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Fatty acid vesicles and coacervates as model prebiotic protocells

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Abstract: The prebiotic organization of chemicals into compartmentalized ensembles is an essential step to understand the transition from inert molecules to living matter. Compartmentalization is indeed a central property of living systems. Fatty acids represent the simplest prebiotic amphiphiles capable of self-assembling into membrane-bound vesicles, and have therefore emerged as valuable molecules to create models of protocellular compartments. Here, the main experimental findings that support this idea are reviewed, together with approaches to increase the stability of fatty acid vesicles in adverse pH, salt or temperature conditions. Recent studies on the self-assembly of fatty acids into membrane-free coacervate micro-droplets are then discussed, providing a promising new paradigm for prebiotic compartmentalization. We last argue that the unique possibility of cycling between fatty acid vesicles and coacervates paves the way to an exciting new hypothesis for the emergence of the first living protocells.

Introduction

"What is Life?" is one of the biggest yet unanswered questions in science that has fascinated myriad philosophers and scientists for centuries. While providing a general and self-sufficient definition of Life has often turned out tedious and unsatisfactory,^[1-3] understanding how Life emerged from an ensemble of inert molecules has attracted growing interest in the scientific community in the past few decades.^[4, 5] This challenging but vibrant research field is exploring the origins of Life from a number of perspectives. Systems chemistry-oriented approaches aim at deciphering general physicochemical rules that guide the assembly of inert molecules – regardless of their prebiotic or biological relevance – into self-organized systems capable of life-like properties, such as energy-fuelled growth, self-replication and, ultimately, population-level Darwinian evolution.^[6-8] In comparison, prebiotic chemistry aims at rationalizing the historical emergence of increasingly complex molecules based on non-enzymatic reactions that could have occurred on the early abiotic Earth.^[9-12]

The idea that Life originated from the synthesis of organic compounds was first put forward by Haldane and Oparin,^[13] who argued that simple chemicals could have been initially synthesized in a prebiotic soup and then undergone a process of gradual chemical evolution. This hypothesis was later supported by the well-known Miller-Urey experiment that showed that amino acids could be synthesized from water and gases such as methane, ammoniac and hydrogen,^[14] giving rise to the field of prebiotic chemistry. Since then, simple amphiphiles such as fatty acids have been synthesized abiotically by Fischer-Tropsch chemistry,^[15-18] and the prebiotic synthesis of more complex molecules, including nucleotides and phospholipids, has also been demonstrated.^[19-24] But what would the first cells made of such prebiotic molecules would have looked like?

By analogy with modern cells, Haldane was also the first to suggest that Life originated within micro-containers^[13, 25] consisting of an aqueous lumen (in which prebiotic reactions could have occurred) and a physical boundary (to delimit an individuated system, restrict surface permeability and control chemical exchange with the environment). Increased knowledge on the cellular lipid membrane^[26] naturally led to the idea that prebiotic cells (or 'protocells') also possessed a lipid-based envelope.^[27] It was hypothesized that this membrane would yet have been much simpler than in

modern cells, and in particular would have lacked proteins or phospholipids. The discovery in the early 1970s that unsaturated fatty acids could form vesicles by self-assembling into bilayer membranes^[28] (initially called “ufasomes for unsaturated fatty acid liposomes”) suggested that prebiotic protocells may have been assembled from such simple single-chain amphiphiles. Other reports demonstrated that fatty acids with different alkyl chain lengths could also self-assemble into vesicles.^[29-35] The large variety of fatty acids found in ancient rocks^[36] further comforted the possible role of fatty acid vesicles as prebiotic protocells: the “lipid world”, or rather, the “fatty acid world”, was born.^[37]

Since then, extensive studies pioneered by the Luisi, Szostak and Deamer groups have explored the plausible role of fatty acid vesicles as prebiotic protocells capable of nutrient uptake, growth and division,^[38-40] and able to host RNA catalysis^[41] and self-replication.^[42, 43] However, fatty acid vesicles are only stable on a fairly narrow pH range,^[32, 44] and readily disassemble or precipitate in the presence of divalent cations^[35, 41] or at physiological salt concentrations,^[45] yet metal ions are often crucial for biopolymer catalysis and folding, and would have likely been present in prebiotic ponds. Other features of fatty acid vesicles include their usually relatively low encapsulation efficiency (suggesting that the probability for prebiotic ingredients to be encapsulated at sufficiently high concentrations would have been relatively low), and their limited permeability to charged or large polar species^[46] (which could have hampered nutrient uptake and waste disposal required to sustain vesicles growth or *in situ* molecular self-replication).

While the instability of fatty acid vesicles is inherent to the chemical nature of these single-chain amphiphiles, the low encapsulation yields and limited permeability are general features of membrane-bound compartments. An alternative protocell model was proposed at the beginning of the XXth century by Oparin as membrane-free microdroplets produced by complex coacervation between oppositely charged polyions.^[47] Such an associative liquid-liquid phase separation has since then been extended to a number of synthetic and natural polymers, and the viability of the resulting microdroplets as protocell models started to be explored.^[48-51] Significantly, despite their lack of a physical boundary, coacervate droplets are capable of spontaneously accumulating a number of solutes by equilibrium partitioning. This peculiar property offers an appealing response to the low encapsulation yield and limited permeability of vesicular compartments that could address the dilution problem on the early Earth (prebiotic chemicals were likely diluted, so reactions would have been unfavorable). Strikingly, amphiphilic molecules can also undergo an associative phase separation process, known as the clouding phenomenon, which produces membrane-free microdroplets able to spontaneously accumulate water-soluble and organic compounds.^[52] Recent studies have reported that fatty acids too could phase separate into coacervate droplets in the presence of specific counter-ions,^[53, 54] and even reversibly restructure into vesicles by changes in pH.^[53] So, could these new fatty acid coacervates be viable alternative prebiotic protocells?

After introducing some key features of prebiotic protocells, we briefly summarize general properties of fatty acid vesicles self-assembly and stability in water, and review experimental evidence that supports their role as prebiotic protocells. We then argue that the recently reported fatty acid coacervate droplets offer a promising new paradigm for prebiotic compartmentalization. Last, we discuss how the unique possibility of inducing reversible transitions between these two types of compartments paves the way to an exciting new hypothesis for the emergence of the first living protocells.

Prebiotic protocells

A protocell can be defined as the most primitive compartment that would have led to the emergence of a living cell. Based on seminal theoretical models of the simplest living chemical systems in the 1970s by Kauffman^[55] (autocatalytic set model), Eigen^[56] (hypercycle model) and Ganti^[57] (chemoton model), it is now acknowledged that a living protocell should contain (at least) three key ingredients: a compartment, to delimit the inner self from the outer environment; a metabolism, defined as an ensemble of out-of-equilibrium reactions coupled to an external source of energy; and a process of self-replication by which the protocell makes copies of itself based on error-prone duplication, paving

the way to population-level Darwinian evolution. The *de novo* self-assembly of a living protocell therefore requires the integration of these three modules to produce a self-sustained growing and duplicating minimal compartment able to store and transfer information to the next protocell generation. While such a complex, integrated system has not been realized yet, a few studies have started coupling two of the three key Life's ingredients.^[58]

Taking a step back, we focus here on the construction of prebiotically relevant compartments as the starting point to assemble living protocells. Compartmentalization is indeed crucial for the spatiotemporal coordination of matter and energy fluxes, since it allows co-localization of reactive partners, confinement and segregation of incompatible species, and sustainment of chemical gradients. The term 'protocell' has been used in the literature to designate various types of compartments with different levels of chemical and structural complexity, going from simple lipid vesicles, possibly hosting elementary chemical reactions, to protein- or particle-based capsules sustaining gene-mediated cell-free protein expression relying on complex supramolecular machineries. Here, we use the term 'prebiotic protocell' to specifically designate among these compartments those that are assembled from prebiotically relevant components, such as fatty acids or other low molecular weight molecules that could have been synthesized abiotically on the early Earth. In this sense, we define a (non-living) prebiotic protocell as a minimal compartmentalized chassis exhibiting rudimentary life-like features, such as selective biomolecule encapsulation, localized reactivity, or growth. With this definition in mind, we highlight below experimental evidence that supports the role of fatty acid-based compartments as models of prebiotic protocells.

Fatty acid vesicles as prebiotic protocell models

Self-assembly and stability of fatty acid vesicles

A fatty acid is a carboxylic acid bearing a long aliphatic chain (at least 4 carbon atoms) that is either saturated or unsaturated (i.e., containing one or more double C=C bonds). Fatty acids vesicles are membrane-bound compartments formed by closed fatty acid bilayers that delimit an aqueous lumen from a continuous aqueous phase (**Figure 1A,B,E**).^[59] Specific pH, concentration, temperature and salinity conditions that we briefly describe below need to be met to form stable fatty acid vesicles (**Figure 1F**).

The formation of fatty acid vesicles first requires close-to-equimolar concentrations of the protonated (neutral, -COOH) and deprotonated (ionized, -COO⁻) forms of the acid, which occurs on a defined pH range close to the fatty acids' pKa^[60] (**Figures 1E,F**). Higher pH values (pH ~ 10 > pKa) favor the formation of micelles of deprotonated fatty acid salts, while low pH values (pH ~ 7 < pKa) result in the precipitation of non-ionized fatty acids or their phase separation into oil droplets. In comparison, at intermediate pH values (pH ~ pKa), protonated fatty acids act as hydrogen bond donors to neighboring ionized fatty acids, which stabilizes bilayer membranes (**Figure 1E**).^[59] Compared to the typical pKa of a carboxylic group (~ 4.8), the pKa of fatty acids is shifted to higher apparent values^[31, 61] (typically ~ 8-9) due to the high local surface charge density and low local dielectric constant around the carboxylic groups in fatty acid self-assemblies.^[31] This high apparent pKa explains why fatty acid vesicles are only observed at a slightly basic pH.

A minimal concentration of fatty acid, known as the critical vesicle concentration (CVC), is also required to produce vesicles (**Figure 1F**). Below this concentration, fatty acids only exist as monomers or micelles in solution. The CVC typically falls in the mM to tens-of-mM range, and increases when the alkyl chain length decreases because longer chains favor more packed bilayers assemblies.^[32, 62] It is important to note that even above the CVC a substantial fraction of free fatty acid monomers are in equilibrium with vesicles.

Fatty acid vesicles are also highly sensitive to temperature (**Figure 1F**). High temperatures disrupt fatty acid vesicles integrity due to increased thermal agitation. Ionized fatty acids also exhibit a lower critical temperature, known as the Kraft temperature or Kraft point, below which they crystallize (precipitate). This temperature increases with the length of the alkyl chain and decreases when the number of unsaturation along the chain increases. Typically, the Kraft temperature falls below room

temperature for short chain fatty acids (< 10 carbon atoms), but is above room temperature for saturated long chain fatty acids, such as sodium myristate (14 carbon atoms, $T_{Kraft} \sim 40 \text{ }^\circ\text{C}$). Last, fatty acids crystallize at high salt concentrations or in the presence of low amounts divalent cations (**Figure 1F**).^[35, 41, 45]

When working above the CVC and Kraft temperature, fatty acid vesicles are spontaneously prepared by dissolving monomers or rehydrating a dry fatty acid film using an aqueous solution adjusted to $\text{pH} \sim 8.5$ or a buffered solution.^[62] An even simpler preparation technique is to decrease the pH of an alkaline aqueous dispersion of micelles under mechanical stirring.^[31] These approaches typically produce polydisperse multi-lamellar fatty acid vesicles. Extrusion through membranes or sonication allows the formation of small (SUV, $\sim 20\text{-}100 \text{ nm}$ diameter) or large (LUV, $\sim 100\text{-}500 \text{ nm}$ diameter) unilamellar vesicles. Although these compartments are much smaller than modern cells, they are still relevant to build protocells, as suggested by studies that showed that 100 nm liposomes could accommodate enough proteins and polynucleotides to sustain cell-free protein expression.^[63] High surface-to-volume ratio in these small compartments could have facilitated fluxes of metabolites to sustain chemical processes within the vesicles. Giant unilamellar vesicles with a size commensurate to modern cells (micrometer size) have also been produced using fatty acids, e.g., by extrusion through larger membrane pores^[64] or fusion of LUVs.^[65] In the following, we discuss general properties of fatty acid vesicles regardless of their size.

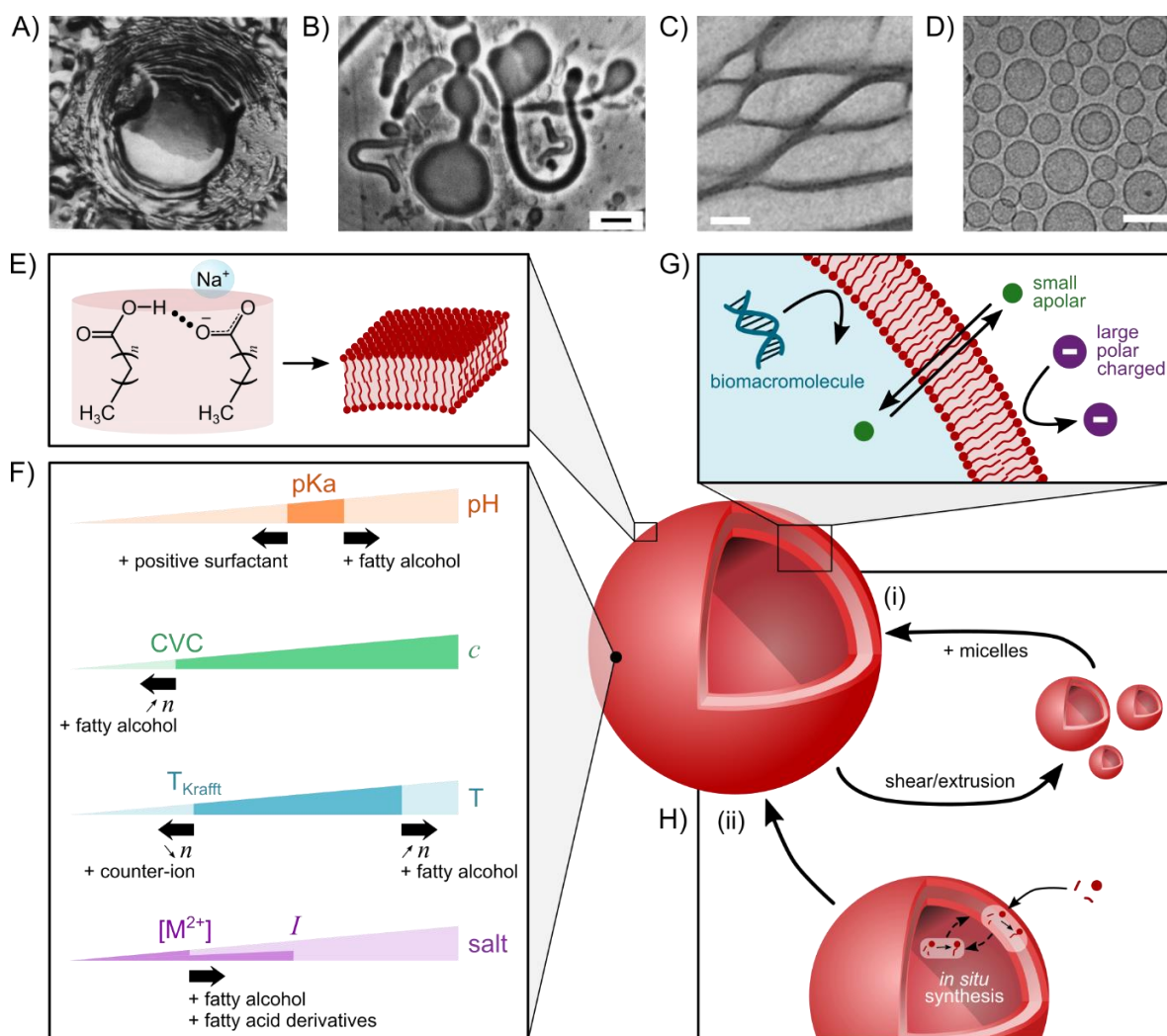


Figure 1. A) Freeze-fracture image of the first reported oleic acid/oleate multilamellar vesicle ($\times 41,650$). Adapted from reference [28] with permission from Springer Nature. Copyright © 1973, Nature Publishing Group. B) Optical microscopy image of a nonanoate/nonanoic acid vesicle. Scale bar, $1 \mu\text{m}$. Adapted from reference [32] with permission. Copyright © 2002 Elsevier Science B.V. C) Interconnected elongated micelles assembled from myristate/guanidinium hydrochloride observed by cryo-TEM. Scale bar, 100 nm . Adapted from

reference [74] with permission. Copyright © 2009 Elsevier Inc. D) Vesicles obtained by decreasing the pH of myristate/guanidinium micelles observed by cryo-TEM. Scale bar, 200 nm. Adapted from reference [76] with permission. Copyright © 2016, American Chemical Society. E) Scheme of the stabilizing hydrogen bond established between the fatty acid carboxylate and carboxylic forms in bilayers. F) Illustration of the pH, concentration, temperature and salt stability domains of fatty acid vesicles (in dark colours) and examples of strategies to increase these domains (black arrows). “*n*” refers to the number of carbon atoms in the acyl chain. “CVC” stands for “Critical Vesicle Concentration”. G) Scheme of the selective permeability of fatty acid membranes and the possible encapsulation of functional biological polymers. H) Possible strategies to achieve vesicle growth (and possibly division): (i) incorporation within bilayers of fatty acids added as micelles followed by mechanical work results in cycles of growth and division; (ii) *in situ* synthesis of fatty acids from simple precursors (within the bilayer or in the aqueous lumen or at their interface) can also produce growing vesicles.

Stabilization of fatty acids vesicles in prebiotically plausible environments

The fairly limited stability of fatty acid vesicles with respect to pH, temperature and salinity may appear as an obstacle to their possible role as prebiotic protocells. Even if one can argue that Life originated in thermally-regulated ponds of pH-buffered salt-free water, more plausible scenarios for the origin of Life on the early Earth involve fluctuating conditions, such as wet/dry cycles or thermal gradients, or extreme conditions, such as alkaline hydrothermal vents in highly saline marine environments.^[66, 67] It is therefore likely that prebiotic protocells would have been submitted to a range of adverse environmental conditions. To maintain their integrity and avoid loss of their content, fatty acid-based prebiotic protocells would have needed to resist these destabilizing conditions.

The chemical nature, and in particular the acyl chain length, of fatty acids can drastically affect the stability range of vesicles (**Figure 1F**). For instance, long chain fatty acids exhibit a lower CVC compared to short chain ones, so that vesicles made from the former would have been more prone to resist dilution in large ponds of water. Vesicles made of long chain fatty acids are also more resistant to higher temperatures, but crystallize more readily at room temperature. These observations may suggest that different vesicles populations (assembled from different fatty acids or mixtures of fatty acids) would have thrived in different environments. Yet, the chemical diversity of fatty acids alone cannot address all the instability issues of vesicles. Notably, all ionized fatty acids are prone to precipitation in the presence of divalent cations, and the formation of vesicles requires a fairly narrow pH range regardless of the chain length of the fatty acid used. These features greatly limit the environmental conditions in which fatty acid-based prebiotic protocells could have emerged.

Since the 1970s, tremendous effort has thus been made to extend the stability conditions of fatty acid vesicles (**Figure 1F**). The addition of small amounts of fatty alcohols was reported to significantly decrease the CVC due to the hydrogen bonds established with the carboxylate groups.^[32] Similarly, stability to more alkaline (typically up to ~ 11) or more acidic (typically down to ~ 7 or even lower) values has been achieved in admixtures with fatty alcohols^[32] or positively charged surfactants,^[67, 68] respectively. It has been suggested that these additional amphiphiles form hydrogen-bonded charged/uncharged dimers (i.e., fatty alcohol/ionized fatty acid or positive surfactant/protonated fatty acid) that stabilize the membrane bilayers similarly to ionized/non-ionized fatty acid dimers.

Other studies have shown that stability to higher temperature can be conferred to fatty acid vesicles using admixtures with fatty acid glycerol esters (monoglycerides),^[69] fatty alcohols^[69, 70] or alkanes.^[71] In a notable example, the resulting increased thermal stability of co-formulated fatty acid vesicles was exploited to demonstrate *in situ* DNA strand separation at elevated temperature,^[69] opening perspectives for template-directed self-replication in fatty acid vesicles subjected to thermal fluctuations. The temperature stability window of ionized fatty acids has also been extended towards lower values by changing the nature of the carboxylate's counter-ion. For instance, studies have reported that when the sodium counter-ions were replaced by bulkier tetrabutyl ammonium cations, the Kraft temperature dramatically decreased so that stable micelles could be obtained at a temperature as low as ~10°C for saturated chain lengths as long as 20 carbon atoms.^[72, 73] Similarly, we found that the addition of guanidinium cations to sodium salts of saturated long chain fatty acids prevented their crystallization.^[74, 75] Rather, elongated interconnected worm-like micelles or gelled bilayers were produced depending on the temperature (**Figure 1C**). Significantly, vesicles of myristic

(14 carbon atoms) and palmitic (16 carbon atoms) acids could then be produced at room temperature in the presence of guanidinium counter-ions by decreasing the pH or by adding a fatty alcohol (**Figure 1D**).^[77] Interestingly, this strategy is compatible with prebiotic conditions since guanidine is a very simple molecule that can be simply produced from the oxidation of guanine.

A last challenging limitation of fatty acid vesicles as prebiotic protocells is the propensity of fatty acids to crystallize at high ionic strength, typically > 200 mM, but also in the presence of divalent cations at low (~ mM) concentrations.^[45] Metal ions are yet likely to have been present on early Earth, and are crucial in modern cells for the folding and activity of biopolymers, such as RNA and metalloproteins. Admixtures of fatty acids and fatty alcohols,^[32] glycerol monoesters^[41, 44] or fatty amines,^[67, 77] but also the addition of citrate chelating molecules,^[42] confer an increased stability to metal ions, albeit often moderate. Recently, amino acids that lack a hydrophobic side chain^[78] (such as serine and glycine) have been shown to bind and stabilize fatty acid membranes against crystallization. Alternative approaches that have not attracted a lot of attention yet would have involved chemical modifications of fatty acids in prebiotic conditions (for instance to form fatty amino acids, fatty nucleobases or other derivatives). Such fatty acid derivatives would not have been sensitive to divalent cations or high ionic strength, and could therefore have self-assembled into vesicles in prebiotic milieus. A recent step has been made in this direction, where single-chain cyclophospholipids produced by chemical coupling of glycerol to nonanoic or decanoic acids and diamidophosphate were shown to form vesicles in the presence of fatty alcohols that exhibited increased stability to monovalent and divalent ions.^[79]

Overall, increased stability of fatty acid vesicles to changes in temperature and pH, or to the presence of divalent cations, relies either on the use of additives (amphiphiles, counter-ions, amino acids, chelators, etc.) or on chemical modifications of fatty acids themselves. These conditions could have occurred on the early Earth where complex molecular mixtures would have likely been the rule rather than the exception, suggesting that the first protocells could have been assembled from mixed amphiphilic molecules rather than pure fatty acids. In this context, mixed fatty acid/phospholipid membranes have been proposed as potential intermediate states in protocellular evolution.^[80]

Encapsulation and selective permeability

Encapsulation of water-soluble molecules within fatty acid vesicles provides a mean to localize species, although encapsulation yields are often relatively limited. Small molecules, such as dyes, but also macromolecules, such as RNA, can directly be encapsulated during the preparation of fatty acid vesicles from alkaline micelles, which requires a step of purification to separate the vesicles with entrapped solutes from non-encapsulated solutes in the continuous solution. This separation can be done by size exclusion chromatography (gel filtration), dialysis or centrifugation, depending on the size of the vesicles, but is usually not straightforward due to the high monomer concentration in equilibrium with vesicles:^[32, 62] the purification buffer has to be saturated in fatty acids to avoid disruption of the vesicles. Larger macromolecules, such as DNA and enzymes, have also been encapsulated by the dehydration/rehydration method.^[32] In this case, the macromolecule of interest is first mixed with vesicles, monomers or micelles before drying, and is therefore captured between dried fatty acid bilayers. Rehydration with the desired buffer produces vesicles that encapsulate approximately half of the added macromolecules,^[32] so that a purification step is still required.

Experimentally measured permeation rates combined with modelling show that fatty acid vesicles can retain the encapsulated molecules (macromolecules or polar solutes) for extended period of times while allowing the diffusion of smaller and less polar species (**Figure 1G**). This selective permeability stems from the physicochemical properties of the fatty acid membrane itself and does not require the need of specific transporters. However, compared to modern cells that rely on a combination of various transport processes across lipid membranes to ensure selective permeability to species (including against their gradients using energy-fuelled machineries), transport of species in fatty acid vesicles only occurs along gradients. In addition, solute exchange across membranes remains limited to small species, which strongly limits the ability of fatty acid vesicles to uptake nutrients (e.g., replenishment of fresh mononucleotides to synthesise polynucleotides) and excrete waste side products.

Despite this limited permeability, various functional macromolecules have been encapsulated within fatty acid vesicles (DNA, RNA, enzymes), and several (bio)chemical reactions have been reported to occur in the aqueous lumen. Notably, Szostak *et coll.* have pioneered the demonstration of RNA catalysis^[41] and template-mediated polynucleotide self-replication within fatty acid vesicles.^[42, 43] Interestingly, fatty acid vesicles have also been shown to chaperon RNA aptamers,^[81] possibly due to crowding effects.^[82] These studies confirm that molecular self-replication is compatible within the physicochemical conditions required to produce a fatty acid bilayer envelope.

Growth and division

Living protocells would have needed to couple *in situ* genome replication with duplication of the vesicular chassis to pass on information to the following protocell generation. As a preliminary step towards volume expansion and division of protocells, efforts have been made to design compartments able to grow and divide.

Fatty acids are particularly suited to implement such dynamic processes. Indeed, although fatty acid bilayers share similar properties with phospholipid membranes, such as a similar thickness,^[83] tensile strength^[84] and thermal stability,^[69] they are much more dynamic at large scales. For instance, the lower hydrophobic surface area on of single-chain fatty acids enhances lipid motions across the two bilayer leaflets (“flip-flop”), and favors the rapid exchange of fatty acids between vesicles and micelles so that the concentration of non-associated (free) fatty acids in equilibrium with vesicles is substantially higher compared to phospholipids.^[46, 59, 85, 86] Therefore, compared to phospholipids vesicles that are kinetically trapped assemblies, fatty acid vesicles behave more like a system under thermodynamic control.^[87]

Various studies have exploited this peculiar property to demonstrate fatty acid vesicles growth via the spontaneous uptake of monomers from micelles added to the solution or via exchange of fatty acids between vesicles (**Figure 1H**).^[39, 88, 89] In a seminal study, Luisi *et coll.* showed that alkaline micelles added to buffered oleate/oleic acid small unilamellar vesicles resulted in vesicle growth due to the uptake of fatty acid monomers from the micelles.^[90] Notably, it was observed that the size distribution of the resulting vesicles was strongly biased towards the size of the seed vesicles, resulting in a much narrower size distribution compared to vesicles formed by injecting sodium oleate micelles into a vesicle-free buffer. This “matrix” effect (seed vesicles act as a matrix to integrate added fatty acids) was also described with kinetics^[89] and molecular dynamics^[91] models. Vesicle growth was further demonstrated under flow, which produced filamentous compartments,^[92] and was shown to generate transmembrane pH gradients.^[86] Other studies also revealed that clay particles (montmorillonite) accelerated the rate of vesicle assembly from micelles due to the high negative charge density of the particles, which was followed by vesicle division by extrusion through small-pore membrane filters, so that repeated cycles of vesicles growth and division were demonstrated.^[93] In another study, the growth of larger spherical multilamellar vesicles into elongated thread-like vesicles upon addition of micelles was coupled to vesicles division under shear forces, resulting in the transfer of RNA molecules from one vesicles’ generation to the next.^[39] Interestingly, membrane asymmetry (e.g., in the presence of mixed fatty acids and other amphiphiles) could produce an intrinsic membrane curvature that would stabilize budded vesicle shapes and thus favor spontaneous division.^[93]

In a notable recent example by Mansy and co-workers, growth of multilamellar vesicle was demonstrated by the addition of LUVs of different lipid composition.^[94] Importantly, the addition of oleate LUVs to myristoleate multilamellar vesicles resulted in spontaneous division, which was attributed to differences in the lipid characteristics. Such a process was extended to shorter, prebiotically relevant fatty acids, and cycles of growth and division were achieved by iterative additions of vesicles.^[94] This study exemplifies a possible pathway to spontaneous vesicle growth and division in populations of different fatty acid vesicles, supporting the idea that mixtures of lipids could have helped to assemble dividing protocells on the early Earth.

The growth and division processes evidenced above rely on the uptake of fatty acids from the environment, possibly coupled to mechanical work or spontaneous physicochemical constraints. In comparison, a living protocell is expected to grow and divide based on self-driven processes.^[95-97]

Growth of a prebiotic protocell would thus require *in situ* prebiotic synthesis of fatty acids from simpler reactive precursors and their integration into existing bilayers (**Figure 1H**). As a step towards this goal, Luisi *et coll.* demonstrated in pioneering studies the autocatalytic formation of caprylic and oleic acid vesicles driven by the hydrolysis of the corresponding anhydrides.^[38, 98] Progress in the direction of self-sustained protocell growth has recently been made in mixed phospholipid/fatty acid or other amphiphile vesicles.^[99-103] In a notable example, Devaraj *et coll.* demonstrated enzyme-driven production of phospholipids from fatty acyl adenylates resulting in membrane growth.^[100] Other promising approaches rely on the use of genetically encoded membranes,^[104] which has been elegantly exploited by Pirzer and co-workers to demonstrate growth of peptide-based vesicles.^[101] Such a vesicle growth process yet relies on a complex machinery, which is not likely to have been present in prebiotic environments. In addition, cell-free protein synthesis within fatty acid vesicles has not yet been achieved to date.^[105]

Fatty acid coacervates: alternative models of prebiotic protocells?

Coacervate microdroplets as protocell models

Coacervation is an associative liquid-liquid phase separation that occurs under specific conditions in dilute aqueous polymer solutions. In this paragraph, we discuss general principles of coacervation (without focusing on fatty acids), together with arguments that support its plausible role in prebiotic compartmentalization.

Two main classes of coacervation can be distinguished: in simple coacervation, a single species – usually a neutral water-soluble polymer or a polyampholyte – undergoes phase separation in desolvating conditions (e.g., upon addition of salt, change of temperature, etc.); in comparison, complex coacervation refers to the electrostatically mediated phase separation of oppositely charged polyions in water,^[106] which is usually driven by the large entropy gain associated to the release of bound counter-ions together with water rearrangements.^[107] Both simple and complex coacervation produce highly hydrated but dense (polymer-rich), liquid-like micro-droplets in equilibrium with a dilute continuous aqueous solution.

At the beginning of the XXth century, Oparin hypothesized that coacervate droplets could be viable models of protocells.^[47] Interest in these systems has recently been reignited by Mann *et coll.* who demonstrated the assembly of coacervate microdroplets using oligopeptides and mononucleotides.^[48] Significantly, due to their lack of membrane, reduced dielectric permittivity, ultra-low surface tension, and molecular composition, these membrane-free compartments readily sequester various solutes based on equilibrium partitioning, including small and large (macro)molecules, and hydrophilic and hydrophobic compounds. Albeit still largely empirical, sequestration by coacervates is usually highly selective so that solutes can either be accumulated within droplets or excluded from them.^[108] Coacervate droplets can also be dynamically formed and dissolved in response to a range of cues,^[109] including light^[110, 111] and enzyme reactions,^[112] offering an additional level of control over solute compartmentalization. Significantly, the local up-concentration of reactive species has been exploited to demonstrate enhanced enzyme-based reactions,^[113] ribozyme catalysis,^[49, 50] or template-directed RNA polymerisation.^[50]

The plausible role of coacervate as functional compartments is also exemplified by the increasingly acknowledged existence of membrane-less organelles (MLOs) in modern cells.^[114] These biomolecular condensates have been described as liquid-like microdroplets assembled from proteins and RNA, and share several features with complex coacervates.^[115] In particular, their multiple functions in cells strengthen the hypothesis that membrane-free droplets could also have acquired complex, emergent functions in prebiotic environments despite their relatively simple structure and much simpler chemical composition than MLOs. The recent observation that complex coacervates could also be produced in mixtures of oppositely charged prebiotically relevant, low molecular weight polyions^[51] reinforce the relevance of these microdroplets as alternative models of prebiotic protocells, as reviewed recently.^[116]

On the downside, coacervate microdroplets are thermodynamically unstable and fuse together upon contact (coalescence), which eventually results in a macroscopic phase separation. The individuality of compartments is thus lost with time. While such a fusion-mediated growth can

generate mixing of material, and exemplifies a simple (passive) mechanism for droplet growth^[95] (**Figure 2H**), efforts have been made to stabilize phase separated aqueous droplets against coalescence,^[117] e.g., with colloidal particles,^[118] phospholipid vesicles^[119] or block copolymers.^[120] These types of assemblies loosely mimic the structure of modern cells as a combination of a semi-permeable membrane and a crowded interior. In a seminal study, Tang *et al.* successfully assembled oleic acid multilayers at the surface of peptide/nucleotide coacervate droplets.^[121] In addition to stabilizing droplets against fusion, the interfacial assembly of such a membrane conferred to droplets a semi-permeable barrier to control material exchange with the environment. By combining the complementary properties of both coacervates (spontaneous and selective solute uptake, dynamic material exchange, crowded environment) and vesicles (long-term encapsulation, selective permeability), this work poses questions on the possible co-emergence and co-evolution of coacervate droplets and fatty acid membranes. But what if coacervate droplets themselves could be made of fatty acid rather than polyions?

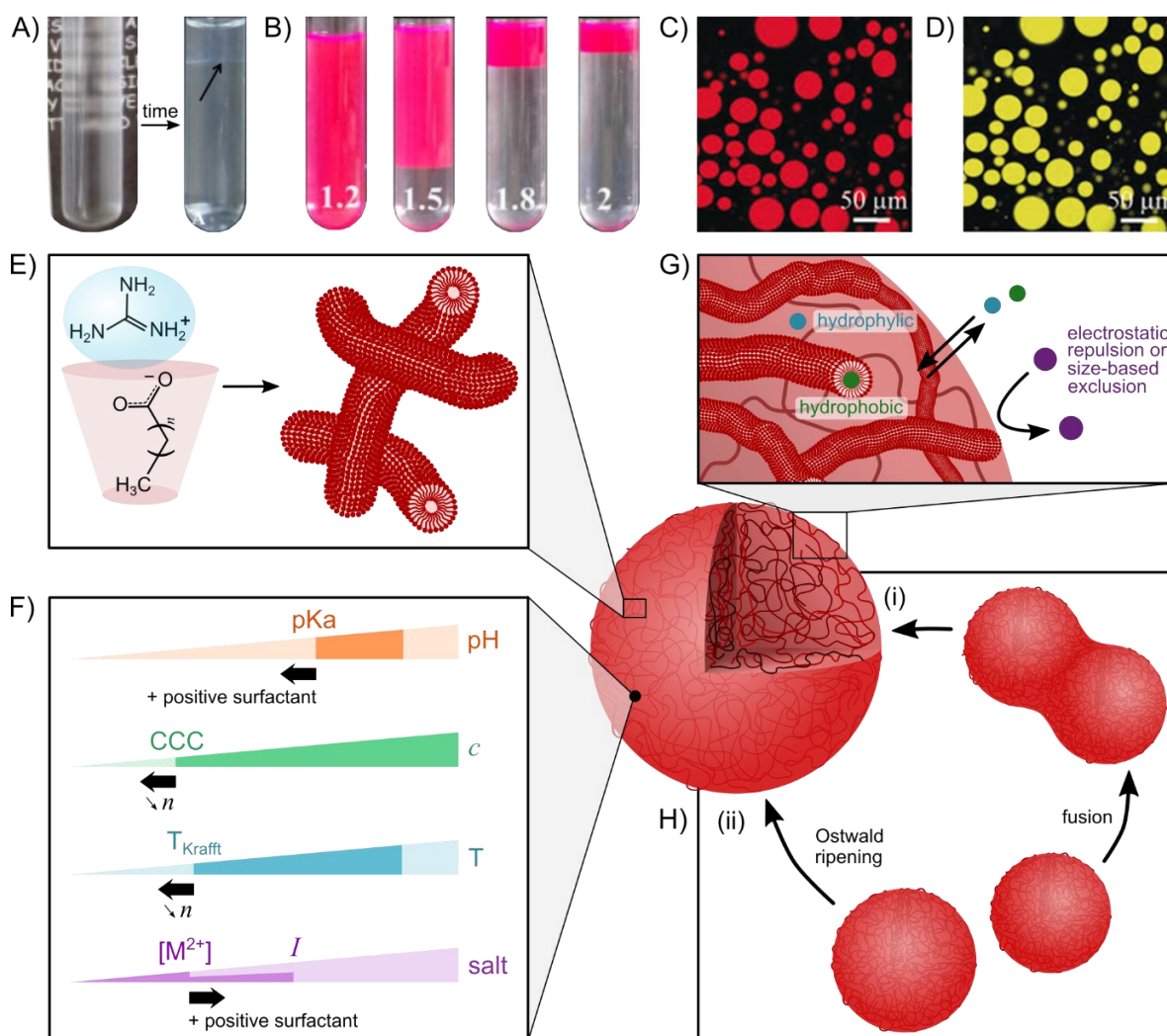


Figure 2. A) Pictures of the turbid suspension of guanidinium myristate coacervate droplets (left) before macroscopic phase separation with time (right). The bulk micelle-rich coacervate phase is less dense than water and is accumulates at the top of the tube (black arrow). Adapted from reference [123] with permission. Copyright © 2016 Elsevier Inc. B) Pictures of solutions of sodium myristate prepared at various guanidinium molar ratio, as indicated, in the presence of Nile Red after resting, showing phase separation when a molar excess of guanidinium counter-ions is used. Adapted from reference [123] with permission. Copyright © 2016 Elsevier Inc. C,D) Confocal fluorescence microscopy images of myristate-guanidinium coacervates in the presence of Nile Red (C) or yellow fluorescent protein (D), showing the uptake of both solutes and their homogeneous distribution throughout the droplets. Scale bars, 50 μm. Adapted from reference [53] with permission. Copyright © 2016 John Wiley & Sons, Inc. E) Scheme of the self-assembly of guanidinium-fatty carboxylate into entangled wormlike micelles forming coacervate droplets. F) Illustration of the pH,

concentration, temperature and salt stability domains of fatty acid vesicles (in dark colours) and examples of strategies to increase these domains (black arrows). “*n*” refers to the number of carbon atoms in the acyl chain. “CCC” stands for “Critical Coacervation Concentration”. G) Scheme of the selective partitioning of solutes in fatty acid coacervates based in hydrophobic and electrostatic interactions. H) Possible mechanism of coacervate growth (and possibly division): (i) spontaneous fusion between contacting droplets (coalescence); (ii) Ostwald ripening.

Fatty acid coacervates: towards new amphiphile-based prebiotic protocells

Beyond oligomers and polymers, coacervation can also occur in aqueous solutions of molecular amphiphiles without any macromolecule. This associative liquid-liquid phase separation is often referred to as “clouding”, and has been observed for different neutral, charged and zwitterionic surfactants.^[52, 122] Here, phase separation is driven by the hydrophobic interaction between amphiphiles to form self-assembled structures, such as interconnected wormlike micelles^[123] or bicontinuous lipid sponge phases,^[124] and the associated rearrangement of water molecules.

Excitingly, the clouding phenomenon has also been reported for oleic acid and saturated fatty acids in the presence of specific counter-ions, such as triethylammonium^[54] or tetrabutylammonium.^[125] More recently, fatty acid coacervation has been extended to saturated long chain fatty acids, such as myristic, palmitic and stearic acids, in the presence of guanidinium counter-ions.^[123] Phase separation was evidenced by the formation of a turbid suspension at $9 < \text{pH} < 10$ that underwent macroscopic phase separation upon resting (**Figure 2A**). Coacervate formation was attributed to the self-assembly of fatty acids into wormlike micelles in the presence of a ~2-fold molar excess of guanidinium ions (**Figure 2B,E**). It was hypothesized that the delocalization of the positive charge on guanidinium favored the bridging between micelles and their phase separation as an entangled network^[123] (**Figure 2E**). Significantly, fatty acid coacervate droplets were shown to selectively sequester hydrophobic and positively charged dyes (**Figure 2B,C**), but also proteins^[53] (**Figure 2D**), while excluding negative dyes. The sequestration of positive solutes and the exclusion of negative ones were attributed to an electrostatic attraction or repulsion with carboxylate anions, respectively.^[123] Such a charge-mediated selective sequestration exemplifies a simple mechanism by which membrane-free fatty acid coacervate droplets can spontaneously and selectively up-concentrate species, paving the way to the compartmentalization of functional, possibly prebiotic, molecules.

The exclusion of negatively charged solutes from fatty acid coacervates limits however the applicability of these membrane-free droplets as protocells able to sequester nucleotides, which is an important step to couple compartmentalization with the emergence of DNA-based self-replication. To overcome this limitation, Douliez *et coll.* developed cationic coacervates at $\text{pH} > 7$ using equimolar charge mixtures of a model, prebiotically relevant fatty acid, decanoic acid, and a positively charged surfactant, cetylpyridinium chloride or cetyltrimethylammonium bromide.^[126] Significantly, and unlike pure fatty acid coacervates, cationic droplets were able to sequester double-stranded DNA and single-stranded oligonucleotides due to electrostatic attractions with positively charged surfactants. Excitingly, cationic coacervates also exhibited enhanced stability to increased ionic strength and magnesium ions since they retained their integrity up to 500 mM NaCl and 100 mM MgCl₂. This enhanced salt stability compared to pure fatty acids was attributed to strong electrostatic interactions between the fatty acids and the oppositely charged surfactant. Since these seminal studies, other coacervate droplets assembled from low molecular weight surfactants have been developed and used as protocells, including lipid sponge phases^[124] and positively charged surfactant coacervates.^[127] These droplets were found to up-concentrate several biomolecules and be compatible, or even enhance, enzyme-based reactions. Although positively charged amphiphiles are rare in biology, they can help building coacervates with useful properties (such as the uptake of nucleotides). Therefore, albeit orthogonal to extant biology, this approach might still be considered prebiotically relevant.

Overall, fatty acid coacervate microdroplets, together with alternative low molecular weight amphiphile-based coacervates, represent interesting alternative models of compartmentalized prebiotic protocells. However, these systems have not yet been shown to be compatible with templated molecular self-replication, ribozyme activity, or other advanced biological machineries such as gene-mediated cell-free protein expression. Future studies should therefore explore the

compatibility of fatty acid coacervates with these processes, together with their stabilization against fusion, dissolution or precipitation in adverse environmental conditions. As a first step, cationic coacervates were shown to resist dilution and macroscopic phase separation after gelation in the presence of alginate.^[126] Although these conditions are not prebiotic, they open perspectives to explore the stability of fatty acid coacervate droplets in different environments. Further studies would strengthen the potential of these alternative compartments as plausible prebiotic protocells.

Bridging the two worlds: reversible transition between fatty acid vesicles and coacervates

We have described two classes of complementary fatty acid-based protocellular compartments. Fatty acid vesicles are delimited by a physical barrier made of one or multiple fatty acid bilayers enclosing an aqueous lumen. Encapsulation yields of biomolecules are often limited and material exchange with the environment is circumscribed to small apolar molecules. The membrane yet ensures selective permeability so that fatty acid vesicles can be described as “closed” or “semi-closed” compartments (**Figure 3B**). In comparison, fatty acid coacervates spontaneously sequester a range of biomolecules by equilibrium partitioning, so that local accumulation of various hydrophobic or charged solutes is readily achieved. Material exchange between these “open” compartments and their environment is therefore favored by simple diffusion and shows selectivity, but it is not easily controlled due to the lack of a physical barrier.

Excitingly, coacervate droplets assembled from fatty acids, including myristate/guanidinium, palmitate/guanidinium or cationic decanoate/positive surfactant systems, have been shown to reversibly transform to vesicles simply by decreasing the pH (**Figure 3A,B**).^[53, 126] Such a pH-mediated reversible coacervate-to-vesicle transition was attributed to the transformation of wormlike fatty acid micelles in the coacervate phase into fatty acid bilayers when the pH reached a value close to the fatty acids' pKa, similarly to the micelle-to-vesicle transition observed for simple fatty acid solutions. Significantly, fluorescently labeled proteins added to the outer fatty acid vesicles solution were spontaneously sequestered in the fatty acid coacervates produced at higher pH, then subsequently transferred within the vesicles formed by re-decreasing the pH (**Figure 3A**). The transition could be induced by direct acid or base addition, or be driven by enzyme-mediated pH changes using antagonistic enzymes,^[128] and multiple transition cycles could be achieved. Using this strategy, various molecules, including dyes and proteins, were transferred from coacervates to vesicles.^[53, 128]

While still rudimentary, this first report of a dual compartmentalised organisation and solute transfer opens promising perspectives for the emergence of metabolically active prebiotic protocells. The transition between membrane-bound and membrane-free compartments could indeed have allowed cycling between “closed” and “open” reactors, and therefore provided a strategy to sustain reactions by spontaneous nutrient uptake and waste release in the coacervate state (via equilibrium partitioning), and biomolecular segregation and localised reactions in the vesicle state (**Figure 3B**).

Future studies will have to extend such a vesicle/coacervate transition to other systems, for instance to produce more stable systems with respect to adverse environmental conditions (temperature, pH, ionic strength), or to design assemblies able to transform between vesicles and coacervates in response to other stimuli. Interestingly, examples of such systems were recently reported using positively charge surfactants that exhibited a reversible coacervate-to-vesicle transition by changes in pH, temperature, or upon dry/wet cycles.^[127] Other studies reported a vesicle-coacervate transition using polyelectrolytes^[129] or block copolymers,^[130] suggesting that this phenomenon may be a general observation that could be implemented with a range of molecular building blocks.

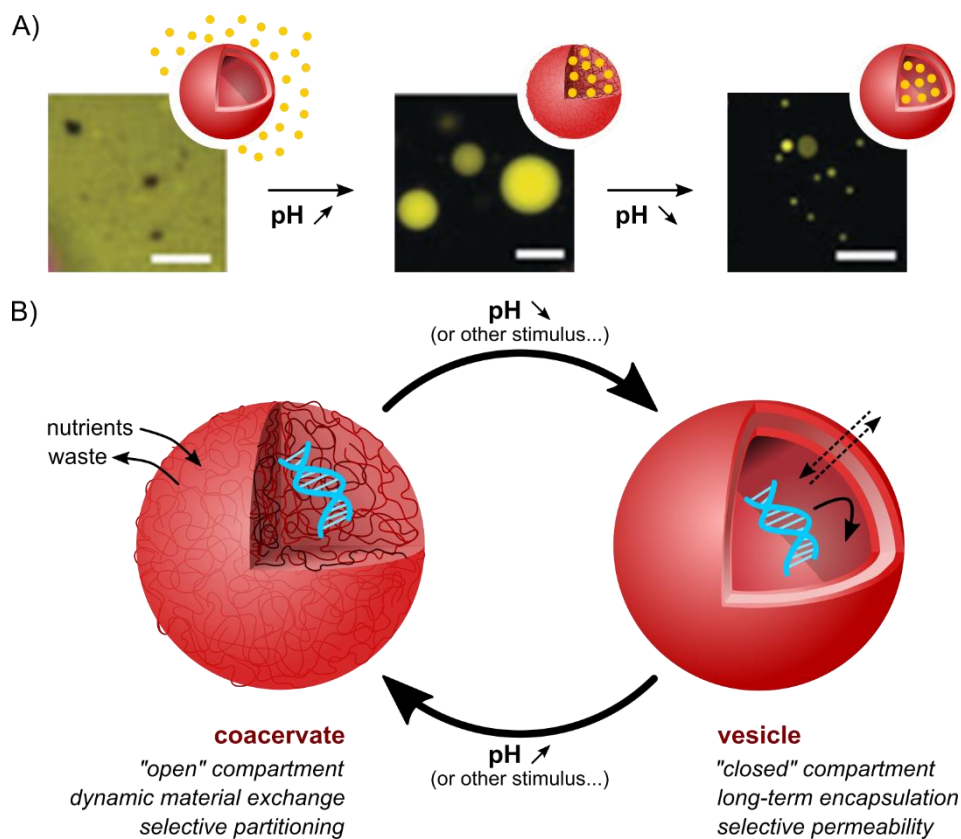


Figure 3. A) Confocal fluorescent microscopy images of myristic acid vesicles supplemented with yellow fluorescent protein in the external solution (left). An increase in pH transforms the vesicles into fatty acid coacervates that spontaneously sequester the fluorescent protein. Re-decreasing the pH converts back the coacervates into vesicles that now contain the fluorescent protein. Therefore, transfer of biomolecules from the external environment into the vesicles' lumen can be simply achieved by the transient formation of coacervates. Scale bars, 5 μm (left and right images) and 25 μm (center image). Adapted from reference [53] with permission. Copyright © 2016 John Wiley & Sons, Inc. B) Scheme of the reversible transition between fatty acid coacervates (membrane-free, "open" compartments) and vesicles (membrane-bound, "closed" compartments). The two types of compartments have complementary sequestration/encapsulation properties.

Conclusions and perspectives

Fatty acid vesicles have long been used as prebiotic protocell models due to their peculiar properties: they recapitulate essential features of the membrane-bound structure observed in modern cells; fatty acids could have been present in the prebiotic soup and are the simplest amphiphiles able to self-assemble into vesicles. While fatty acid vesicles are easily destabilized in adverse environmental conditions (e.g., low or high pH, low or high temperatures, high salinity or presence of low amounts of divalent ions), several experimental approaches have been developed to extend the range of fatty acid vesicles stability. It is interesting to note that most of these approaches rely on the addition of (at least) one secondary component (chelators for metal ions, amino acids, other single acyl chain derivatives...). The chemical diversity likely available in prebiotic environments could thus have favored the emergence of chemically diverse, mixed prebiotic membranes that would have been more stable than pure fatty acids membranes. Another possibility is that fatty acids may have evolved 'chemically', forming covalent derivatives insensitive to high ionic strength or to the presence of divalent ions.

Fatty acid vesicles show selective permeability, and are able to encapsulate large molecules for extended periods while letting small and non-polar solute diffuse along their gradients, but they typically exhibit relative low encapsulation yields. In comparison, coacervate micro-droplets can spontaneously uptake high amounts of solutes by equilibrium partitioning. Therefore, the recently reported fatty acid-based coacervation appear as a new promising platform to build prebiotic

protocells. Fatty acid coacervates are assembled from an entangled network of wormlike micelles at high pH, and can sequester both hydrophobic solutes (in the hydrophobic core of the micelles) and positively charged species. Admixtures with positively charged surfactant favor the uptake of negative biomolecules such as DNA, paving the way to polynucleotide-based catalytic activity and self-replication in membrane-free droplets. However, despite the selective partitioning of species, the lack of membrane impedes a fine control over material exchange with the environment.

Excitingly, fatty acid coacervates have been shown to reversibly transform into fatty acid vesicles by changes in pH. We argue that this phenomenon offers promising new directions to explore the origins of life. Perhaps a plausible prebiotic protocell could be a system capable of cycling between both types of compartments, allowing to sustain reactions into “closed”, membrane-bound vesicles by transiting into “open”, membrane-free droplet able of spontaneous nutrient uptake and waste disposal. This could indicate that populations of fatty acid coacervates and vesicles would have co-emerged and co-evolved until vesicles would have developed more sophisticated transport mechanism to better control material exchange with the environment, which could have offered them a sufficiently selective advantage to thrive and further evolve into more complex membranes. Further research into coacervates made of low molecular weight surfactants could provide insights into the plausible role of such membrane-free compartments as prebiotic protocells.

As a perspective, it is also possible to envision less conventional fatty acid-based protocell models. One of these alternative models could be based on water-in-oil-in-water double emulsion droplets, in which the oil would be made of short chain fatty acids, such as decanoic acid. At acidic pH, decanoic acid would be in its neutral carboxylic form, which forms an oily liquid above 31°C. Emerging from a hydrothermal vent in sea water in the deep ocean, such systems could have formed double emulsion droplets by local mixing, encapsulating nutrients in their aqueous internal lumen. Interestingly, such emulsion-like compartments would not have been sensitive to high ionic strength neither to the presence of divalent ions since fatty acids would be under their uncharged form. An increase of pH could then have yielded the formation of fatty acid bilayers, leading to the self-assembly of vesicles. To our knowledge, such an alternative protocell model has not yet been explored. Other models might be thought of to expand the zoology of fatty-acid based protocells. Studying the interactions between these diverse compartments, such as the reported pH-mediated transition between coacervates and vesicles, opens exciting perspectives for the origin of life as a protocell population paradigm. For instance, it is possible to imagine that different compartments could have co-emerged, competed for the same resources, or collaborated to “survive” until one population would have acquired a selective advantage making it thrive at the expense of the other populations.

To conclude, the fatty acid world has still a lot to offer. Perhaps “the” most plausible prebiotic protocell model is to be searched by investigating synergistic or antagonistic interactions between different types of compartments. The field of protocell populations is therefore an exciting new avenue to be explored to gain insight into the origins of Life on the early Earth.

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Keywords

coacervates • fatty acids • protocells • prebiotic • vesicles

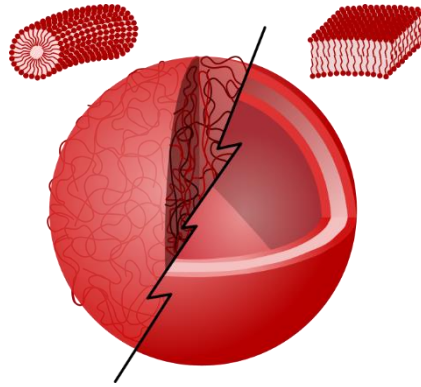
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Table of contents



Membrane or no membrane? Fatty acids are valuable molecules to assemble prebiotic protocells. We here compare two types of fatty acid-based compartments: membrane-bound vesicles and membrane-free coacervates, and show that the latter provide a promising alternative paradigm for prebiotic compartmentalization. We also argue that the dynamic transition between fatty acid vesicles and coacervates offers an exciting new hypothesis for the emergence of the first living protocells.