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Tailoring the appearance: what will synthetic cells look like?

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Abstract

Recently, the bottom-up assembly of a synthetic cell has emerged as a daring novel approach that can be expected to have major impact in generating fundamental insight in the organization and function of actual biological cells, as well as in stimulating a broad range of applications from drug delivery systems to chemical nanofactories. A crucial feature of any such synthetic cell is the architectural scaffold that defines its identity, compartmentalizes its inner content, and serves as a protective and selective barrier against its environment. Here we review a variety of potential scaffolds for building a synthetic cell. We categorize them as membranous structures (liposomes, fatty acid vesicles, polymersomes), emulsions (droplets and colloidosomes), and membrane-less coacervates. We discuss recent advances for each of them, and explore their salient features as candidates for designing synthetic cells.

Keywords: synthetic cell, liposomes, fatty acid vesicles, polymersomes, droplets, colloidosomes, coacervates.

The quest for the creation of a synthetic cell from molecular components is receiving increasing attention from diverse scientific communities. While there is not yet a generally accepted consensus on the concept of a synthetic cell, or even of life itself, we adopt a pragmatic definition of synthetic cells as 'entities that can autonomously replicate both its information-carrying molecules and the container in which these molecules reside, and that can undergo Darwinian evolution' [1]•. Synthetic cells will likely be minimal and simple as compared to biological cells, and will be supported by a highly specific surrounding environment. Although interesting theories for radically different life-forms like surface metabolites [2] and cellular automata [3] have been proposed, here we focus on tangible three-dimensional cell-like objects that can potentially serve as an architectural scaffold for future synthetic cells.

An ideal scaffold should, at minimum, exhibit a number of properties: (i) It should be compatible with biological machinery (biocompatible). (ii) It should be selectively permeable (allowing nutrients and waste products to pass through while retaining indispensable macromolecules). (iii) It should be self-sustaining, with a regulated production and degradation of essential molecules, and be compatible with a life-supporting minimal form of metabolism. (iv) It should be capable of undergoing growth-division cycles. While modern biological cells are the result of billions of years of evolution with historic constraints set by natural environments, synthetic cells may be built using raw materials that can be chosen from a much larger catalog, e.g., mixing components from different organisms and including non-biological elements such as biomimetic nanostructures and nature-inspired synthetic materials.

Note that efforts to establish synthetic cells go significantly beyond approaches that have been published in the origins-of-life field. Both in origin-of-life and synthetic-cell research, one seeks to devise a minimal system of components that jointly leads to live cellular objects. However, while synthetic cell proposals utilize the protein machinery of modern cells as well as synthetic non-natural components, a crucial constraint for the origin-of-life research is that protocells need to be built from prebiotically

plausible components and mechanisms, which limits research on such protocells mainly to fatty acid vesicles.

In this review, we discuss a variety of scaffolds that potentially can be used as the basic carrier for synthetic cells. These can be categorized as membranous structures (liposomes, fatty acid vesicles, polymersomes), emulsions (droplets and colloidosomes), and coacervates (Fig. 1). We further provide examples of valuable combinations of these scaffolds (hybrid systems), and discuss crucial advantages and limitations of each system, with the hope to facilitate the challenging road towards synthetic cells.

Membranous scaffolds

Membranous scaffolds are self-assembled vesicles of amphiphilic molecules that form a sharp boundary layer between the aqueous cell interior and outer environment. The archetype example of such amphiphilic molecules are phospholipids, organic molecules that consist of a hydrophilic head group attached to two hydrophobic carbon chains. Above a certain concentration (termed the Critical Aggregation Concentration, CAC), which can be as low as 10^{-3}-1 nM [4], they spontaneously self-assemble into bilayers that can close to form water-filled spherical vesicles, commonly known as liposomes (Fig. 1A). Phospholipid bilayers form the basis for biological cell membranes across all domains of life [5]. As a result, liposomes are by far the most-studied scaffolds for synthetic cells and provide a standard to which other scaffolds are compared. Their shape is governed by a low bending rigidity of the membrane (~10^{-19} Nm), but they do not tolerate more than ~5% areal strain [6,7]. Liposomes are highly impermeable to charged ions but do allow permeation of small neutral molecules such as water, glycerol, and ribose [8].

Several straightforward liposome production methods (hydration, electroformation, inverted emulsion transfer) are well-established and routinely used. However, these bulk techniques have a low degree of control over important attributes such as lamellarity, encapsulation efficiency, size range, and

monodispersity [9]. Over the past decade, new microfluidic techniques like Octanol-assisted Liposome Assembly (OLA) (Fig. 1A) [10], and glass capillary-based methods [11] have emerged. These are relatively complex methods, but provide a high degree of control and hold great promise for producing and manipulating liposomes. One can reconstitute functional biological machineries inside liposomes, in order to understand them using a bottom-up approach. For example, by encapsulating key proteins that are involved in the bacterial division process (FtsZ and FtsA), septum formation and membrane constriction was reported (Fig. 2B, C) [12,13]•. A wide variety of physical and chemical strategies has been employed to induce division, and to some extent growth, of liposomes (Fig. 2D) [14,15]. Cytoskeletal polymers, which are responsible for maintaining the cell shape and intracellular transport, have proven particularly useful in controlling and reinforcing the liposome shape. Prominent examples are the reconstitution of a contractile membrane-anchored actin-myosin cortex, and the formation of stiff actin bundles inside liposomes that led to severe membrane deformation (Fig. 2E) [16,17]. Similarly, active microtubule-kinesin systems can be used to remodel the liposome shape (Fig. 2F) [18]•. Recently, controlled formation of vesosomes (liposome-in-liposome structures) was demonstrated, opening up the possibility for further sub-compartmentalization (Fig. 2G) [19]•. These vesosomes were shown to effectively encapsulate transcription and translation machinery inside these sub-compartments (Fig. 2H) [19].

Another important class of amphiphilic molecules are fatty acids, which are single aliphatic chains of up to thirty carbon atoms, with a carboxylic acid group on one end. These molecules are attributed a significant role in the membranes of early protocells, as they are prebiotically plausible. Hence, the extensive literature on origin-of-life research provides ample information on their possible use as membrane components of synthetic cells. Similar to phospholipids, fatty acids can also self-assemble to form vesicles (Fig. 1B). They do so within a more narrow pH range between 7-9 [20]. These vesicles can be robust up to temperatures of 90 °C depending on the length of the aliphatic chain [21].

The CAC of fatty acids ranges between 10 μM and 100 mM (i.e., orders of magnitude higher than that of phospholipids), making the vesicles comparatively less robust against dilution, but in turn, more dynamic in terms of the exchange of fatty acids between the vesicle and the aqueous environment. They are also significantly more permeable than liposomes to small cations [8]. While this increased permeability compromises the protective capabilities for the encapsulated content, it allows passive influx and efflux of nutrients and waste without any designated structures such as transmembrane proteins. Fatty acid vesicles have been extensively studied as they are hypothesized to be the plausible containers for the primordial cells on the early earth [8,22,23]. Multiple rounds of a rudimentary growth-division cycle of fatty acid vesicles have been demonstrated (Fig. 2I), where multilamellar fatty acid vesicles grew in size by the incorporation of fatty acids from micelles present in the external environment. This growth caused an increase in surface-to-volume ratio, resulting in the formation of long extended elongated filaments. Under application of a mild external shear flow, the filaments 'divided' into multiple quasi-spherical daughter cells [24]•. Division could also be induced photochemically, through a pearling-like intermediate state (Fig. 1J) [25]. It should be noted that fatty acids tend to precipitate in the presence of even a small concentration (few mM) of divalent cations such as Mg^{2+} [26], which severely limits encapsulating active modern transcription-translation machineries, since they cannot function without Mg^{2+}. Furthermore, fatty acids inhibit the activity of many enzymes such as DNA and RNA polymerase [27] . Both effects discourage their use as the main scaffold for building a synthetic cell.

It is also possible to form hybrid vesicles, comprised of different amphiphiles, which have some important advantages. For example, it was shown that such hybrid vesicles exhibit a significantly increased tolerance for divalent cations, as compared to pure fatty acid vesicles [28]. In addition, it was shown that hybrid vesicles can grow by the uptake of fatty acid micelles and vesicles, as a result of both entropic and chemical effects (Fig. 2K) [22,29]. Next to phospholipids, it is also possible to incorporate

fatty acid glycerols and fatty alcohols into fatty acid bilayers to tune their physical properties (e.g. CAC, pH range, thermostability, permeability, and tolerance for divalent cations) [28,30].

Inspired by these vesicles based on natural amphiphiles, synthetic amphiphilic macromolecular block copolymers were shown to similarly form vesicles. These are termed polymersomes, ranging in diameter from tens of nanometers to micrometers (Fig. 1C) [31,32]. The most frequently used polymers for forming polymersomes are diblock copolymers (consisting of a hydrophilic and a hydrophobic domain) and triblock copolymers (with two hydrophilic domains separated by a hydrophobic domain), although other varieties like multiblock-, grafted- and hyperbranched copolymers can also be used [31]. A major advantage of polymersomes over liposomes is the wider variety of building blocks to choose from, which can be beneficial to tune properties such as thickness, stability, and permeability [33]. The molecular weight of the copolymers is typically an order of magnitude higher than that of lipids, which results in a lower CAC and thicker membranes, making polymersomes more stable in environments of low polymer concentrations and against applied external forces. Interestingly, is has been shown that transmembrane proteins, such as bacteriorhodopsin and F_0F_1-ATP synthase, can be incorporated in polymersome membranes, while retaining their function (Fig. 2L) [34]. Also, membrane-interacting proteins, such as MreB, could be expressed in polymersome lumen and could be further localized to the membrane (Fig. 2M) [35]. The biocompatibility is further highlighted by forming hybrid vesicles, composed of both lipids and block copolymers; such hybrid vesicles were shown to undergo fission (Fig. 2N) [36]. Similar to vesosomes, it is possible to produce polymersomes-within-polymersomes using a microfluidic approach, with controlled and sequential dissociation of different bilayer membranes by tailoring their compositions (Fig. 2O) [37]. Such programmability may provide new opportunities for controlled drug delivery systems. Lastly, fusion and fission of polymersomes has been observed and bears close resemblance to that of natural membranes (Fig. 2P) [38]•. All these demonstrations of

biocompatibility portray a strong potential to design synthetic cells with scaffolds made up of synthetic polymers.

Emulsion scaffolds

Life, as we know it, is water based. One can, however, also think of synthetic cells suspended in an organic phase such as oil. Water-in-oil emulsions are commonly referred to as droplets (Fig. 1D). These well-studied systems can be mass-produced in a highly controlled and robust manner using microfluidic techniques. For example, multi-component droplets can be produced, which may be used as complex micro-reactors (Fig. 3A) [39]. The droplets can also be compartmentalized by forming aqueous two-phase systems within them, that can be used for cell-free expression of proteins [40]. Droplets, having a lipid monolayer at the interface, have been extensively used to study the behavior of biological machinery in vitro, as they provide an excellent encapsulation efficiency of water-soluble biomolecules as well as membrane proteins (provided no extensive transmembrane insertions are essential). For example, crucial protein machineries (FtsZ and MinCDE) involved in bacterial cell division were reconstituted in droplets, where they exhibit the expected spatial anticorrelation between them (Fig. 3B) [41]•. Interestingly, division of droplets induced by chemical reaction at the water-oil interface was demonstrated, resulting in tunable, equal or unequal, daughter droplets [42]. Using three-dimensional printing of picoliter-sized aqueous droplets, tissue-like materials can be created and functionalized with membrane proteins allowing directed communication along specific path (Fig. 3D) [43]•. Such 'synthetic tissues' can display simple neuron-mimic transmission using a light-activated DNA promoter to turn on the expression of genes synthesizing membrane pores [44]. Such emulsion-based synthetic tissues appear very promising as a valuable extension of synthetic cells.

Droplets can also serve as templates that can subsequently be coated with microscopic colloidal particles that self-assemble at the water/oil interface – giving rise to what are known as Pickering

emulsions. Their size, permeability, and mechanical strength can be precisely controlled (Fig. 1E) [45]. When the particles assembling at the interface are inorganic colloids (e.g. colloidal silicate or polystyrene latex particles), the resulting structures are termed colloidosomes (Fig. 3E). These boundary-forming particles can be further interconnected so that the colloidosome can subsequently be transferred into an aqueous phase (Fig. 3F) [46]. Thermosensitive colloidosomes were shown to undergo oscillatory shape changes such as swelling-shrinking and buckling-debuckling [47]. Moreover, a chemically-induced primitive form of self-reproduction of colloidosomes was shown, where the hydrolysis of tetramethoxysilane at the water/oil interface produced silica oligomers (forming a shell at the interface) and methanol (increasing the colloidosome volume) [48]•. This led to an increase in the internal pressure of the original colloidosome until the shell did burst and new droplets budded out, which subsequently got coated with hydrophobic colloids from the surrounding organic solvent, and thus formed daughter colloidosomes (Fig. 3G). Along similar lines, a primitive form of artificial phagocytosis was demonstrated where self-propelled magnetic Pickering emulsion droplets engulfed smaller silica colloidosomes, enabling selective delivery and release of water-soluble payloads (Fig. 3H) [49]. Such chemical-reaction-based mechanisms highlight the potential of purely artificial systems in mimicking life-like properties. Alternatively, instead of using inorganic nanoparticles, Pickering emulsions can also be constructed with appropriately modified proteins to form what are known as proteinosomes [50]. Additionally, one can also consider microcapsules, which are water-filled vesicles with a solid shell made up of polyelectrolytes, as potential synthetic cell containers. Microcapsules can be efficiently generated using microfluidics, and the mechanical and physiochemical properties, such as elasticity and permeability, can be readily controlled [51–53].

Coacervates

Although it is natural to think of a container with a well-defined physical membrane, cells also contain many membrane-less structures (germ granules, Cajal bodies, the nucleolus) formed by a rather counterintuitive liquid-liquid demixing process [54,55]. The driving force behind such phase separation is a higher affinity of particular components to each other, compared with the rest of the system components, thus overcoming the entropy-driven tendency to mix [56]. Coined coacervates, such structures are usually formed through the attractive electrostatic interactions between two or more oppositely charged polyelectrolytes (polypeptides, polynucleotides, polysaccharides) [57], or small multivalent molecules (e.g. ATP [58] and spermidine [59]) (Fig. 1F). However, even like-charged polyelectrolytes can form coacervates by means of cation-π interactions [60].

Almost a century ago, Alexander Oparin had proposed coacervates as the progenitors of modern cells, which would serve as scaffolds facilitating the chemical evolution that eventually would lead to the emergence of life on earth [4,61]. Currently, coacervates are receiving renewed attention in the context of cell-biology and protocell research, particularly because they can provide suitable environments for biochemical reactions by creating highly crowded local environments, resembling in vivo conditions [58]. Like liposomes and emulsions, they can also be formed in a controlled way using microfluidics (Fig. 4A) [62]. Such synthetic coacervates can display rich dynamics under non-equilibrium conditions, such as vacuolarization, size and shape fluctuations, and chaotic growth and fusion (Fig. 4B) [63]. Coacervate formation can be manipulated by altering the interactions between the constituents, such as the phosphorylation-triggered formation and dissolution of RNA/peptide coacervates using a kinase/phosphatase enzyme pair [64]. Furthermore, a pH-triggered rudimentary metamorphosis was displayed in polymer-dipeptide coacervates where the micro-droplets transformed into aster-like micro-architectures (Fig. 4C) [65]. Recent theoretical work demonstrated the possibility of repetitive cycles of growth and division of coacervate-like systems, by maintaining them in a chemically driven non-

equilibrium state (Fig. 4D) [66]•. These examples highlight the fact that, surprisingly, a membrane is not per se a mandatory requirement for synthetic cells, which opens up new avenues for exploration.

Coacervates can be readily combined with other scaffolds to form hybrid containers. For example, coacervation was induced in osmotically shrinking droplets, concentrating the encapsulated cell lysate within them, and substantially increasing the transcription rate (Fig. 4E) [67]•. Furthermore, reversible thermal-responsive coacervation was demonstrated inside liposomes, showing concomitant capture and release of DNA and transcription inside the formed coacervates (Fig. 4F) [68]. Fatty acids were shown to assemble around coacervates to form a multilamellar membrane, with a demonstration of fusion and growth of such hybrid protocells (Fig. 4G) [69]. Using a surface-templating procedure, spontaneous formation of multi-layered micro-compartments with a semipermeable outer membrane, a sub-membrane coacervate shell, and an inner aqueous lumen was achieved. In these containers, the coacervate shell could sequester a variety of molecules (dyes, proteins, nanoparticles), which is potentially useful for spatially localizing and coupling enzyme cascade reactions (Fig. 4H) [70].

Discussion

Building a synthetic cell from the bottom up is a truly exciting challenge in synthetic biology. If accomplished, we expect that it will contribute greatly to the basic understanding of how cells work, while also invigorating applications such as bioreactors and drug-delivery systems [71–74]. Here we reviewed a variety of scaffolds (membrane-based, emulsion-based, and membrane-less) that potentially would be suitable for the task.

Liposomes are clearly closest to modern cell membranes, as they are biocompatible, capable of growth and division, and allow metabolism. However, they require sophisticated machineries such as transmembrane protein channels to control a flux of metabolites. Fatty acid vesicles are less stringent regarding the flux across the membrane, but also much less stable. This compromised stability may be

overcome by addition of other amphiphiles, in particular lipids, thus creating enhanced hybrid vesicles. Polymersomes are a very versatile and surprisingly biocompatible, but so far comparatively less heavily researched and understood. Given their great potential, further study of their use as scaffolds for synthetic cells seems worthwhile.

Fundamentally different to these membranous scaffolds are water-in-oil emulsions which are relatively easy to produce, can act as excellent bioreactors, and have a very high encapsulation efficiency. However, they require a hydrophobic environment that possesses a more limited biocompatibility and the achievement of autonomous cycles of growth and division remains a technical challenge. Droplets can be coated at the interface to generate a cross-linked shell of micro- or nanoparticles to form colloidosomes or proteinosomes, which can be subsequently transferred into an aqueous environment. Taken together, we feel that creating synthetic cells based on emulsion scaffolds will be challenging.

Coacervates are recently rediscovered membrane-less entities, that represent a unique alternative to the other scaffolds, as they provide compartmentalization without a well-defined boundary. As a downside, they inherently fail to provide any selective permeability. However, multiple efforts have already shown that they can be readily combined with other scaffolds, and hence we feel that they have a huge potential to be utilized in future synthetic cells. It will be interesting to see how the field of synthetic biology evolves in the coming years. We speculate that eventually a hybrid system of multiple types of scaffolds, in which strengths are combined and weaknesses are covered, may emerge as the most robust and versatile carrier for future synthetic cells.

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Conflict of interest

Authors declare no conflicting financial interests.

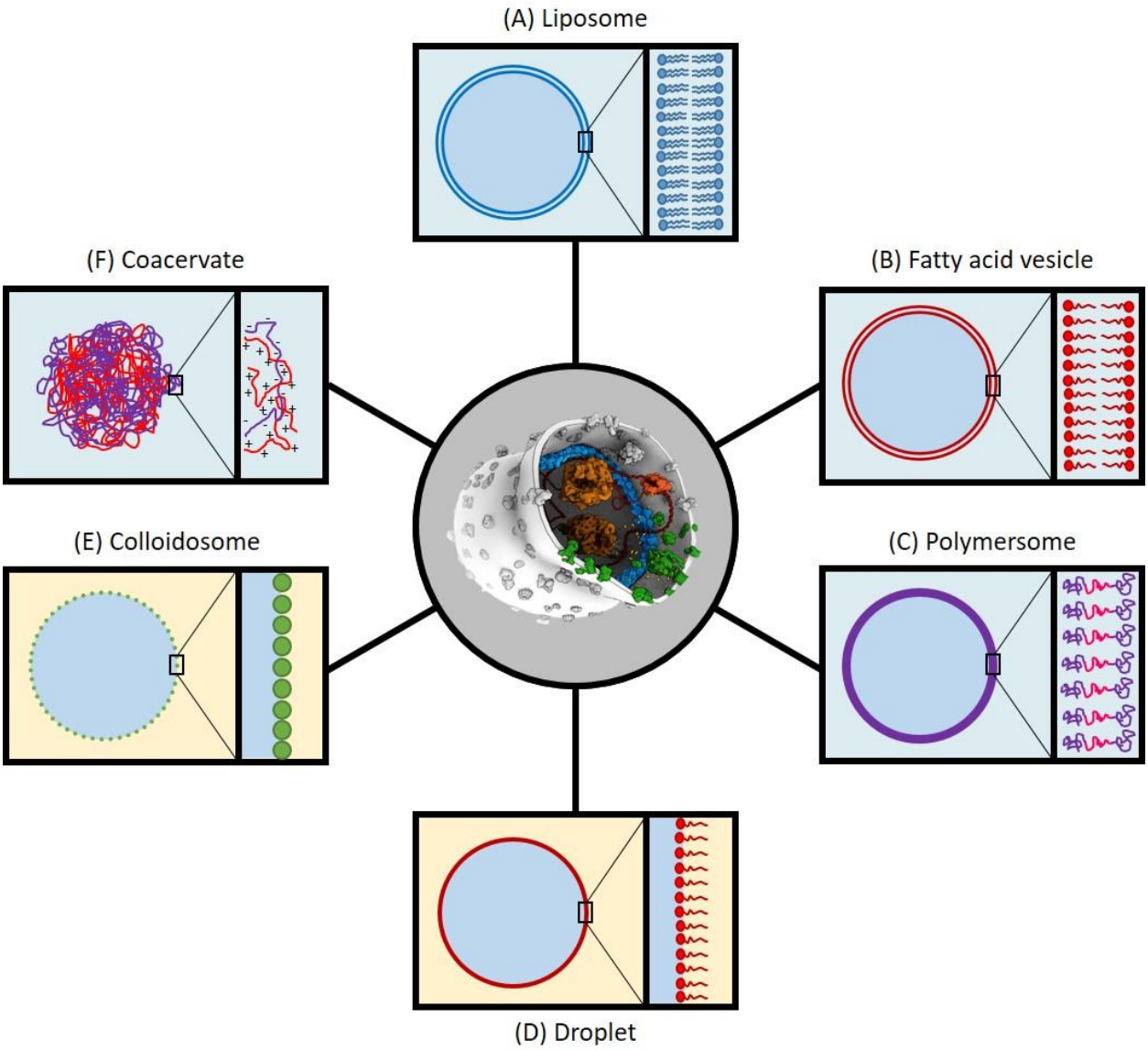


Figure 1: Schematic overview of promising scaffolds for synthetic cells. In clockwise direction, starting from the top, (A) liposome, (B) fatty acid vesicle, (C) polymersome, (D) droplet, (E) colloidosome, and (F) coacervate. Each of these may be suitable for creating a synthetic cell (cf. the artist impression in the center image). The zoom-in on the right of each panel shows the molecular representation of the boundary of each scaffold. Center-image credit Graham Johnson.

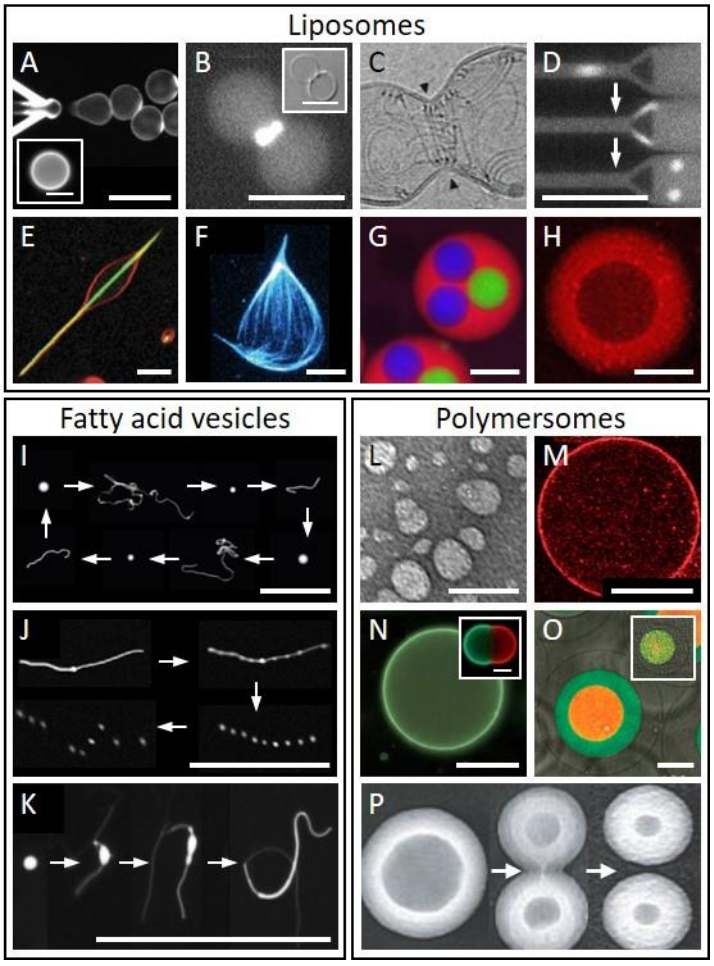


Figure 2: Membranous scaffolds: liposomes, fatty acid vesicles, and polymersomes. (A) Liposome production using Octanol-assisted Liposome Assembly (OLA). Inset shows a fully matured OLA-formed liposome. (B) GUV constriction resulting from an encapsulated Z-ring. Inset shows the corresponding bright-field image. (C) Transmission electron cryomicroscopy showing SUV constriction (denoted by arrowheads) due to the encapsulation of FtsZ and FtsA. FtsZ filaments are clearly visible at the constriction site. (D) Mechanical division of liposomes using microfabricated splitters. (E) Severe liposome deformation due to encapsulation of fascin-induced actin bundles (green: actin, red: membrane). (F) Dynamic liposome deformation resulting from an active, membrane-anchored microtubule-kinesin system. (G) Vesosome showing three stable liposomes encapsulated within a bigger liposome. (H) A functional in vitro transcription/translation system selectively encapsulated within the exterior liposome of a vesosome. (I) Primitive growth-division cycle of fatty acid vesicles. (J) Photochemically driven pearling and division of tubular fatty acid vesicles. (K) Phospholipid-triggered growth of lipid/fatty acid vesicles through the uptake of pure fatty acid micelles present in the environment. (L) Transmission electron micrograph of polymersomes with bacteriorhodopsin molecules embedded in the bilayer. (M) MreB protein expression inside a polymersome and its localization to the membrane. (N) Hybrid vesicle showing a homogenous distribution of phospholipids and synthetic copolymers within the membrane. Inset shows their phase separation and concomitant vesicle deformation when a different ratio of lipids to copolymers is used. (O) Polymersome-in-polymersome-in-polymersome showing encapsulation of different dyes within them. Inset shows mixing of the dyes

due to membrane rupture. (P) Division of a polymersome into two daughters. Panels are adapted as follows: A, Cees Dekker lab; B, [12]; C, [13]; D, [15]; E, [17]; F, [18]; G and H, [19]; I, [24] ; J, [25]; K, [22]; L, [34]; M, [35]; N, [36];O, [37]; P, [38]**.** Permission requests pending. Scale bars: 50 µm (A, D, G, H, I, J, K, M, N, O); 5 µm (A inset, B, B inset, E, F, N inset), 100 nm (L).

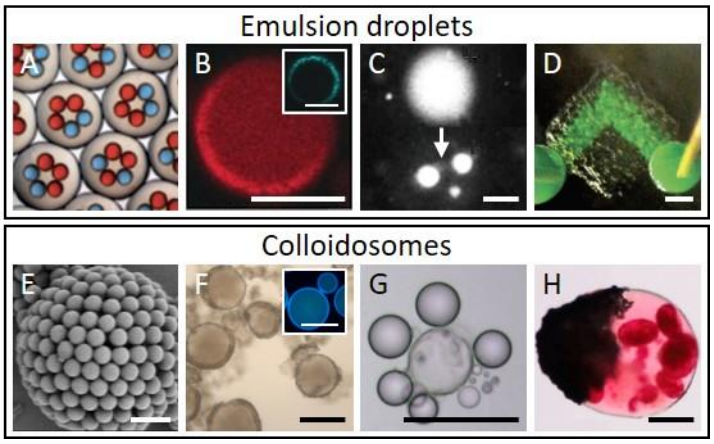


Figure 3: Emulsion-based scaffolds: droplets and colloidosomes. (A) Double-emulsions produced using microfluidic technology, showing blue and red labelled aqueous droplets contained inside oil-in-water droplets. (B) Snapshot of a droplet showing Min protein oscillations at the lipid monolayer. Inset shows the corresponding FtsZ localization in the half where the MinC concentration is low. (C) Interfacial-chemistry-triggered droplet division into multiple daughter droplets. (D) Printed network of droplets, where the green droplets contain membrane pores, enabling ionic current between the electrode-impaled drops. (E) Scanning electron micrograph of a colloidosome. (F) Cross-linked colloidosomes in an aqueous environment. Inset shows the colloidosomes in oil, retaining water-soluble dye. (G) Division of a colloidosome (in the center) into multiple daughters. (H) Artificial phagocytosis of small silica colloids (pink) by a bigger self-propelled Pickering emulsion microdroplet. Panels are adapted as follows: A, [39]; B, [41]; C, [42]; D, [43]; E, [45]; F, [46]; G, [48]; H, [49]**.** Permission requests pending. Scale bars: 20 µm (B, B inset, C); 200 µm (D, F, G, H); 2 µm (E).

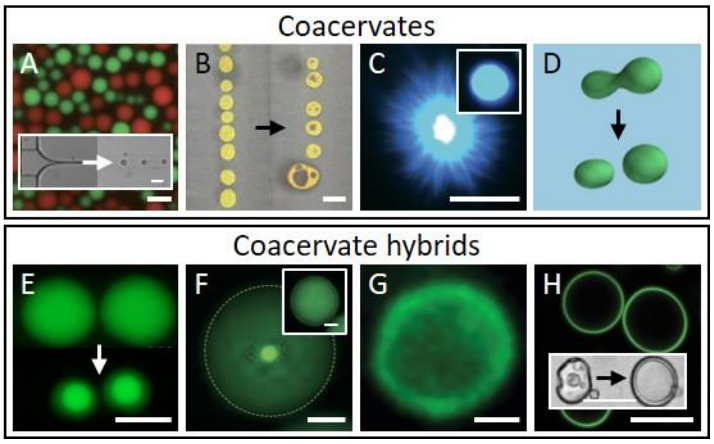
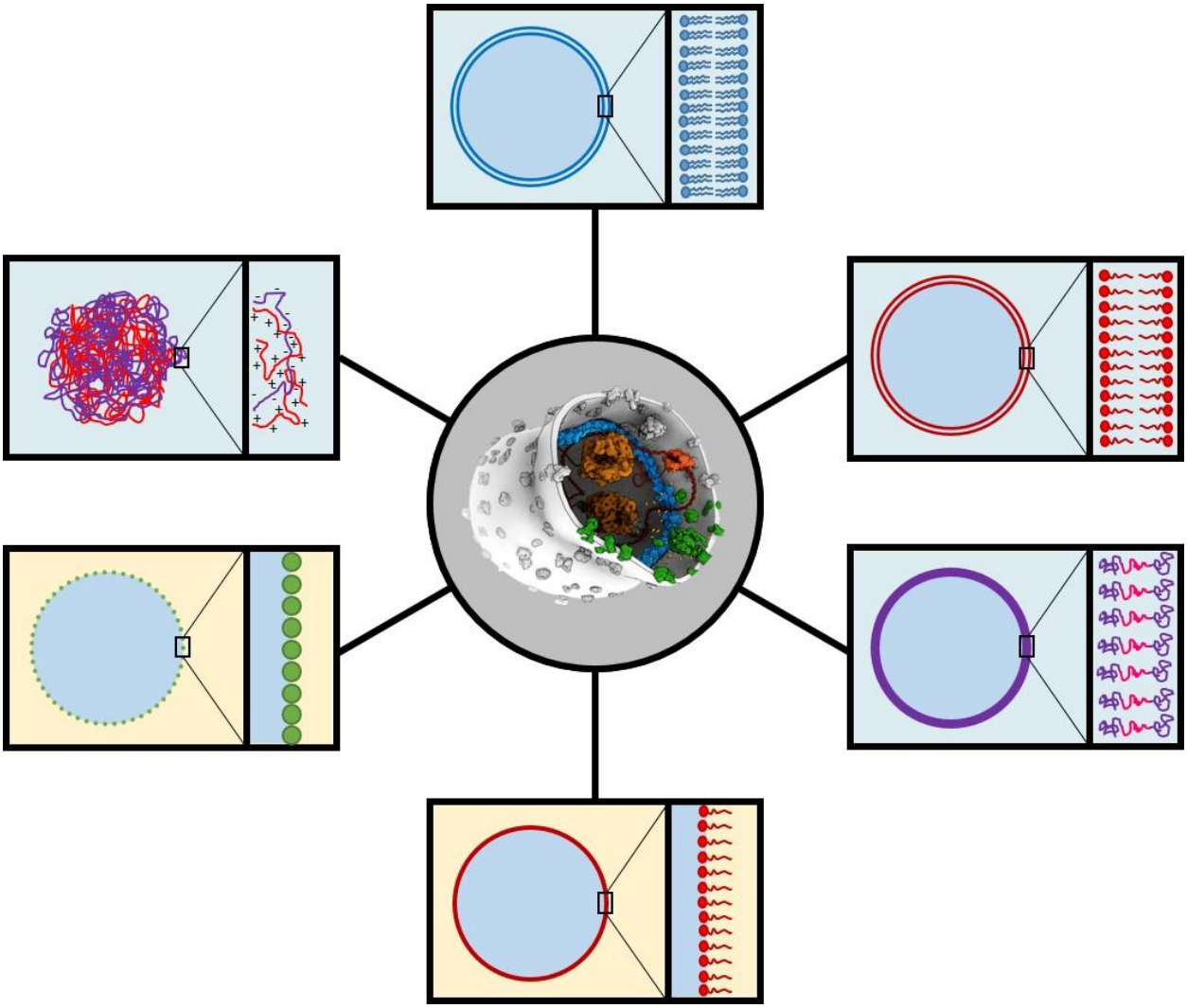


Figure 4: Coacervates as membrane-less scaffolds, and coacervate hybrids. (A) Dual coacervate formation (red and green spheres) using microfluidic technology (inset). (B) Coacervates in a microfluidic channel undergoing vacuolarization in response to an electric field. (C) Aster-like structure formation of an initially spherical coacervate (inset), in response to the lowering of pH. (D) Simulation showing growth and subsequent division of a phase-separated coacervate that is kept out of equilibrium. (E) Coacervate formation inside shrinking droplets, as a result of increase in the concentration of ions and polymers. (F) Temperature-dependent coacervate formation inside a liposome. Inset shows the coacervate dissolution when the temperature is lowered. (G) Fatty acid assembly at the coacervate boundary, visualized through a fluorescent membrane dye. (H) Three-tiered hybrid protocell, retaining a fluorescent dye in the coacervate shell. Inset shows its formation starting with a pure coacervate. Panels are adapted as follows: A, [62]; B, [63]; C, [65]; D, [66]; E, [67]; F, [68]; G, [69], H, [70]**.** Permission requests pending. Scale bars: 20 µm (A, A inset, B, C, E, F, H); 10 µm (H inset); 0.5 µm (G).

Graphical abstract



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