free gene expression[78]. The system was programmed to simultaneously express MreB and MreC, two conserved bacterial proteins that are homologous to the eukaryotic actin protein. Upon expression, the formation of a cortical network composed of flexible fibres was observed inside the vesicles after addition of ATP and Mg^{2+} (Fig. 3i). Interestingly, MreB formed amorphous aggregates in the absence of MreC, which is possibly attributed to the ability of MreC to localise and organise MreB polymerisation in subcellular compartments. This limitation required the design of a MreB-based scaffold with a terminal amphipathic peptide (MreB-18L). The results suggested that the self-association of the 18L peptide at the lipidic membrane triggered nucleation, providing the required domain to direct the formation of protein fibrils. Since lipid membranes display low permeability to macromolecules, this design required the inclusion of the pore-forming protein α-haemolysin to allow the uptake of the required triggers from the continuous phase into the vesicles. In contrast, high permeability of membrane-less coacervates would allow chemical exchange between the condensed and the continuous phases[37,46], overcoming the barrier imposed by lipid membranes. In addition, the transition from a compartment with low macromolecular permeability to a coacervated system would not only avoid the need of pores but could also extend fibrillar growth beyond the boundaries of the compartment. However, dynamic cargo exchange in coacervates comes at the expense of lower selectivity and uptake control. In this line, new strategies to control molecular traffic in fibrillating compartments might open access to new dynamic and collective responses, for example, by designing specific compartment (host)-cargo (guest) interactions[60].

The use of synthetic monomers (e.g., peptides) can help understanding the molecular origins of fibrillation, which can be directly applied to the engineering of biomimetic function through rational molecular design. Our group has pioneered the study of supramolecular fibrillation under confinement with synthetic cyclic peptide building blocks. In particular, the design of cyclic peptides with alternating D/L chirality provides a versatile toolbox to explore intermolecular interactions and assembly modes[79]. Initially, we designed a cyclic octapeptide with alternating chirality that self-assembled into microtubules in response to acidification. Cyclic peptides equipped with histidine residues that enabled pH-controlled self-assembly and a pyrene unit to promote hydrophobic stacking[71]. Upon His deprotonation, the hierarchical supramolecular assembly afforded nanotubes with a high persistence length, which was attributed to a high bending energy in the assembled state. The confined assembly of these filaments deformed the droplet interface and increased the local viscosity, turning the aqueous media into a non-Newtonian fluid with shear-thinning properties. Further, the implementation of microfluidic devices enabled the spatial control of the assembly process within the droplets (Fig. 3ii)[80]. By modulating the uptake of different chemical triggers, the assembly could be controlled to yield fibres of different chemical nature resulting in the localisation of fibres at the cortex or the core of the compartment. Overall, this system provides an autonomous assembly approach to control the rheological properties of an individual aqueous environment by using a stimuli-responsive one-dimensional synthetic polymer. From these studies, lessons towards fibrillation within coacervates could be the extrapolated to control fibre positioning by microfluidics. We also hypothesise that the adjustment of the molecular density or the incorporation of reacting parts at the liquid interfaces of the corresponding compartments could be employed to gain further spatial control on fibre growth and positioning[37].
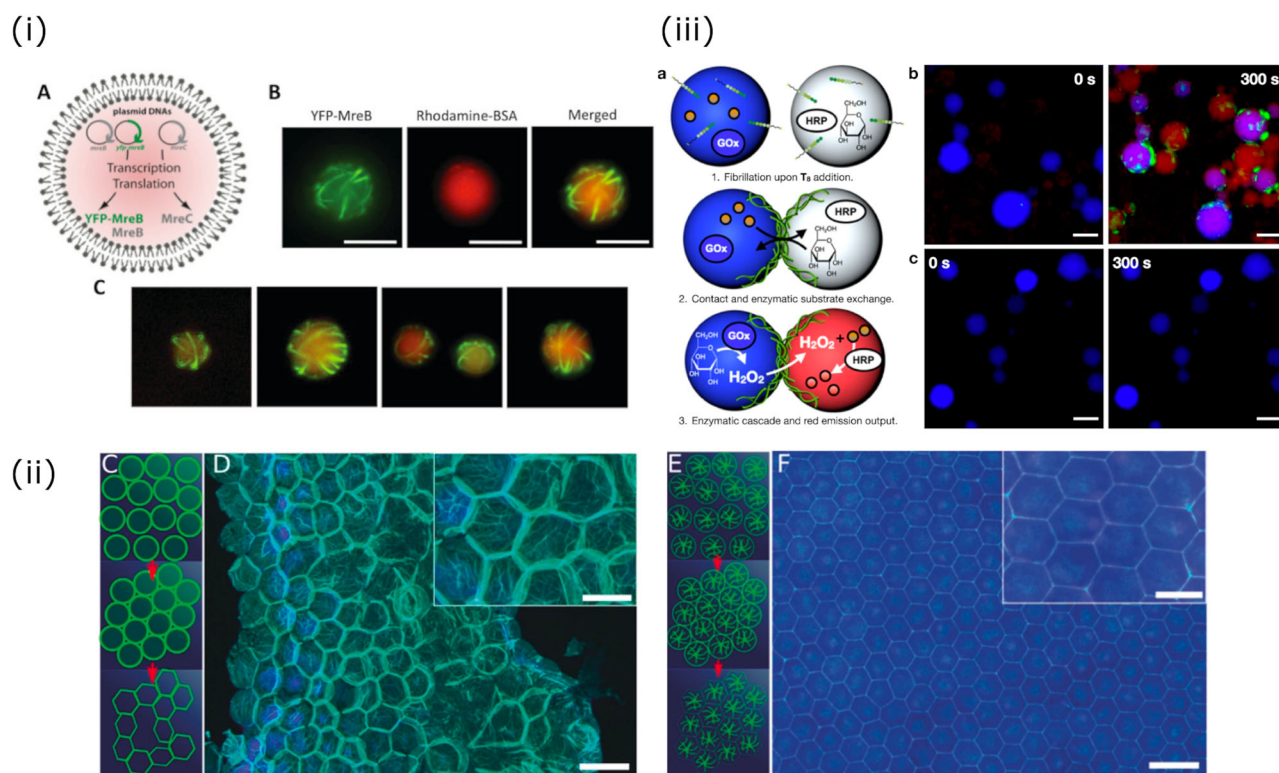
Fig. 3 | Fibrillation processes confined in vesicles and water-in-oil droplets.
i Synthetic biology approach for the expression and assembly of MreB in liposomes:
A scheme of the cell-free expression of MreB and MreC proteins inside vesicles,
and **B, C** epifluorescence micrographs of YFP-labelled MreB assembled into fibres
(green) inside vesicles in the presence of rhodamine-labelled bovine serum albumin
(BSA, red). Scale bar = 10 μm. Reprinted with permission from American Chemical
Society[78]. Copyright 2012 American Chemical Society. **ii** Spatially controlled
fibrillation of cyclic peptides by microfluidics: **C, D** formation of a cortical network
of fibres in alkaline conditions and **E, F** localised formation of aster-like fibres at the
droplet core in the presence of electrolytes. Scale bars = 100 μm (main picture) and
50 μm (insets). Reproduced with permission from John Wiley & Sons, Inc. © 2020
John Wiley & Sons, Inc[80]. **iii** Chemical communication between droplets triggered
by the fibrillation of peptide amphiphiles: **a** schematic representation of the
exchange of contents between droplets, **b, c** fluorescence micrographs of the com-
munication process in **b** the presence and **c** the absence of fibres. Scale bar = 70 μm.
Reprinted with permission from Springer Nature[81].

To investigate the potential consequences of a chemical reaction trigger
in 1D self-assembly under confinement, we also developed a minimal
supramolecular fibrillator inside water-in-oil droplets[81]. A self-assembling
peptide amphiphile sequence was designed with a β-sheet-inducer sequence
at the C-term (VVAAEE) and a short C8 aliphatic chain with an alkox-
yamine terminal group[82,83]. The alkoxyamine dormant peptide reacts with
octanal to amplify its hydrophobic character. The resulting peptide
amphiphile assembled into micellar structures that co-assembled with the
reactive peptide and octanal, thus accumulating monomer precursors and
triggering a physically autocatalytic process that accelerates peptide
amphiphile production rate. The resulting fibrillar bundles accumulated at
the droplet cortex, which promoted selective molecular uptake from the
continuum to the droplet. Contact between adjacent droplets was also
shown to allow the exchange chemical information by coupling a two-step
enzymatic cascade between droplet populations (Fig. 3iii). This example
showed how the confined fibrillation of a simple amphiphile could yield
functional responses, which translated into coordinated collective behaviour
without complex biomolecular machinery. Implementation of these fibrillar
networks in coacervates could potentially extend fibrillation beyond the
compartment given the aqueous nature of both the internal and external
phases, allowing crossed communication and inter-specific relationships
between droplets.

Biological systems have the ability to regulate dynamic functional
networks through complementary interactions between macromolecules[84].
Beyond proteins and peptides, programmable nucleic acids are excellent
building blocks for the bottom-up synthesis of supramolecular networks
through complementary base pairing[85]. Aiming to enable life-like functions,
Agarwal et al. developed nucleic acid-based fibrillar networks confined in

water-in-oil droplets[86]. Excellent sequence specificity required for assembly
allowed the authors to explore conditional assembly diversions using pre-
designed DNA strands in complex fibrillation routes. For instance, using
two complementary strands, a logic gate implemented in the sequence could
drive the co-assembly into a hetero-fibrillar network or the segregation into
two different homo-fibrillar networks. In addition, the introduction of an
RNA polymerase and RNase H could control the in situ (de)polymerisation
of a transient RNA-DNA fibrillar network (Fig. 4i). From these studies, one
can envision the potential assembly of co-existing functional networks that
specifically respond to different stimuli (e.g., strand sequence, pH, etc.)
transiently transferred from the external environment to the compartment.

A particular aspect of biological systems is their capacity to transport
chemical information to specific sites within the cell. Often, this is achieved
through complex molecular machinery, such as kinesin proteins able to
'walk' the cytoskeletal network[87]. The development of artificial mimics will
aid to understand the fundamental aspects of intracellular transport, as well
as to take a step closer into the bottom-up construction of synthetic cells.
Motivated by this challenge, Zhan et al. assembled a de novo fibrillar DNA
network that mimics cytoskeletal functions in water-in-oil droplets[88]. By
programming sequence-specific binding domains, DNA is assembled into
dynamic filaments confined within the aqueous droplets. The use of
modified toeholds at the binding domains introduced the possibility of
displacing the interactions between strands, resulting in the disassembly of
the fibres by invader sequences and the use of an anti-invader strands to
restore the fibre network. The addition of RNA pendants to the DNA
filaments enabled the inclusion of binding sites for a molecular cargo
functionalised with complementary DNA strands to the RNA overhangs.
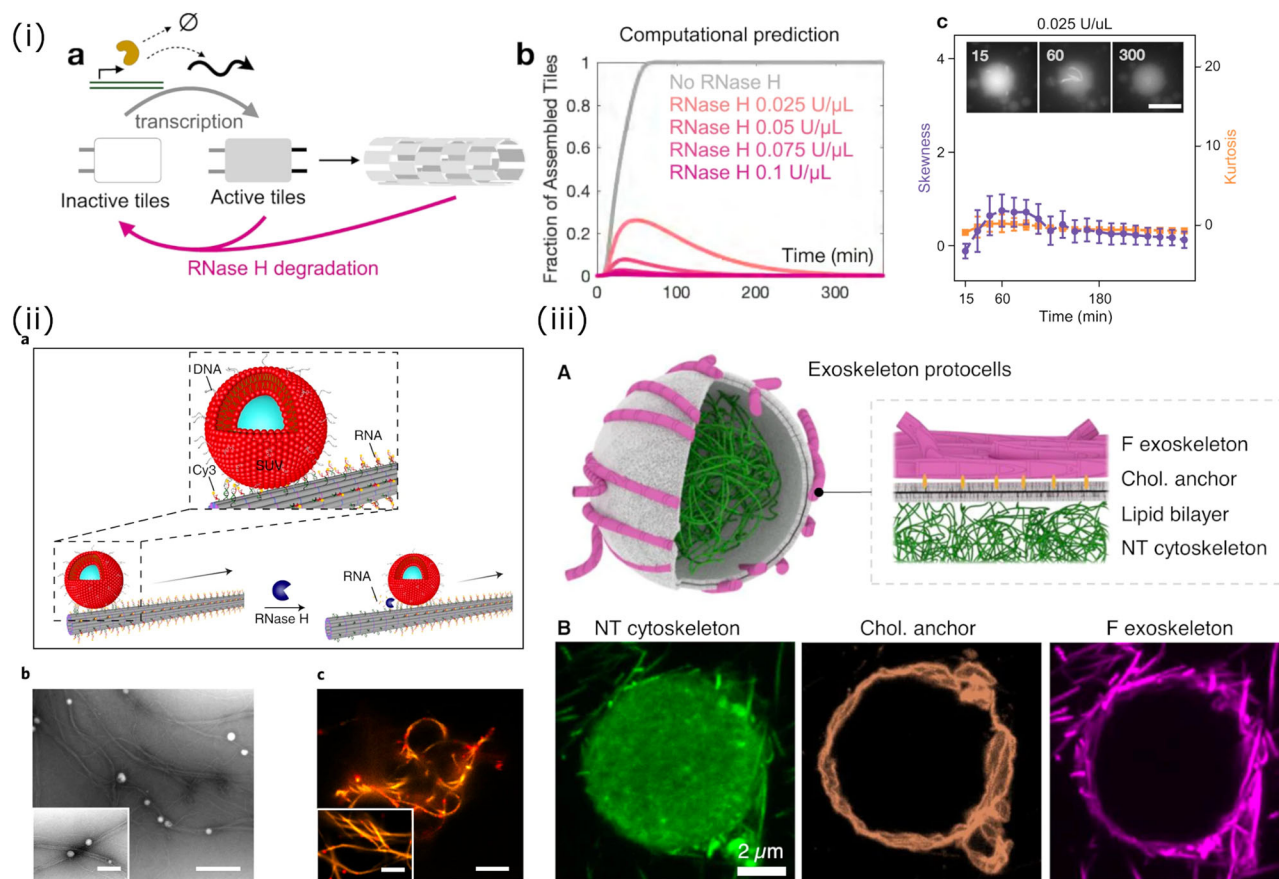Thus, the authors could study the binding and transport of organic (i.e.,

Fig. 4 | Functional roles of artificial fibrillar networks under confinement.
i Enzymatic control over the assembly and disassembly of DNA-RNA fibres in aqueous droplets: **a** schematic representation of the reactions yielding the transient formation of nanotubes, **b** theoretical prediction of the population of nanotubes with varying amounts of RNase, and **c** fluorescence microscopy images (scale bar = 20 μm) and evolution of the fibre population with time. Reprinted with permission from Springer Nature[86]. **ii** Guided cargo transport along DNA filaments confined in aqueous droplets: **a** Scheme of the transport mechanism controlled by RNase, **b** TEM images (scale bar = 500 nm, inset = 200 nm), and (**c**) stimulated emission depletion micrograph (scale bar = 5 μm, inset = 2 μm) of the vesicles attached to the filaments. Reprinted with permission from Springer Nature[88]. **iii** Protocell with programmed cyto- and exoskeleton connected through cholesterol lipid anchors: **A** model of the proposed structure and **B** confocal microscopy images of the cytoskeleton at the vesicle cortex (green), cholesterol anchors at the bilayer (orange), and exoskeleton filaments (pink). Reprinted with permission from Springer Nature[91].

vesicles) and inorganic (i.e., nanoparticles) cargos along the DNA-based filaments (Fig. 4ii). This approach required the addition of RNase to hydrolyse the DNA-RNA hybrids, which promoted the rolling of the cargos and, concomitantly, the guided transport along the DNA fibres. This system hence demonstrates the possibility of developing programmable fibrillar networks, which could assemble and disassemble on demand, with specific functions that mimic natural cytoskeletons, such as the autonomous transport of chemical entities and lower-order assemblies[11,89]. The implementation of fibrillar networks that could transport molecules, both inside and across droplets, could also lead to the development of communicating protocell/tissue models.

While the previous examples explore the behaviour of fibrillar networks in discrete compartments, one key aspect of biological cells is their ability to associate in complex scaffolds and tissues[90]. In this line, nucleic acid nanotechnology was exploited by Arulkumaran et al. to develop functional fibre assemblies confined within giant unilamellar vesicles (Fig. 4iii)[91]. The size and morphology of the assemblies could be controlled by electrostatic condensation, ranging from flexible nanotubes to rigid hierarchical fibres based on counterion concentration (i.e., Mg^{2+}). The system responded differently to changes in osmotic stress: while the nanotubes were barely affected by high glucose concentrations, the higher-order fibres disassembled at high osmolarity. DNA anchoring pendants allowed the complementary association of filaments by incorporating cholesterol-functionalised fibres that adsorb into the

internal membrane leaflet. This strategy was exploited to wrap DNA fibres at the outer part of the vesicle membrane, mimicking the exoskeletal structures observed in certain living organisms[92]. Importantly, the interactions between external frameworks could be exploited to control the organisation of the protocells and promote association into communities and prototissues. Although the formation of coacervate-based prototissues has been reported[93], lipid boundaries are yet to be interconnected through fibrillar bridges while retaining structural integrity. Being membrane-less compartments, coacervates allow inter-droplet connection through fibres without breaking a lipid-aqueous interphase[55]. Such fibrillar connectors between droplets could mediate chemical information exchange across the protocell community as a mimic of a natural endocrine system[81].

Outlook

The development of fibrillar networks with emergent properties in aqueous confinement can provide a versatile tool to mimic the variety of essential processes operating in living cells. Protein condensates obtained through LLPS of natural biomolecules have been shown to localise and regulate the formation of fibrillar structures in cells, where the absence of a membrane allows a dynamic exchange of guest molecules with the cytosol[32,94]. Inspired by these natural events, the roads ahead in the field of in-coacervate fibrillation can be defined from a functional perspective, paving the way for the future development of minimal protocell actuators.

Of particular interest are systems where the same component undergoes LLPS to subsequently form supramolecular assemblies in confinement. This hierarchical process has been identified in cells[53], and mimicked by synthetic monomers to study the interactions and structural determinants that govern coacervate-to-fibre transitions[55,57]. The overall balance of supramolecular interactions, e.g., attractive (hydrophobic effects, π-π stacking, and electrostatic forces) versus repulsive (electrostatic and steric hindrance) interactions, defines the transition from solution to a coacervate phase and, ultimately, to the assembly of fibres. As shown by Lipiński et al., solvophobic effects can induce associative LLPS, but unless directional interactions (e.g., hydrogen bonds) are incorporated to guide the linear propagation of supramolecular fibres[57], monomers will assemble amorphous aggregates within coacervates, as also shown in the assembly of elastin-like proteins[54]. Therefore, new monomer designs should consider adjusting the supramolecular interactions (i.e., attractive *versus* repulsive) with the directionality imposed by molecular design and elongation forces (e.g., by chirality or π-π stacking).

The future development, and perhaps combination, of different synthetic monomers (e.g., peptides, DNA, and other small molecules) provides a large toolbox of non-covalent interactions to rationally design the assembly process and its dynamics, giving access to emergent functions at the chemical, mechanical and biological level. In addition, non-covalent interactions can be easily reversed by displacement or competitive binding, as exemplified for DNA nanotubes[84,86]. These features can be extended to the formation of complex coacervate systems, where non-covalent phenomena (e.g., electrostatic and hydrophobic) define their physicochemical properties and their ability to selectively concentrate molecular and supramolecular entities[2,95,96]. Therefore, there is great potential in artificial self-assembled systems to mimic natural cell behaviour and to access new functions through the de novo design of fibrillating building blocks.

Systems chemistry studies the evolution of supramolecular networks that are controlled by energy inputs, such as chemical, thermal, or light sources[97,98]. The self-assembly into one-dimensional filaments provides an excellent opportunity for certain high-energy monomers to store and transfer chemical information. The coupled processes of energy absorption and self-assembly require multiple components that act simultaneously, which can be complicated to replicate and control in the bulk solution. The alternative study of aqueous compartments as supramolecular assembly hubs has provided some fundamental understanding in stimuli processing and the resulting role of assembled structures[81,88]. However, fibre-mediated signal processing in coacervate systems, particularly between different droplets, remains challenging at the present time. Thus, the development of minimalistic and reproducible synthetic systems capable of transferring chemical signals trough supramolecular self-assembly can shed light on the transition from isolated primitive cells to archaic cell communities, also guiding future developments and applications of synthetic cells in biomedicine[90,99].

A critical aspect of living matter is the communication and functional coordination between compartmentalised entities. The modulation of the collective behaviour of cells develops into the creation of tissues, which can be replicated in artificial mimics in the form of prototissues. Prototissues are constituted by interconnected protocells that display a characteristic collective behaviour. Despite the promising results found in aqueous-in-oil droplets and membrane-based compartments[91,93], more studies are required to understand and exploit the potential collective behaviour of membrane-less coacervate systems[100]. The development of exoskeletal networks beyond the volume of the compartment could, in principle, interconnect LLPS condensates into sophisticated protocell networks that yield synergistic function. As discussed, it is highly likely that the projection of fibrillar networks outside coacervates will be easier than in membrane-based compartments. The study of fibrillating coacervates may spark new ideas in early pluricellular forms and complex protocellular networks.

One of the great challenges in modern chemistry and protocell development is the assembly of a fully functional cell from synthetic chemical precursors[101]. In this pursuit, simple protocellular systems -like the confined fibrillators covered here- have demonstrated that the structural and biochemical complexity of living cells are not always required to emulate some of their functions. Examples in the literature include minimal proto-phagocytes capable of capturing pathogenic bacteria[102], and pancreatic-mimicking protocells that release insulin in response to glucose levels[103]. These systems demonstrate the therapeutic potential of protocellular therapies for cell replacement and biomedical engineering[104]. The high permeability of coacervates poses an advantage over membrane-delimited protocells, like more permissive molecular exchange with their environment, which is yet to be exploited in such biomedical settings[105]. The therapeutic application of stimuli-responsive fibrillating protocells opens exciting opportunities in biomedicine, with potential applications as organised prototissues[91], regulated enzyme depots[106], or antimicrobial neutrophil-like extracellular traps[107]. Combined progress in rational fibrillator design and stimuli-responsive coacervate formulation can crystallise in a collection of functional cell mimics with unprecedented clinical and biotechnological applications.

The overlap between systems and supramolecular chemistry generates opportunities for the development of minimal cells and tissues, as well as building a fundamental understanding of the possible origins of life. Coacervates play a central role in these advances, as they allow the confinement of complex chemical reaction networks and self-assembly processes spontaneously. The plethora of opportunities for the de novo creation of fibrillating monomers and coacervate phases with controlled properties (e.g., polarity, density, permeability, etc.) help unravel the interactions that regulate the proliferation and decay of confined assemblies. Early examples of energy dissipation and mechanical responses in coacervates can already be found in the literature[42]. Overall, these selected reports encourage further studies on compartmentalised self-assembly and biomimetic engineering, bringing together different scientific disciplines and expertise closer towards a deeper understanding of the transition from inanimate molecules to coordinated living matter. Both basic and applied science will benefit greatly.

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Author contributions

All authors discussed together the manuscript structure and key discussion points. A.S.-F. drafted a first version of the manuscript with contributions from all authors during writing and revisions.

Competing interests

The authors declare no competing interests.

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