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# Protocells: Milestones and Recent Advances

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The origin of life is still one of humankind's great mysteries. At the transition between nonliving and living matter, protocells, initially featureless aggregates of abiotic matter, gain the structure and functions necessary to fulfill the criteria of life. Research addressing protocells as a central element in this transition is diverse and increasingly interdisciplinary. The authors review current protocell concepts and research directions, address milestones, challenges and existing hypotheses in the context of conditions on the early Earth, and provide a concise overview of current protocell research methods.

## 1. Introduction

"The doctrine of evolution, while enforcing the fact of spontaneous generation and progressive evolution, gives us no hint as to the physical mechanism of such generation. It does not tell us by what forces, or according to what laws, the simpler forms of life have been produced, or in what manner differences of environment have acted in order to modify them."

Since Stephane Leduc made this statement in 1911 in his book *"The Mechanism of Life,"* the scientific approaches to the origin of life problem have significantly matured. We have learned a great deal about how essential building blocks of life could have possibly emerged under conditions which were in many aspects different from today. We have been able

to assemble astonishing pieces of a complicated puzzle, and realized that further advancement requires input from multiple branches of science, not just biology as the primary life science. Detailed hypotheses have been established about the different scenarios of the emergence of life, including the "RNA world,"<sup>[1]</sup> the "lipid world,"<sup>[2]</sup> "replicator first,"<sup>[3]</sup> "metabolism first,"<sup>[4–6]</sup> and others. Although the origin of life is still surrounded by many open questions, our understanding of chemical,

physicochemical, and biochemical processes possibly involved in the ancient events preceding Darwinian evolution has seen much progress. We have come a long way from Leduc's physicochemical, inorganic matter-centered view on the beginning of the evolution, yet the matter of the transition from nonliving to living matter still remains largely unsolved, and one of the great scientific problems of our time.

The phylogenetic tree of different living domains reflects that life has evolved from simple to more complex structures, i.e., from single- to multicellular organisms. The oldest fossil evidence dating back 3.5 Gy (billion years) comes from stromatolites,<sup>[7–10]</sup> microorganismal residues in sedimentary rocks.<sup>[11]</sup> There appears to be a gap of knowledge regarding the period of evolution between the first primitive hypothetical cells and the fossilized ancient bacteria, which can be considered as an already advanced form of life.<sup>[12]</sup> It is highly likely that intermediate primitive cell precursors preceded the single-cell organisms. The hypothetical prebiotic structures that were the stepping stone to first self-sustaining living cells are commonly termed "protocells." The possibility of a strong link between the formation of protocells and the origin of life can today be reasonably assumed.

One cannot easily proceed in the context of the evolution of cell-based organisms without briefly illuminating the concept of life as we know it on our planet. Over time, different requirements have been proposed for an entity to be considered alive. According to Tibor Ganti's chemoton model,<sup>[13]</sup> a protocell contains three autocatalytic subsystems: a membrane subsystem that keeps the components together and intact, a metabolic subsystem that captures energy and material resources, and an information subsystem that processes and transfers heritable information to progeny. To be considered alive, these subsystems must be unified and function co-operatively for the survival and evolution of the supersystem. Pohorille and Deamer suggested a modified set of 7 criteria related to the chemoton.<sup>[14]</sup> At about the same time, Oro defined the requirements by 10 characteristic features.<sup>[15]</sup> Despite their differences, these descriptions align well with NASA's broader definition of life: "a self-sustaining chemical system capable of Darwinian evolution."

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In order to approach life from that perspective, assuming that there has been a development over time from individual molecules toward living cells with increasing complexity of structure, gain of function and use of energy in metabolic activity, the concept of biomimetic compartments possessing minimal functions of living cells is useful. In his book on prebiotic chemistry,<sup>[16]</sup> Deamer argues that the top-down approaches, on one hand to a minimal genome and on the other hand of back-tracing contemporary enzymes to their simpler versions, are not very promising.<sup>[17,18]</sup> He emphasizes that a first cell carrying hundreds of genes and/or protein enzymes can be safely ruled out, and suggests instead bottom-up strategies as a suitable approach, particularly pointing out the usefulness of compartmentalization to address the problems of metabolism and unlimited heredity, i.e., replication.

In this review, we emphasize compartmentalization, which is based on the transition from small organic molecules with surfactant properties to organized cell-like containers in an aqueous environment. Recent work on essential structural and functional aspects of protocells and related research subjects is reviewed. We also cover important milestones and past developments in the relevant fields of inquiry, particularly major experimental procedures and systems for protocell generation in the research laboratory. We note that although the protocell and synthetic cell models go hand-in-hand, we distinguish for practical reasons the protocell from the synthetic cell by focusing on the environmental conditions and materials specific to the early Earth.

## 2. Relevant Prebiotic Conditions

It is *per se* a hard task to confidently build realistic protocell models since they are supposed to reflect an environment so far back in time that there is no first-hand information available. Models that improve our understanding can only be built if the conditions are sharply defined and in reasonable agreement with what we currently know about that past reality. The existing knowledge on the early Earth conditions is based on limited evidence, which is also to some extent subject to interpretation and assumptions.<sup>[19]</sup>

There are various hypotheses on the conditions during the late Hadean/early Archean eons of the Earth's history, prior to the first fossil evidence of life. It is reasonable to assume that the transition from non-living to living matter occurred during that period. Our planet's evolution has been defined in characteristic time periods covering certain conditions and events.<sup>[20]</sup> **Figure 1** depicts schematically the timeline of prominent events from the beginning of planet formation to the emergence of the first cells around 3.5 Gya (billion years ago).<sup>[7–10]</sup> The terrestrial Earth was formed approximately 4.5 Gya.<sup>[21]</sup> The period of the first 0.5 Gy after the Earth's formation is known as the Hadean era. During this period the conditions and materials that were fundamental for the development of life were established, e.g., water and organic matter. The Hadean period was followed by the Archean eon, dating from 4 Gya until 2.5 Gya. The events that were most influential for protocell emergence occurred specifically during the Eoarchean period, i.e., the first 0.5 Gy of the Archean eon. The boundary between the Hadean

and the Archean period is not very well defined.<sup>[22]</sup> In the following, we will briefly summarize our knowledge on the conditions and materials that could have impacted protocell formation and development, as well as life itself.

### 2.1. Environmental Conditions

In the context of protocell formation on the early Earth, favorable environmental conditions were essential:

- A hydrosphere with certain environmental niches
- A non-toxic stable atmosphere

The composition of the Earth's atmosphere during the first few 100 million years cannot be established with certainty, as many harsh processes occurred during this period. Hypotheses on the development of the early Earth's atmosphere gained momentum in the 1980s, when the long-standing view of the planet as having developed its atmosphere after accretion by outgassing from the interior<sup>[23]</sup> changed towards a hypothesis that outgassing from numerous impacting extraterrestrial bodies was largely responsible for the earliest atmosphere. Intense heat generated by these impacts, forming a magma ocean,<sup>[24]</sup> would allow a dense steam atmosphere to be created and maintained. Competing hypotheses<sup>[25]</sup> propose the arrival of materials in moon-sized, or even larger chunks, suggesting that there were cooler periods which allowed the formation of liquid water. Intermittent heating and cooling periods, which would result from the combination of these hypotheses, infer that liquid water might have formed and evaporated repeatedly, and that a steam atmosphere was present at least part of the time. The atmosphere was likely exchanged again during the giant moon-forming event.<sup>[26]</sup> The environmental nature of this event has been elaborated upon, taking into consideration the ratio of C and N with respect to H in Earth volatiles, which is different from chondrites (non-metallic meteorites). A specific impact mechanism of the moon-forming event, which removed N and C preferentially from the planet, must have occurred during its formation. A more detailed account of the literature on these processes, spanning the entire period from the Hadean era to today, has been presented by Kasting.<sup>[27]</sup> It includes a review of the early atmospheric composition, addressing the interesting questions of the oxidation state of carbon. A high CO<sub>2</sub> content of the early atmosphere would have favored photochemical formaldehyde formation, whereas the Miller experiment, e.g., the electric discharge-induced HCN formation, would require a CO-rich environment. Kasting presents further the relationship between the high UV flux from the sun, which likely would have led to the photolytic decomposition of larger molecules on Earth, and the alleviating effect of a possible early sulfur-based gas atmosphere. The presence of gaseous sulfur compounds, e.g. hydrogen sulfide and sulfur dioxide, together with oxygen, water, methane,<sup>[28]</sup> carbon dioxide, and nitrogen compounds, suggest a rich early atmospheric photochemistry. Moreover, the sulfur compounds may have prevented the light-induced destruction of ammonia and the strong reducing agent hydrazine. The presence of small reactive nitrogen and carbon compounds has been shown to be





water could have been present on the surface already during the Hadean era.<sup>[33]</sup> The cool early Earth hypothesis suggests relatively moderate surface conditions from 4.4 to 4.0 Gya, which would have allowed for liquid water oceans to exist.<sup>[34,35]</sup>

Some researchers suggest specific sets of environmental conditions, which would have been particularly favorable for the development of life. The macrobiont hypothesis comprises a macroscale setting with subaerial ponds ranging from 3 to 300 m in size, where geological, atmospheric, hydrospheric, and extraterrestrial contributors would have been effective.<sup>[36]</sup>

## 2.2. Water

- Reactant and metabolite for life
- Medium for life
- Plays a role in protocell formation due to self-assembly of amphiphilic compounds in water—hydrophobic effect

Life “as we know it” is based on water as a universal solvent and reactant. The biochemical reactions which maintain life take place in aqueous media where water is also the main metabolite.<sup>[37]</sup> Current research is trying to determine when and how water appeared on our planet.<sup>[38]</sup> The source of water on Earth is hypothesized to be of extraterrestrial origin.<sup>[38]</sup> It is suggested that asteroid impacts delivered vast quantities of water ice during the Hadean period.<sup>[39]</sup> The earliest geological evidence for the existence of liquid water on earth is 3.8 Gy old.<sup>[33]</sup>

Oxygen isotopes in the oldest known zircons, a common trace mineral in granitic rocks that is resistant to mechanical and chemical weathering and therefore used in absolute dating, suggest that liquid water may have been present on the Earth's surface as early as 4.3 to 4.4 Gya.<sup>[40]</sup> Analyses from the Greenstone Belt in Isua, Greenland, indicate that  $\approx 3.7$  Gya, permanent oceans existed.<sup>[41]</sup>

In the context of protocells, several unique water environments have been suggested. Deep-sea hydrothermal vents are considered as possible sites for the origin of life, mainly because metals, sulfur, and other compounds that are essential for certain metabolic pathways are ubiquitous in their vicinity.<sup>[42,43]</sup> The “Zinc world” hypothesis, for example, suggests that life emerged within compartmentalized, photosynthesizing ZnS formations of hydrothermal origin, assembled in subaerial settings on the surface of the primeval Earth.<sup>[44]</sup> Similarly, the “Iron-sulfur world” hypothesizes an emerging primitive metabolism based on the formation of small organic compounds from inorganic gaseous precursors by transition metal catalysis, and the reducing action of various sulfides.<sup>[45]</sup>

Regarding the suitability of a hydrothermal deep-sea environment for protocell formation, reasonable arguments challenging this hypothesis have been made. For example, large pH gradients, high concentrations of salt<sup>[46]</sup> and divalent cations are detrimental to the assembly of protocells from fatty acids. In addition, the prevalent high temperatures, which in black smoker-type hydrothermal vents commonly exceed 300 °C, have been considered incompatible with potential protocell-forming chemical precursors.

However, “Lost City” Hydrothermal Fields, which feature temperatures of only around 70 °C, have been discovered in the early 2000s.<sup>[42]</sup> In the section Protocell formation and stability in bulk systems, we discuss a recent experimental study on protocell assembly in the Lost City Hydrothermal Fields, keeping the hydrothermal vent hypothesis alive.<sup>[42]</sup> An alternative setting that satisfactorily addresses the salinity problem is based on fresh water environments such as the “warm little ponds” hypothesized by Darwin.<sup>[47]</sup> In a later study, it was shown via geochemical reconstruction that the ionic composition suitable for the development of primitive cells could not have been present in marine settings, but very well in volcanic pools.<sup>[48]</sup> In contrast to deep-sea vents, warm ponds and volcanic pools can easily go through dry-wet cycles due to evaporation and precipitation. This promotes concentration of initially dilute solutions of organic entities.<sup>[49]</sup>

Damer and Deamer reported on laboratory and field experiments, designed to test a hot spring hypothesis based on protocells aggregating into a hydrogel in the intermediate moist phase of wet-dry cycles<sup>[50]</sup> (Figure 2a,b). This is an alternative to the origin of life at hydrothermal vents and hydrothermal fields. The authors argue that the interaction of protocell constituents with polymers in such a mild environment would establish a pre-Darwinian system of selection by assembling all necessary components for the transition to first microbial communities.<sup>[50]</sup> Wet-dry cycling has also been shown to promote polymerization and solvation of ring-shaped RNA molecules.<sup>[51]</sup> The implications of such circular RNA structures, or “viroids,” for the origin of life were further discussed by Moelling and Broecker.<sup>[52]</sup>

Theories on life elsewhere based on non-aqueous solvents, or chemical foundations other than C/H/N/O, such as silicon, and even gases like CH<sub>4</sub> have been proposed.<sup>[58]</sup>

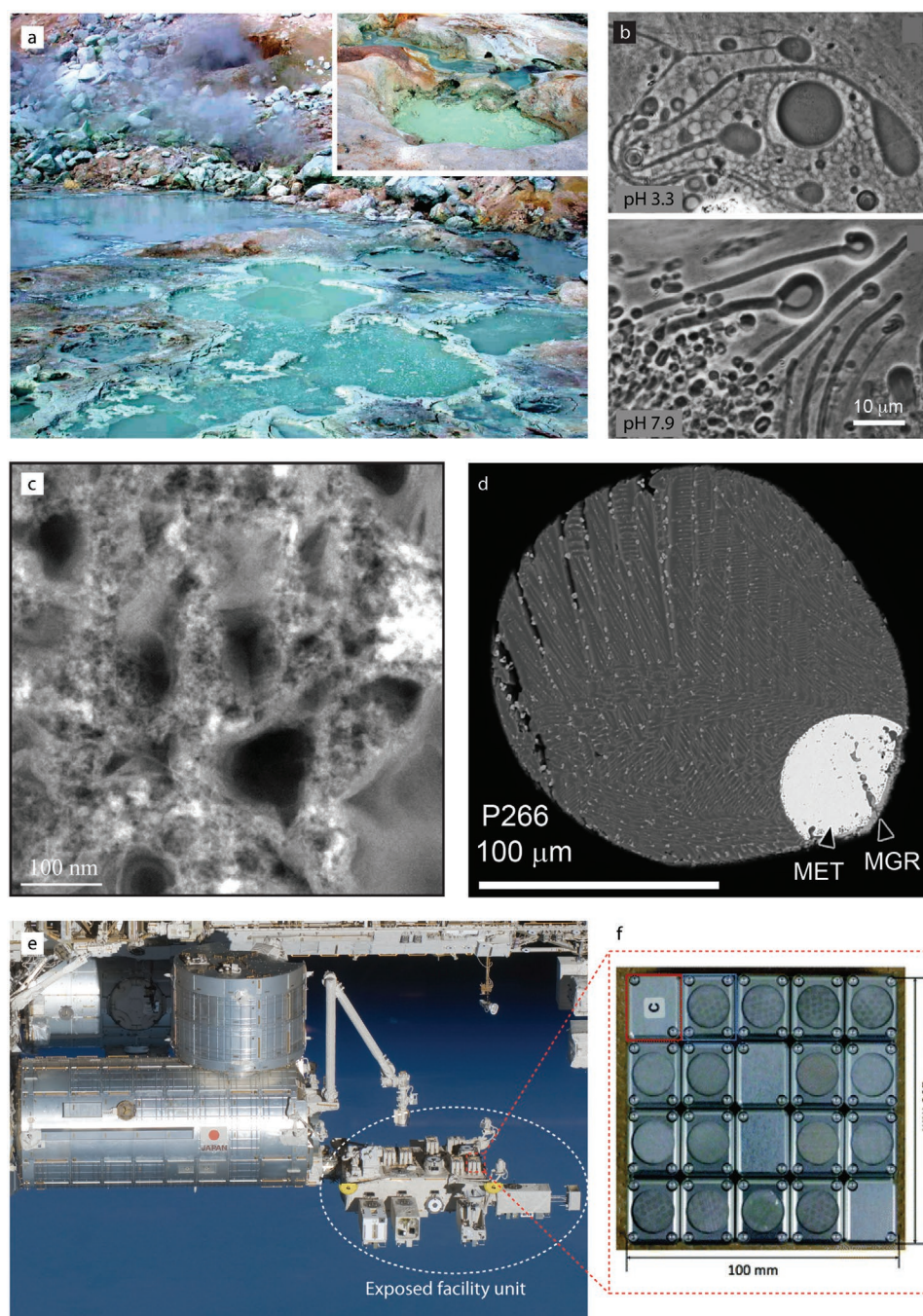
## 2.3. Early Earth Materials

### 2.3.1. Minerals and Rocks

A mineral is defined as a natural, inorganic element or compound with a fixed chemical composition, and a specific crystal structure. Rocks are composed of multiple minerals. In the context of protocell development, minerals and rocks are potentially important for the following reasons:

- Microcavities in rocks have been proposed as solid compartment reactors
- Porous rocks have been proposed to be able to act as extruders for vesicle formation
- Minerals can catalyze organic reactions relevant for protocell development
- Phosphate, which is structurally important for phospholipids, is part of many minerals, e.g., apatite.
- Mineral particles amplify membranous protocell formation
- Mineral surfaces can enhance lipid membrane shape transformations

Today, there are  $\approx 4500$  known naturally occurring minerals, which have evolved through the different eons.<sup>[59–61]</sup> Primary minerals



**Figure 2.** Field experiments and search for extraterrestrial life. a) Bumpass Hell, a hydrothermal field on Mount Lassen in California proposed as an environment suitable for the origin of life. Adapted with permission.<sup>[53]</sup> Copyright 2021, MDPI. b) Mixture of dodecanoic acid and dodecanoyl monoglyceride added to a hot spring water in the Yellowstone at two different pH values lead to self-assembly of vesicular structures. Adapted with permission.<sup>[54]</sup> Copyright 2018, MDPI. c) Microcavities in a Martian meteorite sample as solid compartments for prebiotic reactions. Adapted with permission.<sup>[55]</sup> Copyright 2021, Mary Ann Liebert, Inc. d) Scanning electron microscopy image showing an interstellar dust particle (IDP). MET: metal, MGR: magnetite rim. Adapted with permission.<sup>[56]</sup> Copyright 2017, Geological Society of America. e) Japanese Kibo module from the Tanpopo mission in search of evidence for panspermia, which collects IDPs in sample chambers exposed to the space environment. Public domain, NASA. f) Magnified view of the exterior of the sample chamber array. Adapted with permission.<sup>[57]</sup> Copyright 2016, Mary Ann Liebert, Inc.

were formed from cooling and crystallization of magma, and later transformed by weathering to secondary minerals.<sup>[62]</sup>

It is conceivable that minerals played a role in prebiotic synthesis of organics at the origins of life by uptake and catalysis.

Adsorption of molecules on minerals, especially on phyllosilicates containing parallel sheets of silicates, has been shown to catalyze polymerization reactions.<sup>[63,64]</sup> Minerals such as clay, quartz, feldspar, zeolites, olivine and others have been proposed

as catalysts in prebiotic organic synthesis.<sup>[62]</sup> Franco and da Silva pointed to the possible importance of boron-containing minerals in the prebiotic synthesis of ribonucleotides.<sup>[65]</sup>

Furthermore, it has been suggested that microcavities in rocks<sup>[66,67]</sup> can serve as reaction compartments<sup>[55,68,69]</sup> (Figures 1 and 2c). Hansma formulated the mica hypothesis, which entails that the gaps between mica sheets can act as cell-like compartments. They can be seen as a largely isolated chemical nanoenvironment, allowing dry-wet cycles, and migration and reaction of prebiotic molecules.<sup>[70]</sup> Other phyllosilicates, particularly clay minerals, are structurally suited as containers for concentrating and assembling organic compounds.<sup>[64,71]</sup> Magmatic clay minerals in Martian meteorite NWA5790 exhibit a vesicular texture that forms a network of microcavities or pockets, which could serve as microreactors and allow molecular crowding,<sup>[72]</sup> an advantageous condition for the emergence of life. Figure 2c shows scanning transition electron micrographs of the solid “vesicles” formed by the Fe/Mg clay minerals in NWA5790.<sup>[55]</sup>

Minerals that are similar in composition to the Mars surface (calcite, anhydrite, kaolinite) protect bioorganic compounds (N-heterocycles: purine and uracil) against the effects of UV and cosmic radiation. Exposure experiments demonstrated that organics can survive the radiation dose equivalent to 500 000 years on the Martian surface.<sup>[73]</sup>

Last but not least, minerals have been shown to directly induce protocell formation and energetically support biosurfactant membrane shape transformations. This will be discussed in detail in the section Protocell formation on solid surfaces.

**The phosphate problem:** The phosphate moiety is an essential part of phospholipids<sup>[74]</sup> and genetic polymers. It is commonly found on Earth as apatite, an insoluble calcium phosphate mineral. The prebiotic transformation of the severely insoluble phosphorus source to the water-based protocell constituent is referred to as the “phosphate problem.”<sup>[62,75]</sup> It has been hypothesized that phosphate from iron-rich meteorites reached the Earth during the heavy bombardment period in form of the soluble schreibersite,<sup>[76]</sup> a phosphide mineral. Schreibersite hydrolyses and releases phosphorous species of various oxidation states. When exposed to UV light, it can form phosphate to become available for prebiotic reactions.<sup>[77]</sup> Recently, Hess et al. proposed lightning strikes as another source of prebiotic phosphorous. They showed that lightning strikes form fulgurite glasses from clay-rich soil. These fulgurites feature abundant schreibersite inclusions.<sup>[78,79]</sup>

### 2.3.2. Organic Materials

A large variety of organic matter is considered important for protocell formation and development:

- Fatty acids and phospholipids are likely building blocks of protocells
- Organic compounds interact with the protocell membrane; they can be encapsulated and function as reaction precursors
- Genetic fragments central to protocell replication are organic

There are three scenarios regarding the major sources of generic organic matter on the early Earth:<sup>[80]</sup> delivery via comets

and meteorites, geological and atmospheric synthesis, cosmic delivery of pre-existing living organisms, i.e. panspermia.

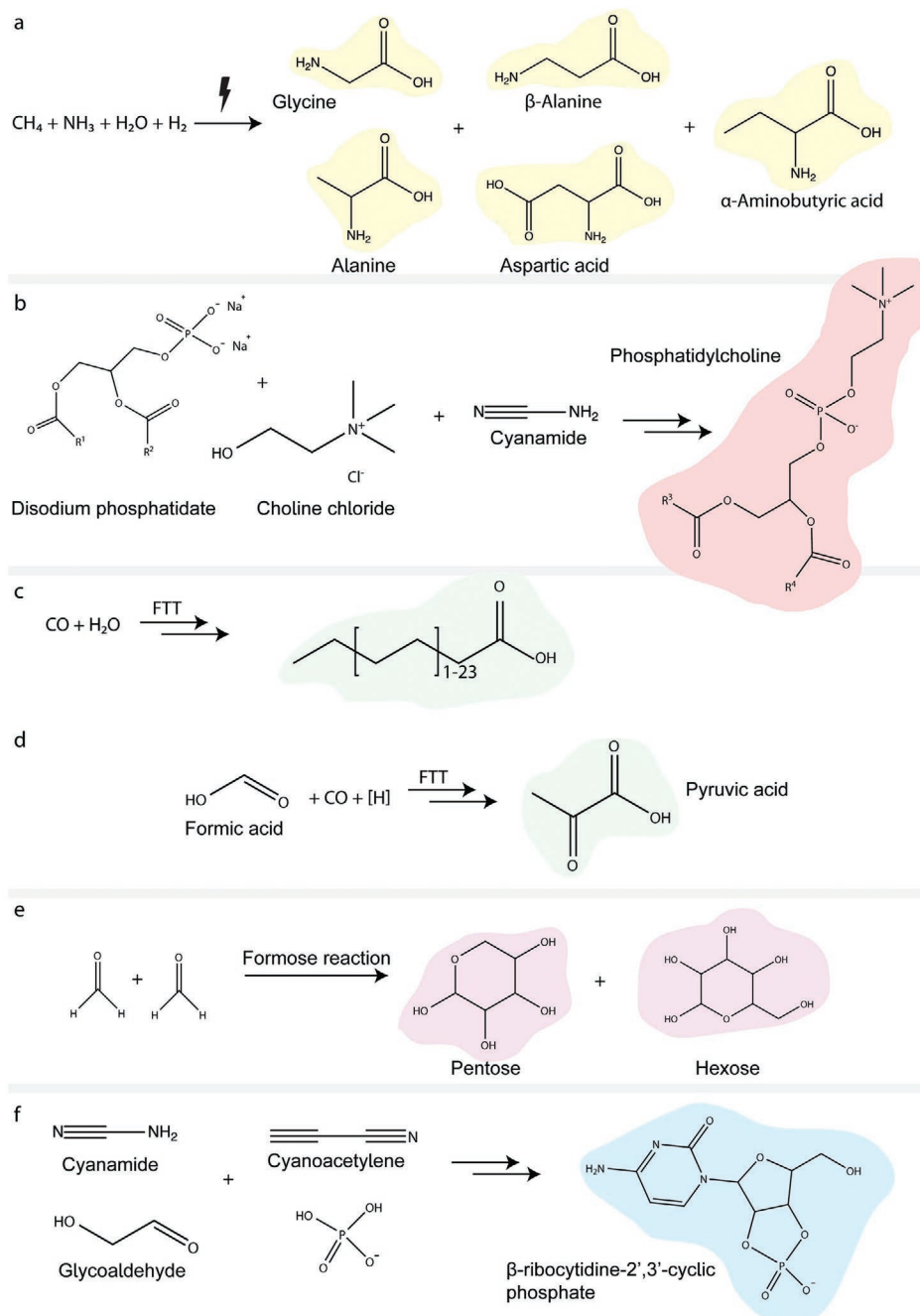
It is generally accepted that at the end of Hadean eon the Late Heavy Bombardment (LHB) occurred, a time period in which the meteoritic impact rates increased. Organic molecules were directly delivered, or synthesized from endogenous organic molecules triggered by shock waves of meteor impacts.<sup>[81]</sup> The timing of the LHB within the Hadean eon is being increasingly questioned. According to Mojzsis and Werner, the LHB might have happened earlier than 4.48 Gya.<sup>[82]</sup> This would have provided an extended time span of favorable “cooler” environmental conditions, suitable for prebiotic chemistry to develop towards abiogenesis.

Influx of extraterrestrial material to the inner solar system is a potentially viable source of small organic molecules, including essential chemical building blocks of life. Already at the beginning of the 20th century, American geologist Thomas Chamberlin advanced the idea that organic material which “could have favored organic synthesis” might have entered the atmosphere of the early Earth on planetesimals, small solid bodies traveling in the solar system. The hypothesis of delivery has been developed further over the decades, and increasing amounts of experimental evidence was gathered.<sup>[83–87]</sup> Exogenous delivery can occur via comets, asteroid-type bodies and micrometeorites (interstellar dust, Figure 2d–f).<sup>[56]</sup> In several independent studies, analyses of meteorite material, especially of carbonaceous meteorites, have been performed, and numerous organic compounds were detected.<sup>[85]</sup> Carboxylic acids, hydrocarbons, nucleotides, amino acids and membrane-forming lipids, all being relevant prebiotic, potentially life-enabling chemical precursors, have been extracted from objects of extraterrestrial origin. Recently, the coma of comet 67P/Churyumov-Gerasimenko has been analyzed in conjunction with the Rosetta mission, and various quite complex molecules including hydrocarbons, oxygenated carbon moieties, and nitrogen-containing molecules were detected.<sup>[88]</sup> Micrometeorites have been identified relatively recently as a constituent of interstellar dust, and it was established that they are ubiquitous on the Earth’s surface.<sup>[89]</sup> Organic matter has also been identified in such particles.<sup>[90]</sup> Ten tons of intact organic matter is estimated to reach the atmosphere in the form of interplanetary dust particles, meteorites etc. every year.<sup>[91,92]</sup>

If not provided through exogenous delivery pathways, the organic matter available on the early Earth must have been synthesized on-site randomly from simpler precursors, e.g., carboxylic acids or nucleotides<sup>[93,94]</sup> (Figure 3). The assumption of the availability of these precursors is based on several hypotheses on the existence of small reactive organic molecules, such as hydrogen cyanide,<sup>[95]</sup> formamide,<sup>[96]</sup> and amines. A classic example is the Miller-Urey experiment<sup>[29,97]</sup> (Figure 3a) which results in formation of several amino acids. The spectrum of proposed chemical scenarios is quite broad. Prominent examples are Oparin-Haldane’s prebiotic soup hypothesis,<sup>[30,98]</sup> the Thioester world,<sup>[99]</sup> the Iron–sulfur world<sup>[100]</sup> and the Zinc world hypotheses.<sup>[44]</sup>

Of relevance for membranous protocells are primarily lipidic membrane constituents, e.g., fatty acids and phospholipids. Formation of phosphatidylethanolamine and phosphatidylcholine were observed under simulated primitive Earth conditions





**Figure 3.** Prebiotically plausible key chemical reactions. a) Miller-Urey synthesis of amino acids. b) Phospholipid synthesis. c,d) Fatty acid synthesis. e) Formose reaction and synthesis of ribose sugars. f) Ribonucleotide synthesis.

at temperatures ranging from 25 to 100 °C, mimicking the environment of evaporating ponds<sup>[101,102]</sup> (Figure 3b). In a similar setting, at temperatures of 100–400 °C, lipids up to C<sub>33</sub> were formed from oxalic acid<sup>[103,104]</sup> (Figure 3c,d). Phosphatidic acid and phosphatidylglycerol were obtained in a dry hot pond-like environment in the presence of silicate minerals as condensing agents.<sup>[105]</sup> In a recent review, Deamer has assembled the experiments performed under natural conditions that are presumably analogous to prebiotic environments.<sup>[53]</sup> Recently, Devaraj

and co-workers have prepared natural diacyl phospholipids in water in the absence of membrane-embedded enzymes.<sup>[106]</sup> Fiore and Strazewski reviewed newer work on prebiotic lipidic amphiphile synthesis and related condensing agents.<sup>[107]</sup>

Genetic material has been considered particularly important for protocell development<sup>[108]</sup> (*cf.* chemoton criteria). Synthesis of nucleotides, the building blocks of genetic polymers, could have possibly occurred under early Earth conditions.<sup>[109,110]</sup> However, the genetic polymers themselves, e.g. DNA, require



more sophisticated synthesis pathways. This challenge is at the heart of the “RNA world hypothesis”<sup>[1]</sup> (Figure 3e,f).

The assumption of a LHB renders the time period between a subsequent moderate, cool Earth and the first appearance of microbial communities relatively short, perhaps too short to allow for the transition between non-living and living matter. In this light, the Panspermia hypothesis, based on the thought that life originated “someplace” where the conditions were favorable, and arrived on Earth through extraterrestrial space, was suggested.<sup>[111,112]</sup> According to Nicholson,<sup>[113]</sup> the term panspermia is attributed to the Greek presocratic philosopher and scientific enquirer Anaxagoras. It refers today to a set of hypotheses on the delivery of living organisms of extraterrestrial origin to Earth. The concept of panspermia has over the years been entertained by many scientists, including the Swedish Nobel prize winner Swante Arrhenius; and the astronomers Hoyle and Wikramasinghe, who in the mid-1970s first attributed an anomaly in the infrared spectrum of cosmic dust to the possible presence of bacteria. A significant increase in interest became notable at the end of the 1990s, when first experiments on microorganism survival were carried out during space missions. Open questions include survival upon escape from the original host planet, during travel in an interstellar environment for extended periods of time (comets, radiation pressure), and upon entry into the Earth’s atmosphere. In a review by Kawaguchi, a number of interesting aspects of the Panspermia hypotheses are discussed.<sup>[112]</sup>

There is a body of research showing that the presence of other organic matter can influence the fate of lipid-based protocells. For example, latest work by Keller and co-workers has shown that prebiotic amino acids bind and stabilize prebiotic fatty acid membranes in the presence of salt and  $Mg^{2+}$ .<sup>[114]</sup>

### 3. Model Protocell Systems

Protocells are self-organized micro- or nano-sized compartments, which are considered as intermediate structures between non-living weakly structured precursors, and living cells. Protocells are not as dispersed as a liquid medium, but they are also not as well-organized as modern biological cells. Protocell research is focusing on building synthetic structures from prebiotically relevant materials, and the step-wise implementation of evolving biological function. The aim is foremost to discover and explain the transition to life. Accordingly, protocell models aim to generate information on how exactly this transition might have occurred, in search for the pending answer to one of the great questions of our time.

The simplest protocell model structures are membrane-free droplets consisting of largely hydrophobic compounds, held together by weak interactions. More sophisticated model structures feature a self-assembled lipid membrane envelope encapsulating an aqueous volume. Protocells and biological cells have a common basic feature, which is the barrier function of their boundary. Both establish a set of chemical environments separated from the surrounding aqueous medium. In contrast to this predominantly structural feature, which is largely a subject of physical chemistry, dynamic processes such as growth,

replication, metabolism and evolution, are evidently experimentally harder to implement.

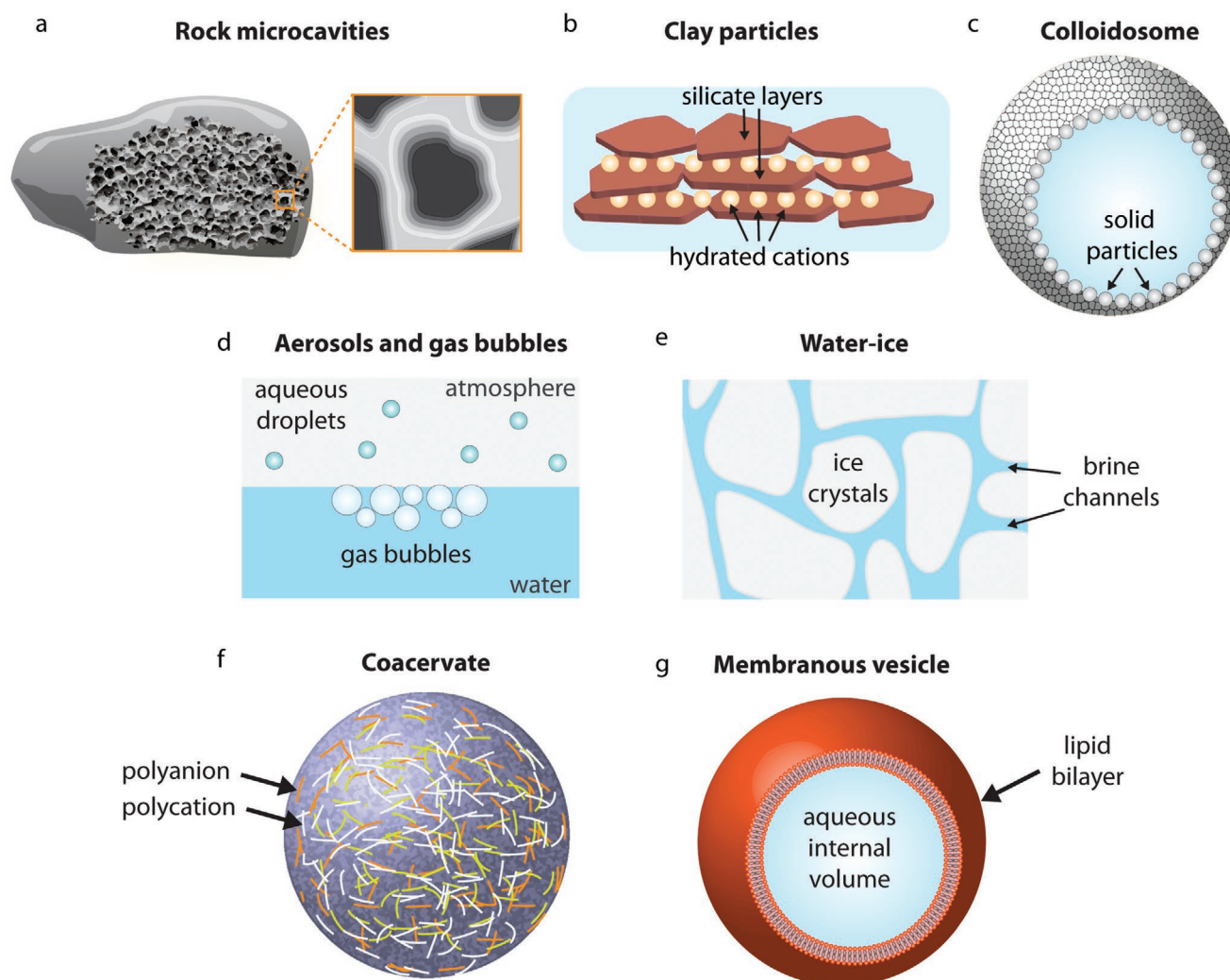
In this section, we will describe different protocell models (Figure 4). We concentrate on membranous protocells, and only briefly cover inorganic protocells and coacervates. The latter are intriguing for several reasons including comparatively simple building blocks, and unique possibilities to self-control their interface permeability.<sup>[115,116]</sup>

#### 3.1. Inorganic Compartments

Abiogenesis has occurred in a crude environment, with a limited range of organic molecules, but rich in water and minerals. It is therefore appealing to consider protocells that are derived from inorganic building blocks, which via organic/inorganic hybrids might have eventually led to the development of life. Inorganic protocell models involve minerals, or mineral-derived entities in several forms. This includes microcavities (Figure 4a) which can be viewed as “solid vesicles”; phyllosilicates, for example clay nanoparticles (Figure 4b); and colloidosomes, consisting of droplets or coacervates surrounded by solid nanoparticles (Figure 4c). Since the early 2000s, there is a body of new works appearing at a steady pace, addressing protocell environments based on predominantly inorganic components. Mizuuchi et al. reviewed possible inorganic environments for compartmentalization, including gas bubbles, atmospheric compartments and ice crystals<sup>[117]</sup> (Figure 4d–e). Very recently, Laneselli et al. showed evidence that trapped gas bubbles in heated micro-sized rock pores can affect coacervate protocell populations and support growth, fusion, division and selection of the droplets.<sup>[68]</sup> Moreover, the formation of multi-compartmentalized layers, consisting of gas bubbles entrapped in a mixture of surfactant-like molecules near the water surface has been introduced as the “scum hypothesis.”<sup>[118]</sup>

In addition to solid-walled vesicles, which can act as micro-reactors (Figures 1, 2 and 4), it was discussed if porous rocks could support vesicle extrusion. Since extrusion is a facile preparation procedure in the laboratory, such process does not seem unreasonable, provided that proper conditions with respect to pore size, pressure gradient etc. existed. Arguments were brought forward against the feasibility of this scenario, among them the loss of encapsulated vesicle contents to the environment during vesicle extrusion through small pores.<sup>[119]</sup> However, there is recent experimental evidence in its favor.<sup>[120]</sup>

Li et al. proposed a new model of solid protocells in the form of self-assembled graphene capsules containing selective ion channels.<sup>[121]</sup> It was reported that on these capsules, the L-amino acids exhibited higher reactivity than D-amino acids to form peptides, and under the influence of graphene the peptides would be transformed into a secondary structure, promoting the synthesis of left-handed proteins. This may be illuminating regarding the unsolved problem of biomolecular homochirality.<sup>[122]</sup> Conventional organic synthesis of chemical compounds carrying an asymmetric carbon center results in racemic mixtures. Living organisms, however, synthesize biomolecules favoring one of the two enantiomers. How and when that distinction was implemented in nature in the first place, is currently not established.



**Figure 4.** Compartment types. a) Microcavities in rocks constitute solid compartments. b) Phyllosilicates can accommodate chemical species in between smectic layers. c) Aqueous droplets are stabilized by a layer of nanoparticles (colloidosomes). d) The gas–water interface can provide two types of compartments: water particles suspended in air (aerosols), and gas bubbles suspended in water, which can accommodate biosurfactants at the interface. e) A frozen water–ice matrix upconcentrates solutes. f) Gel-like aqueous entities composed of macromolecules (coacervates). g) Spherical lipid bilayer compartments fully enclose and encapsulate an aqueous volume.

While most laboratory studies use silica nanoparticles as constituents of colloidosomes<sup>[123]</sup> (Figure 4c), natural clay materials associated with the early Earth are predominantly investigated in the context of colloidosome protocells.<sup>[124,125]</sup> Sun et al. presented a colloidosome membrane-based protocell model comprising Fe(III)-rich montmorillonite clay particles as multifunctional building blocks, featuring self-directed membrane remodeling, and signal-induced protocell communication. The clay colloidosomes exhibit size- and charge-selective permeability, and show catalytic functions applied for in-situ polymer synthesis.<sup>[126]</sup>

Inorganic models comprise a relatively small part of the protocell model variety. It is apparently more complicated to extend these models and make the transition to organic compartments. Given the fact that the world was predominantly inorganic on the early Earth, such models nevertheless have merits. As shown above, the studies on inorganic compartments reveal

potential important implications on key aspects of the origins of life, e.g., the emergence of homochirality.

### 3.2. Coacervates

Coacervates, introduced to the origins of life debate already by Oparin,<sup>[30]</sup> are gel-like aqueous entities composed of macromolecules,<sup>[127,128]</sup> primarily polymers,<sup>[129]</sup> peptides,<sup>[130]</sup> proteins,<sup>[129,131]</sup> nucleic acids,<sup>[132]</sup> or surfactants<sup>[133]</sup> (Figure 4f). The number of publications on coacervates in the context of the origins of life has been steadily growing since the 1950s.<sup>[134,135]</sup>

Coacervates lack an envelope, and therefore show lower structural stability compared to other models.<sup>[136]</sup> They are generated by a liquid–liquid phase separation process, and have found extensive use as a tunable dynamic model for artificial cells or organelles.<sup>[137–141]</sup> Particularly interesting are “active”

coacervates, governed by an incorporated organic reaction which supplies the droplet with de novo synthesized material for autonomous growth.<sup>[142]</sup> The possible role of coacervates in the origin of life has been recently repeatedly reviewed.<sup>[117,134,143]</sup>

Coacervates are suggested as precursors to membranous vesicles with various levels of hierarchical complexity.<sup>[144–146]</sup> For example, Tang et al. proposed the spontaneous self-assembly of a continuous fatty-acid membrane at the surface of preformed coacervate microdroplets.<sup>[147]</sup> Martin and Douliez reviewed fatty acid vesicles and coacervates, and emphasized that coacervates can reversibly transform into fatty acid vesicles due to changes in pH, suggesting to consider this phenomenon as a new direction to explore the origins of life.<sup>[143]</sup> Deshpande and Dekker laid out different strategies to generate and manipulate coacervates in liposomes and polymersomes.<sup>[148]</sup>

Furthermore, coacervates are proposed as model structures for membraneless organelles in modern biological cells<sup>[127]</sup> which are nowadays referred to as “cellular condensates”. Prominent examples are the nucleolus, paraspeckles, and the Cajal bodies therein. Similar to such cellular components, metastable coacervates can exist in various phases such as liquid, gel-like, and aggregated.<sup>[127]</sup>

Compared to membrane-encapsulated protocells, routes to multi-compartmentalization can be more facile for coacervates, with the possibility to generate several layers of substructures. Such organized models create advanced opportunities to perform coupled or competing reactions.<sup>[146,149]</sup> Some recent examples include complex coacervation, where phase separation of mixtures of oppositely charged polymers provides a direct route to compartmentalization.<sup>[141]</sup> Moreover, capture of enzyme-encapsulating proteinosomes by fatty acid coacervates was shown to produce multi-compartmentalized host–guest protocells capable of antagonistic chemical and structural coupling.<sup>[150]</sup> Compared to membranous protocells, coacervates can facilitate the transfer of biologically relevant molecules over the phase boundary. For example, by binding Ni<sup>2+</sup>-nitrilotriacetic acid to His-tagged proteins, control over the loading of macromolecules was achieved.<sup>[128]</sup> In another example, it was shown that coacervate droplets were able to uptake and retain numerous intact plant chloroplasts, and accommodated light-induced electron transport.<sup>[151]</sup>

In the context of the origin of life, if a fraction of coacervates is outperforming another fraction in the population, it could be considered advantageous from an evolutionary point of view. One recent example illustrating this aspect involves coacervates made from short polyions. They were shown, in comparison to those formed from longer polyions, to more efficiently generate distinct pH microenvironments, to accumulate RNA, and to preserve duplexes.<sup>[152]</sup> In another example, coacervates comprising sugars and amino acids were reported to facilitate RNA cleavage, compared to coacervates free of these constituents.<sup>[141]</sup> Following a theoretical study on how chemically active droplets grow and divide,<sup>[153]</sup> gas bubbles inside heated rock cavities were shown to promote the growth, fusion, division and selection of coacervate microdroplets consisting of polyanionic (carboxymethyl dextran, ATP) and polycationic (poly (diallyl dimethyl ammonium chloride), poly (l-lysine)) species.<sup>[120]</sup>

Between coacervates and lipidic protocells exists a considerable range of membranous and membrane-like structures that

can form an interface. Membrane material of such interfacial assemblies can consist of inorganic nanoparticles, proteins, amphiphilic block copolymers, or mixtures of bio-macromolecules and polyelectrolytes. They are established by self-assembly or phase separation.<sup>[154,155]</sup>

### 3.3. Membranous Compartments

A major obstacle to unraveling the path to the earliest living cells is the complexity arising from the necessity to satisfy the chemoton or equivalent conditions, i.e., to unify metabolism (energy), inheritance (function) and container (structure). In the early Earth environment, where the inventory of materials was rather limited, any simple material that could critically contribute to satisfying all three of these conditions would have been of advantage towards the development of life. We have pointed out earlier that there is evidence supporting the presence of lipid material on the early Earth. Lipids have favorable properties: they are structurally relatively simple, and can self-assemble to 2D fluids (membranes) which easily accommodate a number of different molecular species. Lipid membranes readily form closed compartments (Figure 4g), which have the ability to undergo non-trivial shape deformations: grow, bud, tubulate, divide, form dynamic pores, etc. Contemporary biological life universally features and utilizes membranes. This is a strong argument in support of membranous compartments as capable protocell models, but not the only one. Lipids are capable of more than just forming compartments and membranes. In a milestone publication, Segre et al. formulated the hypothesis of a “lipid world”, pointing to the potential of the unique chemical and physico-chemical (collective) capabilities of lipids and other small amphiphiles to form catalytic networks, perform molecular information processing, and give rise to self-reproduction and compositional inheritance.<sup>[2,156]</sup>

The combination of membrane-forming and chemical capabilities could have been the foundation of functionally advanced protocells that came close to the transition to life. At some point, structural “upgrades” and the incorporation of other chemical systems must have occurred. Such chemical combination systems encapsulated in lipid membranes make excellent candidates for prebiotic protocells, because they address several of the chemoton criteria at the same time. Some of the most interesting unanswered questions are: when did they appear, how did they develop, and how did they reach the transition to life? Segre et al. made the argument that the lipid world is simpler and more probable to have preceded the “RNA world”,<sup>[1]</sup> a putative stage in which RNA, before proteins and DNA; displayed and evolved new catalytic activities through a molecular type of Darwinian selection.

The literature shows numerous angles from which to look at membranous protocells, both experimentally and theoretically.<sup>[157–160]</sup> This includes compartmentalization, encapsulation, hosting chemical reactions, recognition, signaling, shape transformations, and others.

A widely employed, easy-to-produce model features self-assembled lipid compartments freely suspended in bulk; solid particle- and surface adhesion-based models have also been reported.

### 3.3.1. Protocell Formation and Stability in Bulk Systems

A majority of protocell models are based on bioamphiphile compartments in aqueous solutions/suspension. The water environment provides the physico-chemical conditions for the amphiphiles to self-organize into compartments and become an independent entity, which is assumed to have been the basic container and template for developing chemical processes of increasing complexity, and eventually achieved the transition to life.<sup>[157]</sup> Chen and Walde emphasized in their review article<sup>[161]</sup> the outstanding importance of thermodynamics in governing the fundamental process of biosurfactant self-assembly in bulk media.

Spontaneous formation of membranes from amphiphile solutions is a concentration-dependent process, in which a significant critical aggregate concentration (cac) must be reached.<sup>[162]</sup> The concentrations of simple bioamphiphiles, synthesized under prebiotic conditions, would probably be too low to lead to self-assembly, therefore discovery of autonomous upconcentration mechanisms is crucial for identifying suitable origin of cellular life conditions. Narrow, vertically oriented channels within mineral and rock formations have been proposed to have acted as thermal diffusion columns, in which temperature gradients concentrate dilute molecules through the coupling of thermophoresis and convection. This concept was experimentally investigated using microcapillaries as thermal diffusion columns to concentrate a solution of oleic acid to the point where vesicles were formed.<sup>[163]</sup> Early protocells were likely composed of single-chain amphiphiles. However, the stability of pure single-chain amphiphiles is, apart from concentration, highly dependent on environmental conditions, for example, pH, temperature and divalent cation concentration.<sup>[164]</sup> Compositional diversity benefits the stability and robustness of membrane assemblies towards multiple selection pressures experienced by protocellular life.<sup>[165]</sup> Vesicles composed of fatty acids mixed with fatty alcohols, fatty amines, glycerol monodecanoate or polycyclic aromatic hydrocarbons have a broader window of environmental conditions in which they are stable.<sup>[166]</sup> For example, adding fatty alcohols increases durability over a large pH range;<sup>[166,167]</sup> adding glycerol monoester, citrate, isocitrate, and oxalate makes the vesicles more  $Mg^{2+}$  tolerant;<sup>[168,169]</sup> and mixtures of alkyl amines and fatty acids form vesicles at strongly basic and acidic pH, which are resistant to the effects of divalent cations up to 0.1 M.<sup>[170]</sup> Citrate allows  $Mg^{2+}$ -dependent RNA synthesis within vesicles, while at the same time protecting RNA from  $Mg^{2+}$ -catalyzed degradation.<sup>[169]</sup> A recent study has shown the formation of vesicles from fatty acids of different chain length, e.g.,  $C_{10}$  to  $C_{15}$ , under alkaline hydrothermal conditions, with high salt content and at  $\approx 70^\circ C$ .<sup>[167]</sup>

$C_{10}$  to  $C_{12}$  monocarboxylic acids and their monoglycerides were shown to form stable membrane compartments in hydrothermal pool water from hot springs in the Yellowstone National Park (USA). Compared to pure fatty acids, this mixture was less temperature-sensitive and assembled into membranes at both acidic and alkaline pH, but not in seawater samples.<sup>[54]</sup>

Fatty acid vesicles compete with each other to incorporate fatty acid monomers from the surrounding aqueous buffer.<sup>[171]</sup> In extension, the effects of a gradual transition from fatty acids

to phospholipids were shown by Budin et al. A low amount of phospholipids enhances the growth of vesicles (10 mol%) in the membrane by consuming vesicles with less phospholipid contents, or vesicles consisting of pure fatty acids.<sup>[172]</sup> Similarly,  $Mg^{2+}$  in the preparation buffer preferentially removes fatty acids from mixed fatty acid/phospholipid membranes.<sup>[173]</sup>

Fatty acid membranes are highly permeable to solutes in the aqueous environment. The transition to phospholipid-rich membranes decreases membrane permeability.<sup>[172,174]</sup> Mixed fatty acid-phospholipid membranes were shown to selectively retain  $K^+$ , but allow the passage of  $Na^+$ . The  $K^+/Na^+$  selectivity of the mixed fatty acid-phospholipid semipermeable membranes suggests that protocells could have established electrochemical  $K^+/Na^+$  ion gradients in the absence of macromolecular transport machinery or pumps, thus potentially facilitating a rudimentary metabolism.<sup>[175]</sup> In a study where four fatty acid mixtures were subjected to laboratory buffers and liquid samples obtained from hot springs, only mixtures of fatty acid and its glycerol derivatives were found to form vesicles in natural water.<sup>[176]</sup> The authors advise caution that the transition from the laboratory to a more realistic natural environment can lead to dramatic changes in behavior.

While fatty acids self-assemble to micelles before forming unilamellar bilayer vesicles, phospholipids typically form small, nanosized unilamellar vesicles, i.e., liposomes.<sup>[177]</sup> Cell-sized unilamellar vesicles, which are most interesting for laboratory investigations of protocells, generally require templating or surface support. A new report on self-assembly of giant unilamellar phospholipid vesicles in bulk describes their properties and fabrication conditions in detail.<sup>[178]</sup>

Several investigations focusing on RNA encapsulation and activity utilize membranous protocells.<sup>[108]</sup> In a recent article, it was shown that the encapsulation of ribozymes inside phospholipid compartments in bulk led to faster evolutionary adaptation compared to ribozymes free in solution.<sup>[179]</sup> Nonenzymatic primer extension inside fatty acid compartments was also reported.<sup>[180]</sup>

### 3.3.2. Protocell Formation on Solid Surfaces

To create bulk assemblies, only two essential components are required: amphiphiles and water. Both must have co-existed at some point on the early Earth. Solid surfaces were also present in the form of various minerals and rocks. The predominantly passive role of solid interfaces in accommodating dry-wet cycles and promoting protocell formation through lipid hydration was suggested by Deamer.<sup>[50]</sup> It appears, however, that certain high-energy surfaces can contribute more directly, as their intrinsic surface energy is on the same order of magnitude as the energy that is required for membrane shape transformations.

A distinct set of studies in this context has been concerned with interactions of mineral and mineral-like micro- and nanoparticles with lipid monomers and micelles in bulk. It was shown that if lipids are combined with mineral particles in aqueous media, liposome formation is enhanced as compared to a solution environment free of particulates.<sup>[62,173,181,182]</sup> This was confirmed by determining changes in turbidity of the solution, and by transmission electron microscopy. Several



different types of mineral particles were investigated, including silica and hydroxyapatite. Montmorillonite microparticles were shown to accelerate the conversion of myristoleate ( $C_{14}$ ) fatty acid micelles to vesicles by a factor of up to 100.<sup>[181]</sup> The decisive parameters for promotion of lipid compartment formation were determined to be surface charge density,<sup>[182]</sup> the isoelectric point of the mineral particles, and their reactive surface area.<sup>[183]</sup> In a related study, photocatalytic mineral particles were used to perform a primordial metabolic reaction.<sup>[184]</sup> The mineral particles in the extra-vesicular medium harvested light energy to generate a transmembrane pH gradient, and reduced nicotinamide adenine dinucleotide (NAD<sup>+</sup>) to NADH within vesicles. Such a proton gradient or a chemiosmotic potential created by a mineral particle surface could have potentially led to adenosine triphosphate (ATP) synthesis under prebiotic conditions.<sup>[184]</sup>

Another set of studies focused on protocell assembly on extended areas of flat interfaces. Sets of conditions for the formation of well-defined lipid films by self-spreading of multilamellar reservoirs on solid surfaces has been established earlier.<sup>[185,186]</sup> Lipid films generated in that fashion can be monolayers, single- or double bilayers depending on the magnitude of the surface energy. Solid supports do not necessarily have to be macroscopically flat, some reports featured the assembly of bilayers on micro-sized glass beads, and subsequent transformation into budding vesicles.<sup>[187,188]</sup>

Köksal et al. utilized high-energy surfaces to transform spreading double lipid bilayers into giant membranous compartments interconnected by lipid nanotubes.<sup>[189,190]</sup> In this autonomously occurring process, common lipid agglomerates are consistently driven to non-trivial lipid structures by surface free energy minimization. Similar to bulk assembly, very few fundamental assumptions are required for this process in a prebiotic setting, essentially the availability of water, biosurfactants and suitable environmental parameters. Access to natural surfaces can be reasonably assumed. Mildly elevated temperatures, similar in range to temperatures measured in the Lost City Hydrothermal fields,<sup>[42]</sup> facilitate this transformation.<sup>[191]</sup> This could have implications in the context of the origin of life.

Mineral surfaces are considered of having been a co-factor in many early biosynthetic reactions.<sup>[192,193]</sup> Fontecilla-Camps reviewed primordial bio-energy sources and argued that a transition between surface-based catalytic chemistry and equivalent chemical systems within protocells must have happened, pointing to a likely role of ATP phosphorylating agents in this process.<sup>[194]</sup> It seems reasonable that surface-adhered protocells, whether formed on the surface or attached there after formation, would have benefitted in their evolution at some point directly from co-located interfacial chemistry. That gives surface-based systems a possible advantage in early evolution towards life.

#### 4. Protocell Subcompartmentalization

Compartmentalization is a fundamental organizational principle of all forms of life. Confinement of biochemical reactions into distinct intracellular membrane-enveloped

volumes, the “organelles”, allows cells to simultaneously accommodate otherwise incompatible reactions. Each organelle has a specific structure, composition, environment and function.

Membrane-bound organelles are still by definition exclusively associated with eukaryotes.<sup>[195]</sup> However, membrane-enveloped compartments have also been identified in bacteria and archaea,<sup>[196–198]</sup> which led to awareness of the possibility that similar membranous structures might have already existed much earlier in time, for example in the last universal common ancestor (LUCA),<sup>[199]</sup> or even in simple protocells, provided that they had the ability to form subcompartments in a consistent way. In addition to increased versatility in co-hosting chemical reactions,<sup>[200]</sup> such systems would be more robust towards mechanical and osmotic stress in an unpredictable, shifting environment.

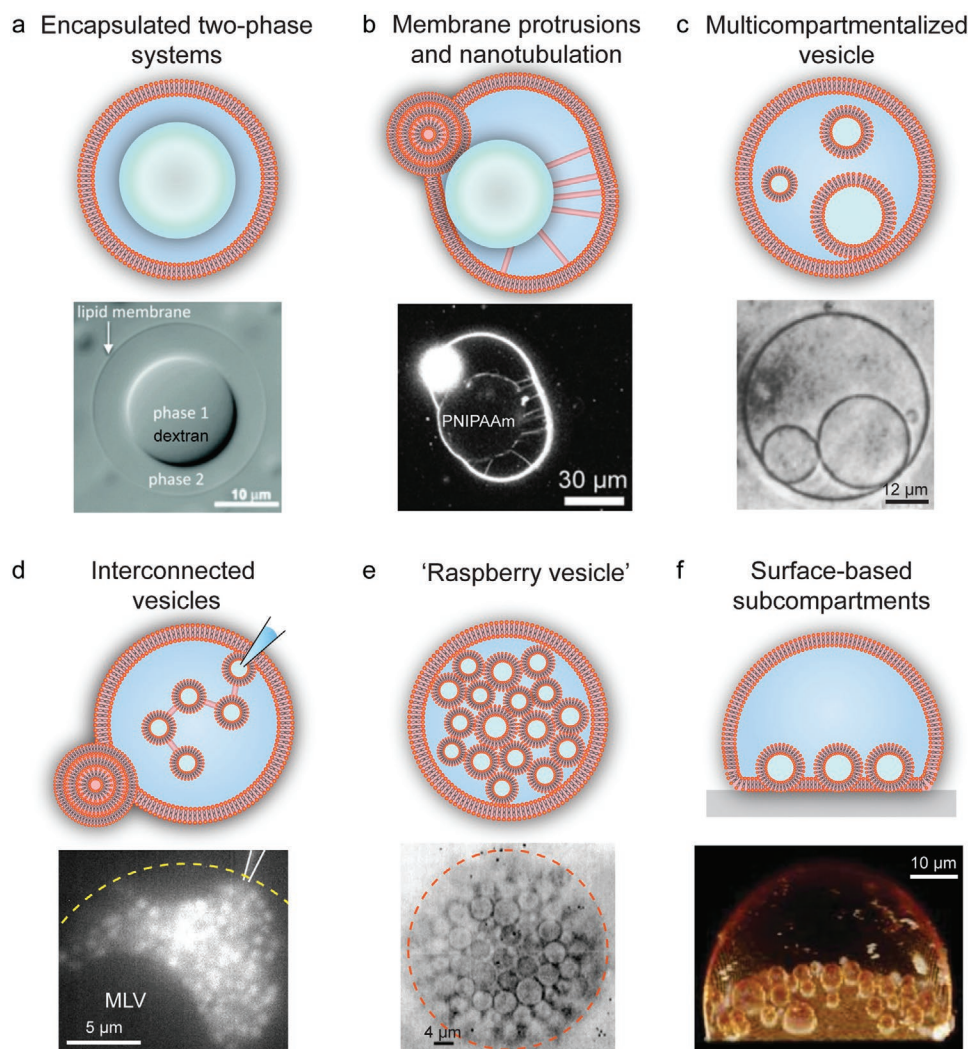
The membrane enveloped two-phase systems, described in the coacervate section above, qualify easily as subcompartmentalized protocells<sup>[116,201]</sup> (Figure 5a). In one example of membrane-enclosed two-phase systems, nanotubes bridging the vesicle membrane to a contracting hydrogel were retained and transformed into smaller vesicles, reminiscent of cellular endosomes<sup>[202]</sup> (Figure 5b). The encapsulation of small membranous vesicles in coacervates opens opportunities for creating cytoplasm mimics, crowded with organelle-like lipid structures.<sup>[201]</sup>

A similar system is the reported bottom-up assembly of subcompartmentalized protocells by spontaneous encapsulation of semipermeable polymersomes inside cell-sized coacervates.<sup>[146]</sup> The microdroplets in this study were assembled and finally enveloped in a polymer outer shell.

The equivalent of a biological cell with internal membrane-bound organelles is known as a multivesicular vesicle, or vesosome,<sup>[207,208]</sup> a membrane envelope encapsulating free-floating vesicles of smaller size (Figure 5c). Typically, the boundaries of the internal vesicles are not physically connected to the membrane of the enveloping container. There is one notable exception where the internalized vesicles are interconnected by nanotube sections in a pearl chain-like fashion<sup>[204]</sup> (Figure 5d). The compartments and the nanotube membranes remain connected to the outer bilayer. Vesosomes can form randomly as a result of mechanical perturbation during lipid self-assembly, or due to osmotic stress<sup>[205]</sup> (Figure 5e). They can be prepared with a variety of laboratory techniques.<sup>[207,209]</sup>

Subcompartmentalization of giant vesicles incorporating biotinylated lipids was induced on avidin-decorated solid surfaces driven by avidin–biotin interaction.<sup>[210]</sup> In this case, the surface acted as a solid support for membrane pinning and immobilization. Somewhat closer to early Earth conditions is the new work reported by Spustova et al. on a subcompartmentalization mechanism which harvests the surface free energy for autonomous membrane shape transformations<sup>[206]</sup> (Figure 5f).

A review by Schmitt et al. covers subcompartmentalization of various membranous structures, comprising a large range of materials including polymers and gels, and aspects of intravesicular communication.<sup>[209]</sup> This work summarizes promising approaches to combining structural with functional features which go beyond the mechanistic aspects of subcompartment formation.



**Figure 5.** Subcompartmentalization of protocells. a,b) Lipid membrane-bound two-phase liquid coacervates. In (a) the condensed liquid phase is dextran, and in (b) the thermo-responsive polymer PNIPAAm. Lipid nanotubes form between the droplet and the protocell membrane during phase transition of the polymer to a gel droplet. a) Adapted with permission.<sup>[116]</sup> Copyright 2012, American Chemical Society. b) Adapted with permission.<sup>[202]</sup> Copyright 2011, American Chemical Society. c) Multicompartmentalized/multivesicular giant unilamellar compartments. Adapted with permission.<sup>[203]</sup> Copyright 1994, American Chemical Society. d) Nanotube-connected, membrane-bound subcompartments inside a membranous protocell (dashed lines) formed via exposure of the membrane to a  $\text{Ca}^{2+}$  flow. Adapted with permission.<sup>[204]</sup> Copyright 2020, Springer Nature Limited. e) A “raspberry vesicle” with multiple, disconnected subcompartments. Adapted with permission.<sup>[205]</sup> Copyright 2002, Elsevier. f) Subcompartments formed at the basal membrane of a surface-adhered lipid compartment. Adapted with permission.<sup>[206]</sup> Copyright 2021, John Wiley and Sons.

## 5. Protocell Growth and Division

Size regulation of living organisms down to the cells and their substructures is an intricate research problem in biology.<sup>[211]</sup> Biological cell division in this context is a combination of regulated growth limitation, which establishes a balance between form and function of the cell, and reproduction. For a protocell, however, growth and division are energy-controlled processes,<sup>[212,213]</sup> where the membrane composition is also important.<sup>[214]</sup> In a thought experiment, if a spherical protocell grew by incorporation of new membrane material, its surface-to-volume ratio would decrease. This is not possible in the physical world, since the lipid membrane has low water permeability and the volume cannot increase proportionally. Thus, the

container needs to adopt a non-spherical form. This has been confirmed experimentally:<sup>[119,215]</sup> a pearling phenomenon was observed, which resulted in reduced mechanical stability, such that the vesicle chain could be readily divided into individual “daughter” vesicles by weak external forces.

Spontaneous division of an unperturbed protocell, even in pearled form, is unlikely because the edge energy of the two pores generated in this process is too high to be overcome spontaneously without external forces or molecular machinery. If sufficient lipid material is available, a closed membrane compartment would grow, deform and eventually decay or divide. Growth and division processes are thus closely coupled, which is reflected by recent studies, for example on growth mechanisms,<sup>[216]</sup> and autonomous or stimulated division.<sup>[217]</sup>

The combination of these fundamental processes to achieve continuity was most likely a development milestone on the path to living cells. Sophisticated examples of certain prebiotic relevance have emerged.<sup>[218,219]</sup> It is rather unlikely that molecular machinery was available to early protocells, but external cues and energy sources that could have caused growth and division events must have existed. Szostak recently reviewed the ongoing efforts to create advanced protocell models which combine replicating compartments and genetic materials.<sup>[220]</sup>

### 5.1. Lipid Reservoirs

Direct growth of protocell compartments can occur if the compartment has physical access to a lipid reservoir (Figure 6a), e.g. a multilamellar vesicle or a multilayered lipid film. Preparation of vesicles from multilamellar reservoirs and films is the most commonly employed technique for vesicle/protocell preparation in the laboratory, and is discussed further down in detail (cf. *Laboratory fabrication of protocell models*). Unilamellar protocell compartments attached to lipid droplets, i.e., an oil droplet containing dispersed lipid monomers, use the reservoirs to grow or shrink, and dilute or concentrate its contents in order to adjust to different osmotic conditions.<sup>[221]</sup> Lipid nanotubes can also serve as membrane reservoirs, and increase the size and robustness of vesicles during changes in the osmotic conditions<sup>[222]</sup> (Figure 6b).

### 5.2. Incorporation of Precursors

Insertion of lipid monomers, especially fatty acids, from the ambient solution into protocells can lead to rapid growth (Figure 6c,d).<sup>[172]</sup> Kurihara et al.<sup>[228]</sup> demonstrated that GUVs encapsulating DNA could spontaneously grow and divide after addition of membrane precursors. Amplification of DNA later accelerates the division process.<sup>[228]</sup> A similar effect was observed in RNA-encapsulating fatty acid vesicles. RNA creates osmotic pressure and tension in the membrane which in turn drives membrane growth by incorporation of fatty acids into the vesicle membrane.<sup>[229]</sup> Tension-mediated material incorporation and growth was demonstrated in a study by Deshpande et al., where small unilamellar vesicles were the source of material, and transmembrane osmotic pressure generated the tension.<sup>[230]</sup> Zhu et al.<sup>[119]</sup> also grew large multilamellar fatty acid vesicles by feeding them with fatty acid micelles, and even repeated this process in consecutive cycles. Growth by adding fatty acid monomers to the external monolayer of pre-existing phospholipid vesicles exhibits a “matrix effect.”<sup>[231]</sup> Insertion is followed by flip-flop to the internal monolayer. The free fatty acids in the environment later interact preferentially with fatty acids in the bilayer. This leads to the growth of elongated unilamellar structures (Figure 6c). The pearl chain-like vesicles can split upon sonication into similarly sized vesicles, keeping the size distribution strongly biased towards the original vesicles. Results are consistent with a model that involves growth and subsequent fission of the mixed vesicles. The study provides further support for the matrix effect.<sup>[232]</sup> The pre-existing DMPC vesicles act as a kind of seed to control the behavior of the system in the presence of added fatty acid anions.

Supplying fatty acids in ethanolic solutions to suspensions of microsphere-supported bilayers (cf. section Protocell formation on solid surfaces), causes membrane area growth and subsequent formation of vesicles.<sup>[233]</sup>

Growth of a protocell does not necessarily occur by direct area enlargement of its membrane. When oleic anhydride is added into the aqueous suspension of oleic acid/oleate vesicles, its hydrolysis is catalyzed within the bilayer,<sup>[225]</sup> leading to formation of a second internal vesicle which eventually separates (Figure 6d).<sup>[119,224]</sup> The processes of formation of a gap for “birthing” of the vesicle, and subsequent healing of the membrane, have been previously described.<sup>[203]</sup>

Continual growth was reported for a sophisticated protocell model that has the ability to synthesize catalytically active precursors of additional membrane material, which is produced and incorporated in the membrane.<sup>[234]</sup> A similar experimental observation was made for oil-in-water droplet systems comprising amphiphilic molecules that catalyze their own formation by lowering the interfacial tension between droplets of the reaction mixture and the aqueous phase, eventually causing them to divide.<sup>[235]</sup>

### 5.3. Vesicle Fusion

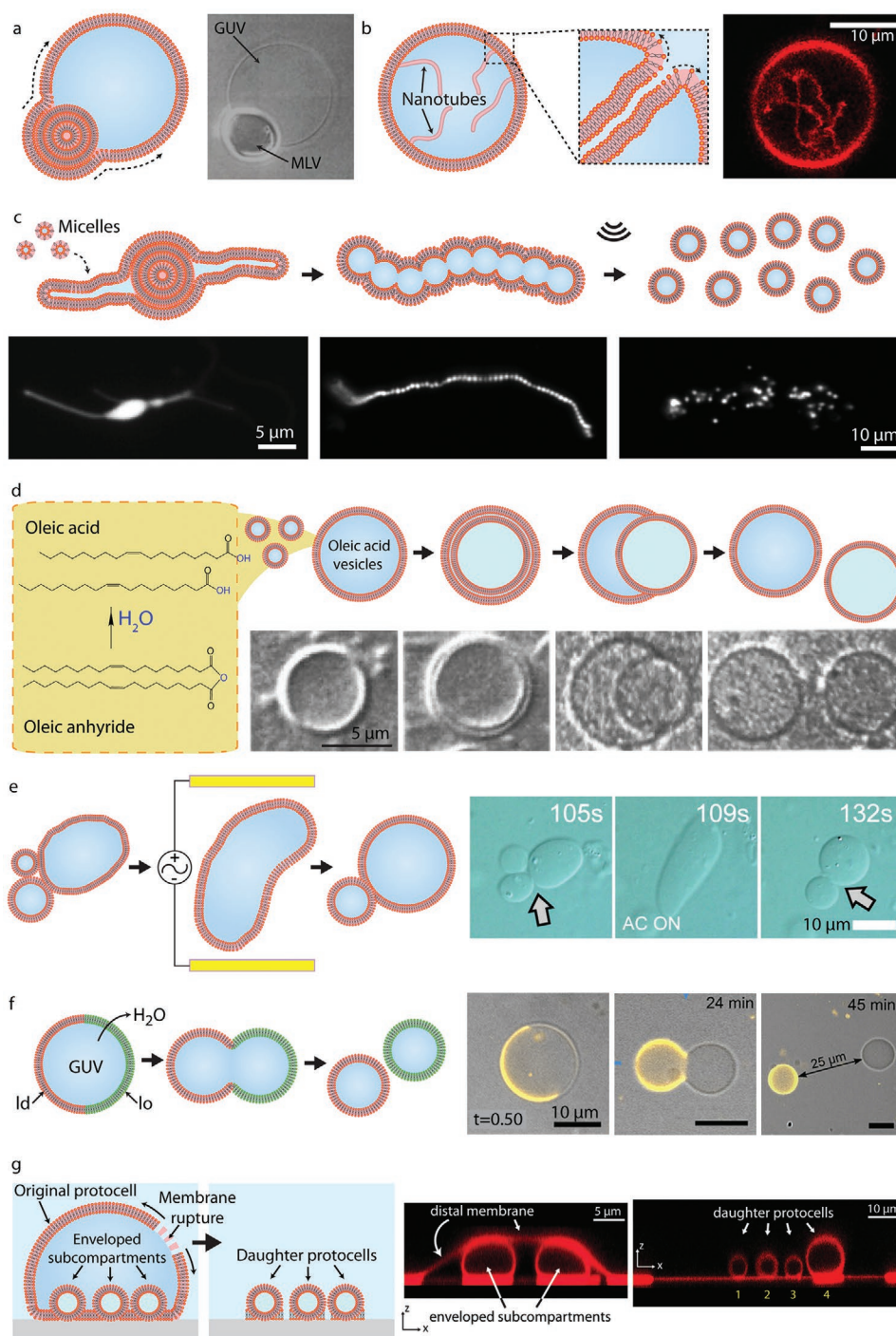
The literature on vesicle fusion, a process which naturally increases the membrane area by combining the areas of the fusing entities, is extraordinarily extensive due to the importance of small unilamellar vesicle fusion in biology.<sup>[236]</sup> The concept of merging a vesicle with a model protocell is highly relevant, since this process is not only increasing the size of the original container, but is also combining the contents.<sup>[237]</sup>

A recent review provides details on fusion mechanisms with relevance for model prebiotic compartments.<sup>[238]</sup> Protocells consisting of membranes of opposite charge can particularly rapidly fuse.<sup>[239]</sup> In one study, fusion and division of polymer-containing membranous protocells were reversibly induced by electrical currents<sup>[226]</sup> (Figure 6e). In another example, fusion of surface-adhered giant lipid compartments containing RNA, and merging of their content, were induced by increasing temperature from RT to 90 °C.<sup>[191]</sup>

### 5.4. Division

On the path to life, protocells eventually have to gain the ability to divide.<sup>[217,237]</sup> To be able to perform this transformation, membrane deformation and fission need to occur.<sup>[217]</sup> This requires energy, which in contemporary cells is produced from chemical sources. Evidence suggests that even under early Earth conditions, where energy storage molecules were not yet available, division of protocells in bulk could have possibly occurred,<sup>[216,227]</sup> provided that a suitable stimulus was applied, such as a surge in available membrane material.<sup>[119,178]</sup> Caspi et al. reported on a range of abiotic mechanisms for the division of lipid-membrane-encapsulated vesicles due to physical or chemical principles.<sup>[240]</sup> Another review presented by Murtas discusses possible early division and self-reproduction mechanisms in protocells.<sup>[216]</sup>





**Figure 6.** Protocell growth and division. Lipid compartments can grow using the material in membrane-bound a) lipid reservoirs, or b) lipid nanotubes. a) Adapted with permission.<sup>[223]</sup> Copyright 2011, Springer Nature Limited. b) Adapted with permission.<sup>[222]</sup> Copyright 2018, American Chemical Society. c) Incorporation of fatty acid micelles from the solution into a multilamellar vesicle results in tubular growth. The tube exhibits pearly instabilities. Upon mechanical agitation the pearled compartments divide into individual daughter cells. Left panel Adapted with permission.<sup>[172]</sup> Copyright 2011, National Academy of Sciences. Middle and right panels. Adapted with permission.<sup>[119,224]</sup> Copyright 2012, National Academy of Sciences. d) Addition of oleic anhydride to an oleic acid vesicle suspension causes the hydrolysis of oleic anhydride to oleic acid, which assembles into small vesicles and incorporates into giant vesicle membranes. The growth due to vesicle fusion leads to formation of a daughter vesicle, followed by “birthing.” Adapted with permission.<sup>[225]</sup> Copyright 1995, American Chemical Society. e) Alternating electric current induces vesicle fusion which is a rapid form of growth. When the current is off, the protocell divides into two compartments. Adapted with permission.<sup>[226]</sup> Copyright 2012, National Academy of Sciences. f) Membrane phase separation and osmosis drives protocell division. A giant membranous compartment consisting of liquid disordered and liquid ordered phases of lipids, divides spontaneously upon loss of water due to osmotic imbalance. Adapted with permission.<sup>[227]</sup> Copyright 2021, John Wiley and Sons. g) Pseudo-division occurs when protocell subcompartments become individual daughter cells upon rupturing of the enveloping membrane. Adapted with permission.<sup>[206]</sup> Copyright 2021, John Wiley and Sons.



Division by extrusion, i.e., size reduction by pushing larger lipid assemblies through pores with smaller cross-sections or across physical obstacles,<sup>[217]</sup> has been hypothesized to be a protocell formation mechanism on the early Earth, supported by the presence of porous rocks and pressurized fluids, e.g. in hydrothermal vents. A study tested this hypothesis by investigating the fate of solutes of low- and high molecular weight during division of large vesicles by means of extrusion.<sup>[241]</sup> The investigated process led to a loss of internal content to the solution environment, but macromolecules were partially retained. These findings suggest that there might be a size-discriminating mechanism operating on internalized molecules during the critical step of vesicle growth and division, which could have contributed to primitive evolution.

Further above we described how rapid membrane expansion upon incorporation of lipid material can drive shape transformations and pearling, as a step towards division.<sup>[119,215]</sup> Protocell contents appear to also influence the division process. One study reported that the division of lipid compartments depended on the length of encapsulated DNA; this may provide an explanation of how the presence of nucleic acids could have directly affected the division of prebiotic protocells.<sup>[242]</sup>

Thermally-driven division mechanisms might also be relevant for early prebiotic replication. Examples include thermally driven fission of protocells<sup>[243]</sup> and division by “birthing” of an internally assembled daughter vesicles due to membrane contraction and pore formation in the mother vesicle upon cooling.<sup>[244,245]</sup> The presence of the synthetic lipid DLPE appears to be necessary for the latter process. However, this does not strictly exclude that other lipid species or lipid mixtures would support similar pathways in nature. A recent study shows how lipid membranous protocells consisting of two different lipid phases, e.g., liquid disordered ( $l_d$ ) and liquid ordered ( $l_o$ ) domains, can divide due to an osmotic imbalance leading to loss of water from the internalized aqueous medium (Figure 6f).<sup>[227]</sup> Vesicle birthing following membrane phase separation and formation of pores was also reported.<sup>[246]</sup>

A newly published unique pathway for growth and division exploits differences in the compositions of fatty acid membranes.<sup>[247]</sup> In this experimental study, growth is driven by the thermodynamically favorable exchange of lipids between two populations, and division occurs as a result of growth-induced curvature.

Instead of a single compartment dividing into two smaller compartments, other, non-trivial protocell models have been proposed for division. For example, pseudo-division can occur by disintegration of a protocell membrane due to increased tension, and transformation of its original sub-compartments into individual daughter cells<sup>[206]</sup> (Figure 6g). In another example, a protocell network in which lipid compartments are interconnected via lipid nanotubes could be considered as a pre-divided protocell, which is able to transfer contents to adjacent nodes, which can subsequently separate from the population, for example, due to gentle flow.<sup>[189,248]</sup> The “birthing” mechanism, where the size of the mother vesicle is not changing, is also a pseudo division phenomenon.<sup>[244,245]</sup> Besides these experimental works, there are computational division models.<sup>[249]</sup> Theoretical work on protocell growth and division will be discussed further in the section *Protocells in silico*.

## 5.5. Heredity of Genetic Information Across Protocell Generations

The above-mentioned examples of vesicles encapsulating genetic polymer RNA or DNA, and undergoing growth and division, are prominent among the few examples of self-replicating protocell systems available in the literature.<sup>[218,224–226,228]</sup> Transfer of genetic information between generations is an essential requirement for protocell evolution. The design of a model with self-replicating ability is very challenging, given the absence of protocellular transport and replication machinery. In one rare instance Rubio-Sanchez et al.<sup>[250]</sup> have demonstrated “content reshuffling” with thermally-driven membrane phase transitions. Parent fatty acid-based protocells containing 10-nucleotide RNAs disintegrate at higher temperatures and release their contents. At lower temperatures, fatty acid membranes reassemble and encapsulate genetic material in a new generation of protocells. One important practical aspect in these studies is the unavoidable dilution of contents across generations in the absence of amplification or synthesis. A notable exception is a study by Kurihara et al.,<sup>[228]</sup> who implemented an amplification reaction in a protocell prior to division.

## 6. Possible Interactions between Protocells

Modern cells predominantly communicate through chemical signaling,<sup>[251]</sup> and to some extent directly through physical interconnections.<sup>[252,253]</sup> This is valid for mammalian cells and plant cells as well as for bacteria, and even Archaea.<sup>[254]</sup> Combarrous and Nguyen pointed out that chemical means of communication between individual entities might have already been an inherent feature of early protocells.<sup>[255]</sup> Such an assumption has merits, as the earliest fossils known consist of remnants of multicellular structures composed of densely packed individual bacterial cells (cf. stromatolites in the Introduction section). Since it is likely that a transition from a primitive protocell to a colony of bacterial cells was gradual rather than sudden, it is also likely that large populations of densely arranged primitive cells existed on the early Earth. This infers the possibility that the cells in such populations communicated with each other in some form, and derived benefits from it.<sup>[256]</sup>

The concept of dynamic kinetic stability (DKS), for example, describes a driving force acting on the step-wise development and evolution of a collective of entities that are able to reproduce either chemically autocatalytically, or by replication.<sup>[256]</sup> Hypothetically, protocells that have gained the ability to reproduce autocatalytically would acquire a collective form of stability, a benefit which supports survival in the intermediate steps of an incremental development process.

Deamer and Damer proposed the possibility of “progenote” species,<sup>[50]</sup> protocell aggregates forming a hydrogel in the intermediate phase between wet and dry cycles. Since progenotes are protocell populations, not isolated cells, they are hypothesized to have performed information sharing and might have collectively evolved into the first microbial communities.

Experimental efforts with respect to protocell interaction and communication have been made in several different directions.

Examples include studies focusing on the release of chemical compounds from one compartment and uptake by a nearby compartment, work showing physical protocell interactions by vesicle colony formation, and investigations on the transport of materials among protocells via tunneling nanotubes. The field of cell-free gene expression in model protocells is vast,<sup>[257–260]</sup> here we limit the discussion mostly to the prebiotically more plausible materials and mechanisms.

The most rudimentary form of communication is “content sharing” by transport from one protocell to the other via inter-membrane crossing. Two experimental examples are depicted in **Figure 7a,b**. In **Figure 7a**,  $\text{Ca}^{2+}$  ions released from one compartment are encapsulated in an adjacent compartment upon addition of ionomycin, a membrane permeable  $\text{Ca}^{2+}$  ionophore. The receiving compartment contains Rhod2, a  $\text{Ca}^{2+}$  indicator which fluoresces upon  $\text{Ca}^{2+}$  uptake and binding.<sup>[261]</sup> In the second example, enzyme-free DNA displacement reactions are employed to monitor protocell-to-protocell communication.<sup>[262]</sup> A single-stranded DNA is transferred between coacervates which are enveloped in a stabilizing polymer (**Figure 7b**).<sup>[263]</sup> Briefly, one type of DNA strand was released from one species of protocells due to initiation of the reaction, and taken up by the other protocell species. In the second compartment, the DNA hybridized to its complementary DNA strand. Uptake and binding was confirmed with increasing fluorescence intensity of Cy5 in the receiver protocells (encircled in green in **Figure 7b**). The fluorescence of the donor protocells also increases due to the displacement and release of the single strand from the nano-scaffold (encircled in orange in **Figure 7b**). One other recent example shows communication in protocell populations by creating *DNA circuit*-based reaction networks.<sup>[262]</sup> The study takes advantage of the modularity and scalability of enzyme-free DNA strand displacement reactions to develop protocell populations that can sense, process and respond to, DNA-based messages.

Not all protocell interactions lead to mutual benefits.<sup>[268,269]</sup> Competition for resources among the same or different species is important in natural selection. One example of predatory behavior was reported for a population of protease-containing coacervates, and proteinosomes. The two species have opposite charges and initially interact based on electrostatic attraction. The coacervates act as killer protocells, destroy proteinosomes by lysing their protein–polymer membranes, and take over their content including single-stranded DNA. The newly formed compartments are also capable of releasing contents and killing.<sup>[268]</sup>

Formation of model protocell colonies, i.e., densely packed communities of membranous containers within a limited space, was achieved by attaching containers to each other with molecules or ions acting as “molecular glue”, such as  $\text{Mg}^{2+}$ <sup>[174]</sup> (**Figure 7c**) or poly-L-arginine (**Figure 7d**).<sup>[264]</sup> The affected vesicles in these studies formed stable irregular 2D assemblies. Another mechanism of aggregation relies on attractive interactions of membrane-embedded molecules, such as complementary DNA strands<sup>[265]</sup> (**Figure 7e**), streptavidin/biotin couples,<sup>[265]</sup> or oppositely charged biopolymers.<sup>[270]</sup> The morphology of such vesicle aggregates can be further altered by adjusting the concentration of the membrane components, and by choice of method of assembly.<sup>[270]</sup> One reported assembly method pro-

viding a high degree of control over colony structure is acoustic trapping<sup>[266]</sup> (**Figure 7f**).

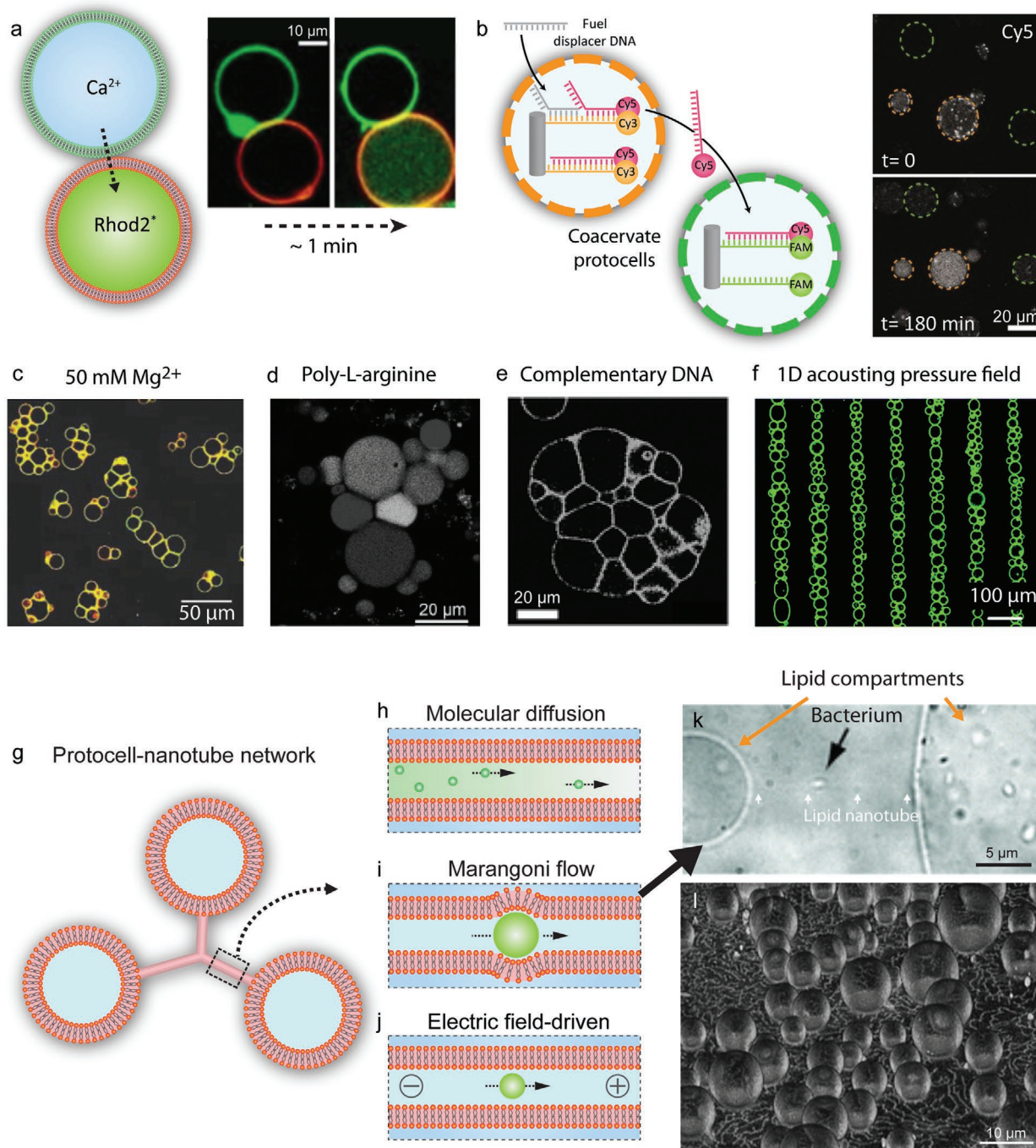
Colonies were demonstrated to exhibit increased mechanical stability in comparison to individual vesicles. Closely packed, colony-like protocells reacted to the external hypotonic (pure water) osmotic shock as a collective<sup>[271]</sup> and also displayed enhanced mechanical stability against forces produced by turbulent fluid flow.<sup>[265]</sup>

It is likely that the proximity of neighboring vesicles aided prebiotic chemical communication. Membranes of colony-associated vesicles were shown to be more permeable to negatively charged small molecules (e.g., ADP) and macromolecules (e.g., tRNA, ferritin), as compared to unassociated protocells. These vesicles also showed an increased tendency for fusion.<sup>[265,270]</sup> Uptake of larger or charged molecules occurs via transient pores, which open due to increased membrane tension,<sup>[206,246,272]</sup> or as a result of membrane components acting as “cell-penetrating peptides” (CPP).<sup>[265]</sup> Protocells hold the molecules for a prolonged time without loss of membrane integrity.<sup>[270]</sup> Li and co-workers developed a method of magnetic manipulation of vesicles filled with a ferromagnetic solution to engineer protocell aggregation models, so-called prototissues.<sup>[271]</sup> Such large assemblies, featuring intervesicular connections and embedded functional molecules, are examples of promising bottom-up approaches toward functional biomimetic tissues.<sup>[273]</sup> In this context, Gobbo et al. exploited inter-linked proteinosomes to generate prototissue spheroids capable of reversible contractions and relaxations.<sup>[274]</sup>

The earlier mentioned discovery of tunneling nanotubes between mammalian cells<sup>[252,253]</sup> was preceded by reports of synthetic vesicle-nanotube networks.<sup>[275]</sup> Different transport modes for molecular exchange between interconnected containers, and containers and biological cells, were investigated (**Figure 7g**). Driving forces were predominantly diffusion, but also membrane tension<sup>[267]</sup> and electric fields.<sup>[276]</sup> Cascade enzymatic reactions were also segregated in multi-compartment lipid vesicles,<sup>[266,277,278]</sup> which in one example contained membrane protein pores to facilitate transport of substrate and product molecules between neighboring compartments.

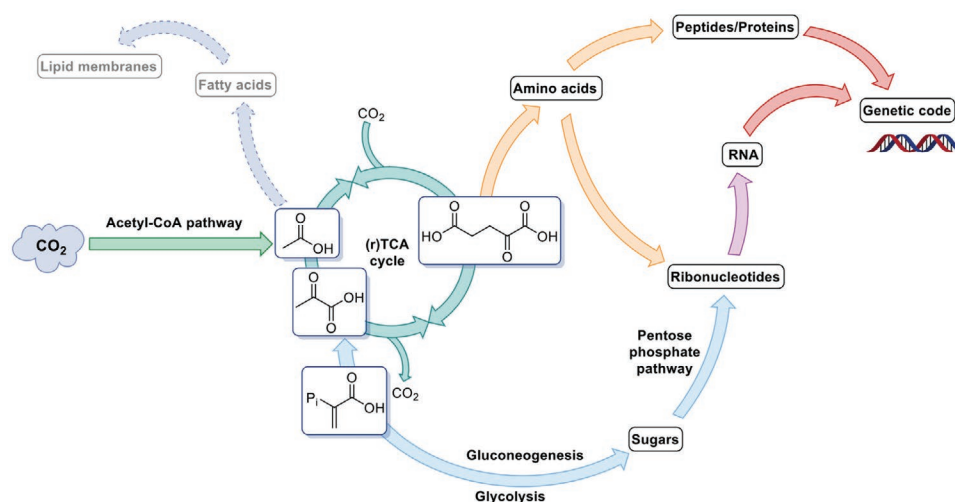
## 7. Prebiotic Chemical Reactions and Reaction Networks in Protocells

The ultimate purpose of a model protocell is to reveal a viable pathway from abiotic matter to life, which is only possible if integration of chemical, and eventually biochemical functionality can be achieved to a point where the minimal criteria (cf. Introduction section) are fulfilled. A satisfactory model system, from which a mechanism of the transition to life can be derived,<sup>[279]</sup> is still not available. Its construction requires, above all, that any protocell-internalized chemical processes and the supramolecular enveloping container are compatible, and can coexist. Under the assumption that a defined set of reactive chemical precursors and conditions existed in the prebiotic world, only a limited subset of all possible chemical and physicochemical interactions would have been compatible with, and could therefore have been actively involved in, protocell development, and eventual transformation to life. Higgs



**Figure 7.** Possible interactions between protocells. a,b) Primitive chemical signaling via release of molecules from one protocell, followed by uptake by another. a)  $\text{Ca}^{2+}$  released from one compartment is taken up by an adjacent compartment encapsulating the  $\text{Ca}^{2+}$  indicator Rhod2. The micrographs show the donor and acceptor compartments before and after addition of ionomycin, a membrane permeable  $\text{Ca}^{2+}$  ionophore. Adapted with permission.<sup>[261]</sup> Copyright 2019, Royal Society of Chemistry. b) Single-stranded DNA released from one population of protocells (orange) are taken up by a second population (green), which contains the DNA complementary to the released single-stranded DNA. Adapted with permission.<sup>[263]</sup> Copyright 2020, American Chemical Society. Vesicle colonies can form due to bilayer-to-bilayer adhesion mediated by c)  $\text{Mg}^{2+}$ , d) a positively charged synthetic amino acid poly-L-arginine, e) complementary DNA strands, f) an acoustic field. c) Adapted with permission.<sup>[174]</sup> Copyright 2018, John Wiley and Sons. d) Adapted with permission.<sup>[264]</sup> Copyright 2012, John Wiley and Sons. e) Adapted with permission.<sup>[265]</sup> Copyright 2021, Royal Society of Chemistry. f) Adapted with permission.<sup>[266]</sup> Copyright 2019, Royal Society of Chemistry. g) Protocells can communicate via interconnecting lipid nanotubes. Mechanisms of transport of contents vary: h) molecular diffusion, i) adhesion of constituents to the lipid membrane and Marangoni (tension-driven) flow, j) electric field-driven. k,l) Micrographs showing surface-supported protocell-nanotube networks. k) Nanotube-interconnected GUV network manually created by a microneedle injection method. Adapted with permission.<sup>[267]</sup> Copyright 2008, Royal Society of Chemistry. l) Extensive protocell-nanotube network spontaneously formed from a multilamellar reservoir on a flat  $\text{SiO}_2$  substrate. Adapted with permission.<sup>[189]</sup> Copyright 2019, American Chemical Society.





**Figure 8.** Relationships between coexisting biochemical subsystems in a “metabolism first” prebiotic chemical reaction network. Adapted with permission.<sup>[286]</sup> Copyright 2020, American Chemical Society.

formulated criteria for chemical reaction networks to be valid contributors to growth and division of protocells.<sup>[280]</sup> These conditions strongly involve the physical and materials features of protocells, for example, their distinct ability to retain reactants at high concentrations, establish concentration and reaction rate gradients, and exclude unsuitable components. He argues that, although the complexity of small molecule reaction networks<sup>[281]</sup> could have become high already early on, simple rather than complex chemical mechanisms should be considered for the onset of replication.<sup>[280]</sup>

Especially over the last few decades, research went way beyond thoughts and hypotheses, and increasingly sophisticated experimental work emerged. The design of chemical reaction systems that might have contributed to the development of life has advanced into a progressive field within the origin of life research. Ruiz-Mirazo et al. most extensively reviewed prebiotic chemistry under the systems chemistry umbrella, covering systematically the available research from the synthesis of simple protocell constituents to chemical networks of greater complexity, including a method overview.<sup>[282]</sup> Islam and Powner<sup>[283]</sup> as well as Lopes and Fiore<sup>[284]</sup> assembled a similarly comprehensive set of resources, but with a more specific focus on the protocell as prebiotic chemical reactor. Loakes and Holliger gave an account on the diverse aspects related to “Darwinian chemistry:” synthesis concepts for an artificial cell, considering minimal systems and their adaptation to different evolutionary challenges. Similarly, Toparlak and Mansy reviewed progress in synthesizing sophisticated protocells.<sup>[285,286]</sup> The extensive work presented in these reviews documents the power of the systems approach, which has led to the progressively solidifying understanding that individual classes of metabolites cannot be considered in isolation.

Krishnamurthy points out that our biology/biochemistry-driven Early Earth hypotheses, such as the RNA-, protein-, lipid- or metabolism-first prebiotic environments, are top-down concepts based upon tracing back the discovered internal mechanisms of admittedly broad, but still isolated subsets of cellular chemistry. Consequentially, the focus has predominantly been only on the origins of directly related chemical

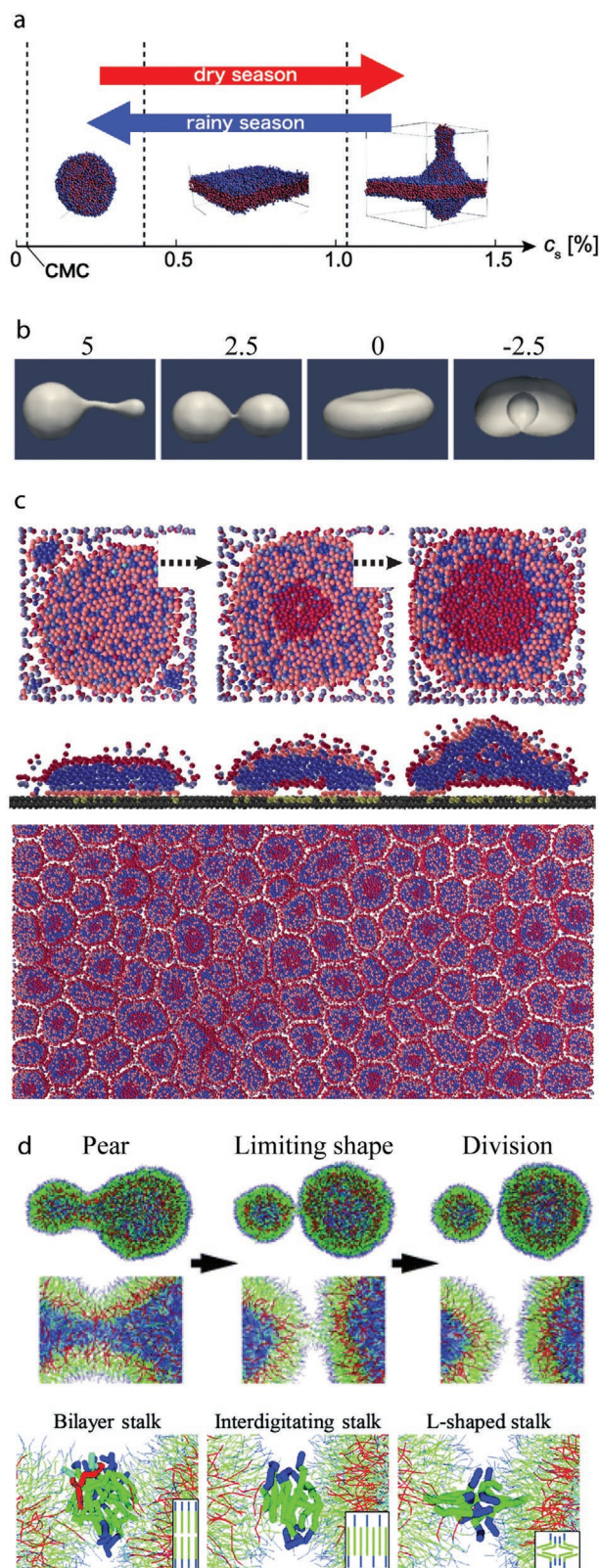
and biological building blocks. This is gradually being superseded by systems approaches in prebiotic chemistry—leading to true “bottom-up” systems based on increasingly complex interactions of a multitude of different molecules in networks of interacting entities.<sup>[287]</sup> Muchowska et al. presented a view at life’s origins as self-organized reaction networks by looking at different chemical sub-systems in non-enzymatic “metabolism-first” approaches to prebiotic chemistry.<sup>[286]</sup> The authors give a comprehensive overview of plausible biochemical pathways and existing models of their evolution, and emphasize that the different metabolic pathways need to be viewed as working together as ensemble (Figure 8). The enzyme-free chemistries of the individual branches and pathways have to be considered concurrently with their constraints and mutual interactions.

However, the individual subsystems of reaction networks of prebiotic chemistries are typically investigated under laboratory conditions, neither taking into consideration the container compatibility requirement, nor cross-reactivity and the effects of resulting by-products. Bonfio et al. argue that in order to provide a more complete description of how prebiotic cellular entities could have emerged, reservoirs of coexisting reactive species present on early Earth need to be experimentally investigated, without disqualifying potential cross-reactivity and the associated by-products, which may have even played fundamental roles in protocell development and maturation towards the transition to life.<sup>[288]</sup> The authors showed that vesicles composed of plausible amphiphile constituents support methyl isocyanide-mediated activation of amino acids, peptides, and nucleotides. Moreover, this “activation chemistry” approach drives the synthesis of cyclic phospholipids and lipidated active species, and thus supports the hypothesis of phospholipids having been likely constituents of protocell membranes.

## 8. Protocells In Silico

Computational model studies have proven useful to test hypotheses on chemical environments and systems, development





**Figure 9.** Protocells in silico. Snapshots of computer simulations: a) Coarse-grained simulations showing the transition from spherical compartments (relatively low amphiphile concentration, wet season) to compartments growing nanotubular protrusions (high amphiphile con-

and evolutionary processes.<sup>[159]</sup> 4.5 billion years of time distance to the early Earth, also considering the absence of direct fossil evidence from that period, is a significant obstacle to identifying realistic conditions, and accordingly designing experimental protocell model systems. Additionally, the ubiquity of organic and biological matter in today's environment makes it challenging to perform chemical reactions free of contaminants in field studies. Modern computational models and simulations allow for the generation of complete chemical and physicochemical environments that go beyond what is experimentally possible. For example, ab initio molecular dynamics simulations have been used to explore the reaction space of prebiotic chemistry of life that could have originated from two simple inorganic molecules (HCN and water).<sup>[289,290]</sup> Atomistic computer simulations are also emerging as a powerful tool to explore potential prebiotic reaction pathways.<sup>[291]</sup> Several reviews present the state of the art of theory and computational methods developed for origin of life research, in particular prebiotic chemistry.<sup>[292–295]</sup>

In general, molecular simulations of amphiphile self-organization into membranous assemblies is an established research area in the context of drug research and structural biology, which has proven fruitful also in the early Earth context.<sup>[296]</sup> A variety of studies focus on the virtual visualization of protocell assembly and shape transformations at the mesoscale.<sup>[297,298]</sup> A coarse-grained molecular simulation study found two membrane morphologies depending on the concentration of fatty acid monomers in dry or wet seasons: spherical vesicles, and vesicles with extending nanotubes<sup>[299]</sup> (Figure 9a). Prebiotic vesicles with an elongated membrane tube were also captured in numerical simulations along with a large diversity of other morphologies<sup>[297]</sup> (Figure 9b). A particle dynamics model focused on self-organization of lipid micelles and bilayers in bulk and on solid surfaces, and predicted the cavities forming on the basal membrane of solid adhered membranous protocells, and the formation of colony-like protocell entities<sup>[300]</sup> (Figure 9c). The results align with the experimental work reporting on similar lipid structures.<sup>[206]</sup> Using coarse-grained molecular dynamics models, the influence of lipid geometry on protocell division was investigated. Lipids with negative spontaneous curvature were determined to induce division by favoring the formation of stalk intermediates at the membrane neck connecting a bud and a vesicle.<sup>[212]</sup> (Figure 9d). In a similar study, based also on

centration, dry season). Adapted with permission.<sup>[299]</sup> Copyright 2016, Royal Society of Chemistry. b) Finite element simulations capturing protocell shape transformations in bulk liquid medium at different spontaneous curvatures, from left to right tubulation, budding, pancake-like geometry, tubulation inwards. Adapted with permission.<sup>[297]</sup> Copyright 2019, Springer Nature Limited. c) Coarse-grained simulations showing surface-adhered protocell dynamics. Upon reduced affinity to the surface, the compartments lift off and bulge inwards. Increasing the concentration of amphiphiles (particle number in the simulation) leads to confluence of the surface and colony/tissue-like protocell populations. Adapted with permission.<sup>[300]</sup> Copyright 2017, Elsevier. d) Coarse grained simulations capturing protocell division. Lipids with negative spontaneous curvature were determined to induce division by favoring the formation of stalk intermediates at the membrane neck connecting a bud and a vesicle. Adapted with permission.<sup>[212]</sup> Copyright 2018, Royal Society of Chemistry.

a coarse-grained model, it was reported that in hypertonic solutions, small solutes drive budding and fission of membranous vesicles, a feasible mechanism for division of prebiotic compartments in alternating osmotic conditions.<sup>[249]</sup> One recent study focused on characterizing protocell motility using cellular automata.<sup>[301]</sup>

Functional coupling of chemical subsystems under consideration of cross-reactivity in a suitably structured container architecture is the largest experimental challenge for creating a protocell model that is representing the state of development at the crossing of the non-living and living worlds. A comprehensive overview of *in silico* approaches in the context of artificial chemistry is given by Banzhaf and Yamamoto.<sup>[302]</sup> One prominent example of a systems-level implementation, aimed at overcoming the challenge of coupling functional chemical subsystems in a viable protocell, is the “Los Alamos bug,” a nanoscale protocell model named after its place of origin, the distinguished U.S. national laboratory. The model comprises dissipative particle dynamics simulation, and to satisfy the chemoton model, combines the self-assembly processes with chemical reaction networks. The model does not provide quantitative aspects but defines systemic processes required for the life cycle of a minimal protocell.<sup>[303]</sup> Similar models incorporating basic metabolism into protocell compartments have been established.<sup>[304–306]</sup>

## 9. Laboratory Fabrication of Protocell Models

In this section, we describe established methods for membranous protocell generation along with some of the recent technological developments. Contemporary laboratory fabrication protocols are either based on dehydration/rehydration of unstructured lipid films, or utilize microfluidic devices for direct vesicle generation from lipid solutions.<sup>[307]</sup>

### 9.1. Dehydration/Rehydration

Dehydration/rehydration-based methods are the most commonly employed preparation procedures, due to their simplicity, low-cost and high yield. They rely on swelling of initially dry lipid films in aqueous media<sup>[284,308–310]</sup> (Figure 10). A main drawback is poor control over vesicle size and lamellarity, and the ratio between the multilamellar and unilamellar compartments produced. In Figure 10, four examples of dehydration/rehydration-based methods are presented: gentle hydration in bulk (Figure 10a,b), electroformation (Figure 10c,d), gel-assisted hydration (Figure 10e,f), and heat-induced formation (Figure 10g,h).

The dehydration/rehydration (gentle hydration) method (Figure 10a,b) starts with a dried lipid film originally formed by reduced-pressure evaporation of organic solvent from a lipid solution, typically in chloroform (Figure 10a). Hydration of this lipid film with buffer, preferentially with the help of ultrasonic agitation, creates a membrane suspension containing vesicles of varying size and lamellarity, often as pairs of uni- and multilamellar vesicles (Figure 10b). Slow dehydration of this suspension, for example by means of a vacuum desiccator,

concentrates salt in pockets between the lamellae. In a subsequent rehydration step, the salt pockets cause an osmotic pressure gradient across the lipid layers, leading to the swelling of the pockets, and formation of vesicles.

The easy implementation and high efficiency makes gentle hydration one of the most commonly utilized laboratory methods for model unilamellar protocell formation. However, there is poor control over lamellarity and monodispersity, and the solvent removal step is considered time-consuming (approximately hours). This method was first implemented in the 1960s<sup>[313]</sup> and has since been used abundantly.<sup>[310]</sup> It was shown that if doped with PEGylated lipids and sugars, separation of lipid bilayers during swelling, and thus vesicle yield, can be improved.<sup>[314]</sup> Giant lipid vesicle formation in the presence of sucrose, glucose, and sorbitol was recently thoroughly characterized.<sup>[315]</sup> A new review<sup>[207]</sup> compares hydration methods for multivesicular, i.e., compartmentalized, vesicles.

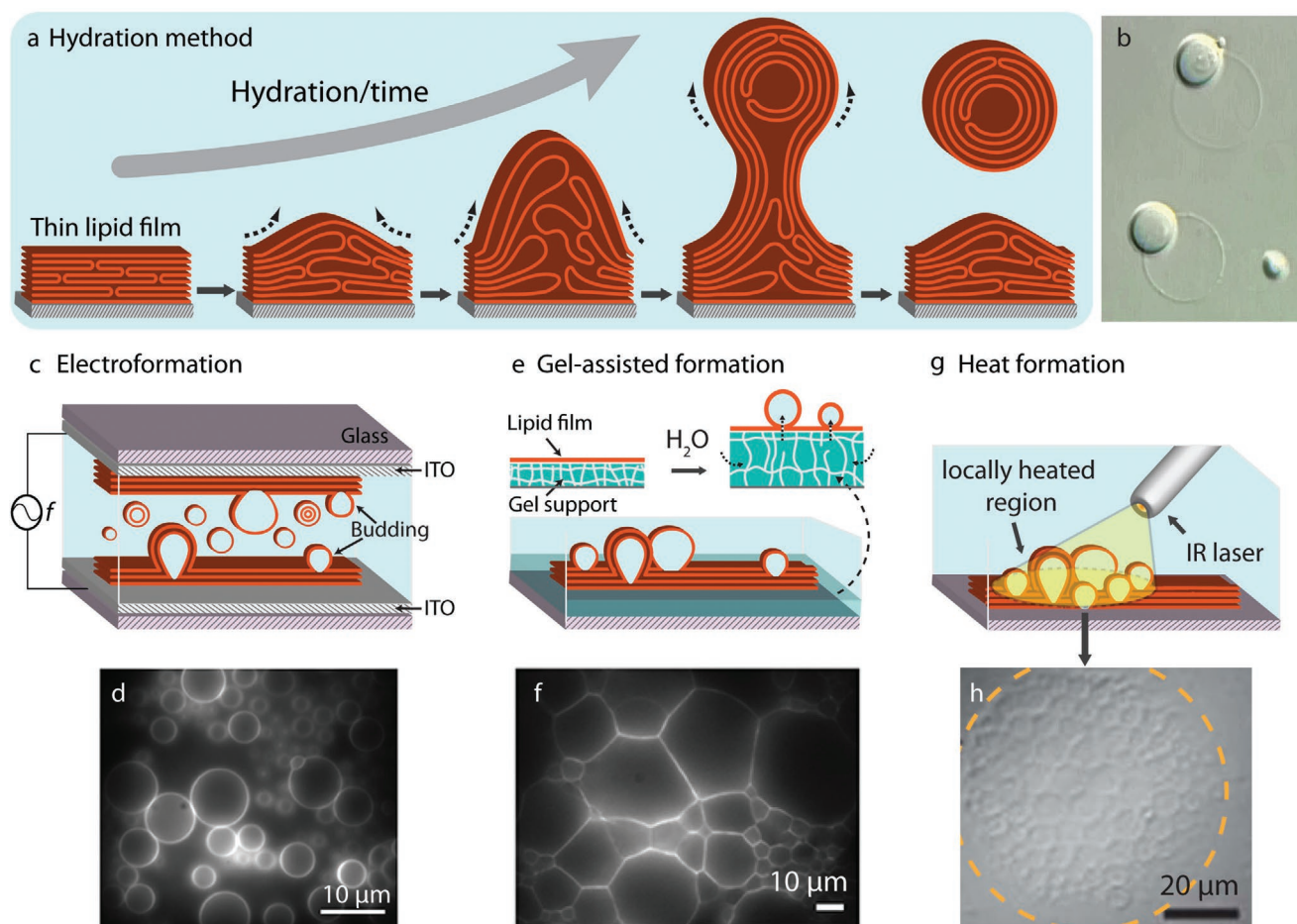
Electroformation (Figure 10c,d) combines the hydration method with the application of an AC electric field with a frequency of 1–100 kHz. This method rapidly leads to unilamellar vesicle formation in high yield, but still over a large size range. A detailed overview including materials and setups is given in a recent review by Stein et al.<sup>[311]</sup> Thorough characterization of electrical parameters<sup>[316,317]</sup> revealed that dielectrophoretic and electrohydrodynamic effects have a profound impact on the yield. A modification, where large droplets comprising liposome suspension were deposited repeatedly on a small electrode area, was reported<sup>[318]</sup> to reduce membrane defects,<sup>[319]</sup> and improve the yield of unilamellar vesicles further. By tuning the electrical parameters or lipid composition<sup>[316,320]</sup> it is possible to induce fusion events and budding,<sup>[321]</sup> or form double bilayer vesicles in a consistent manner.<sup>[322]</sup> Electroformation is not limited to lipid material. Giant polymersomes consisting of block copolymers with phosphate headgroups have also been prepared with this technique; Rideau et al. established a library of precursors for the fabrication of polymer containers.<sup>[323]</sup>

For gel-assisted vesicle formation (Figure 10e,f), a glass substrate is pre-coated with a thin polymer gel film, e.g., poly (vinyl alcohol), before depositing the lipids, which promotes the formation of vesicles. Porous hydrogel benefits the formation of GUVs, affecting sizes and yield.<sup>[324]</sup> Schultze et al.<sup>[325]</sup> prepared a micropatterned gel structure with a terpolymer, obtaining monodisperse anchored vesicles with about 10% variation in diameter. Liu et al.<sup>[326]</sup> combined the gel-assisted method with extrusion by coating the gel with a microporous polycarbonate membrane, producing freestanding liposomes with an upper size limit. Parigoris et al.<sup>[327]</sup> used poly-(acryl amide) substrates, and obtained predominantly unilamellar GUV populations. Pazzi and Subramaniam<sup>[328]</sup> employed hydrophilic nanocellulose fibers with a nanoscale surface curvature for cost reduction and increased yield. Electroformation has also been applied in conjunction with gel substrates to incorporate proteins.<sup>[329]</sup>

### 9.2. Microfluidics-Based Techniques to Fabricate Protocells

The second group of protocell fabrication methods utilizes microfluidic device technology. Microfluidic setups require special design (Figure 11a) and assembly, and often involve



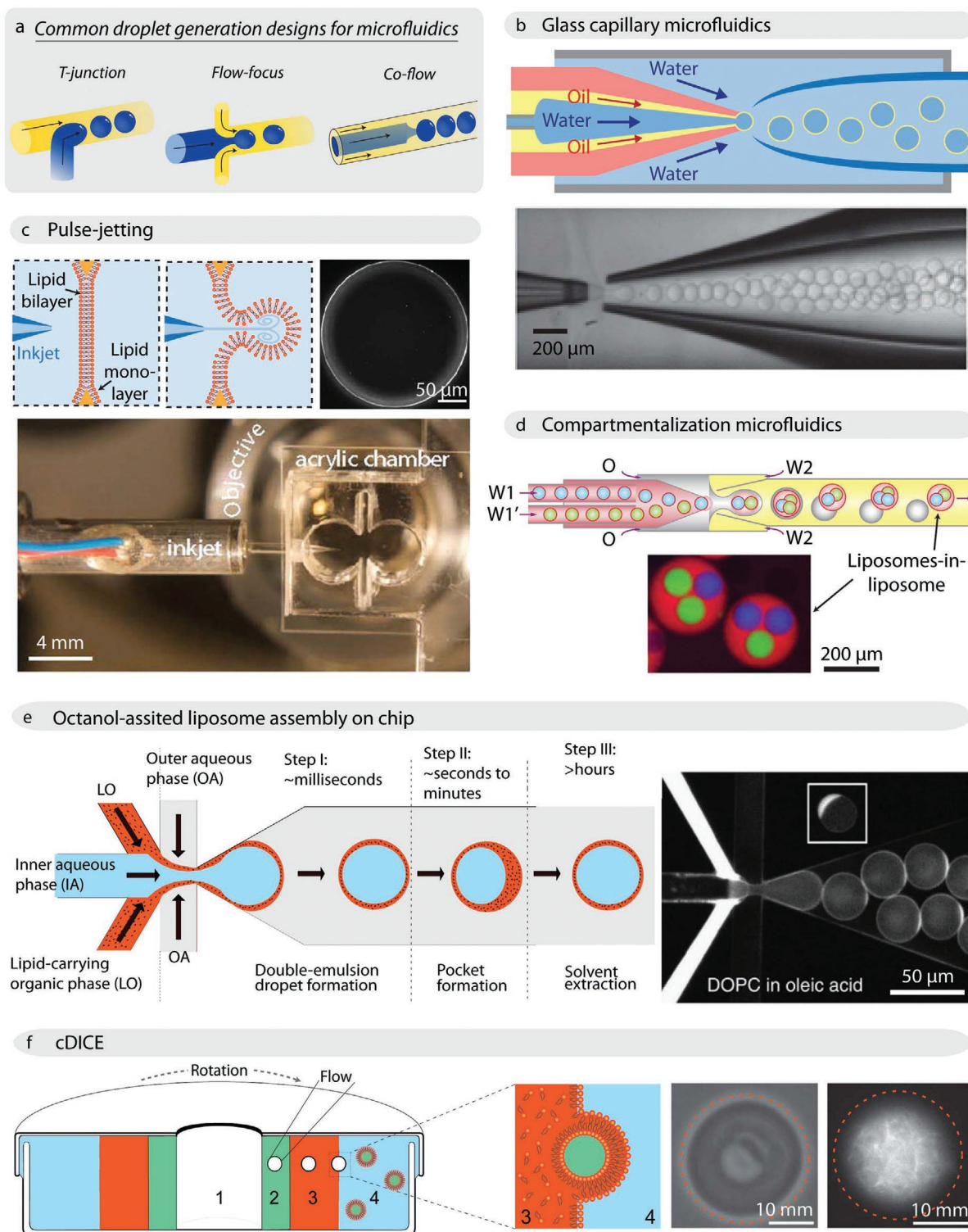


**Figure 10.** Dehydration/rehydration-based membranous protocell formation methods. a) Gentle hydration: a schematic drawing showing a stack of thin lipid films, i.e., dry lipid cake, swelling over time and transforming into a multilamellar lipid vesicle/lipid reservoir. b) Differential interference contrast micrograph of a sample obtained from the process depicted in (a) which contains multi- and unilamellar lipid compartments. (a). Adapted with permission.<sup>[223]</sup> Copyright 2011, Springer Nature Limited. c) Schematic drawing showing the principle of electroformation, in which an alternating electrical field is applied to disordered lipid films positioned on electrodes to induce rapid swelling and vesicle formation. ITO: indium tin oxide. d) Fluorescence microscopy image of lipid compartments formed by electroformation. e) Schematic drawing showing the principle of gel-assisted vesicle formation. The gel support takes up water upon hydration, promoting the entry of water into the lipid film from the bottom, leading to swelling of the film and its transformation into vesicles. f) Fluorescence microscopy image of vesicles formed with gel-assisted (poly-(vinyl alcohol)) formation. d,f) Adapted with permission.<sup>[311]</sup> Copyright 2017, Frontiers. g) Schematic drawing showing heat-induced vesicle formation. Infrared laser light is guided to the vicinity of a solid-supported lipid film by means of an optical fiber. The resulting local temperature increase causes swelling of the film, rapidly generating vesicles. h) Bright-field microscopy image of vesicles produced by locally applied temperature increase on a lipid cake submerged in aqueous buffer. Adapted with permission.<sup>[312]</sup> Copyright 2012, Royal Society of Chemistry.

microfabrication. They enable membranous protocell formation in high throughput with superior monodispersity and encapsulation efficiency. The earliest designs used to produce lipid compartments were based on glass capillary microfluidics<sup>[330]</sup> (Figure 11b), where rigid microneedles embody the microchannels. Figure 11b shows an example of a co-flow setup, where membranous compartments are formed from water/oil/water (w/o/w) emulsion droplets created by a collinear capillary arrangement. Arriaga et al.<sup>[331]</sup> designed a capable glass capillary device that allows w/o/w layers to become ultrathin membranes, reducing the amount of oil in the system. Guerrero et al. recently reviewed the underlying technology of capillary-based microfluidics.<sup>[332]</sup>

An elegant and unique method is “pulse-jetting,”<sup>[333]</sup> where a planar bilayer is assembled inside a chamber, and GUVs with

controlled content were generated through microfluidic jetting (Figure 11c). The pulse jet flow technique<sup>[337]</sup> was inspired by the transformation of a soap film into soap bubbles, and is essentially the most simple microfluidic approach. Conceptually related to the classic ink-jet print head, pulse-jetting is also useful for the preparation of membranous compartments.<sup>[338,339]</sup> Pulse-jetting provides high control over lipid composition and spontaneous curvature, oriented membrane protein incorporation, and encapsulated contents. Kamiya et al.<sup>[340]</sup> generated a jet flow interacting with an asymmetric planar bilayer, which produced elongated membranes that split into vesicles of two different sizes: 100–200  $\mu\text{m}$  and 3–20  $\mu\text{m}$ . A recent development from this group features the creation of vesicle-in-vesicle asymmetric lipid compositions.<sup>[341]</sup> Maktabi et al.<sup>[342]</sup> used water-in-oil multiphase flows to



**Figure 11.** Microfluidics-based membranous protocell formation methods. a) Schematic drawings depicting common microdroplet generation designs used for protocell fabrication. b) Glass capillary microfluidics. Adapted with permission.<sup>[330]</sup> Copyright 2011, Royal Society of Chemistry. c) Pulse-jetting. Adapted with permission.<sup>[333]</sup> Copyright 2011, National Academy of Sciences. d) Microfluidic vesosome assembly, i.e., compartmentalization microfluidics. Adapted with permission.<sup>[334]</sup> Copyright 2017 American Chemical Society. e) Octanol-assisted vesicle assembly based on the “flow-focus” design principle. Adapted with permission.<sup>[335]</sup> Copyright 2016, Springer Nature Limited. f) The continuous droplet interface crossing encapsulation (cDICE) technique. The drawing depicts the different interfaces which a droplet crosses inside a centimeter-scale centrifuge from the center outwards: hydrocarbon solvent, oil, aqueous phase. The micrographs show vesicles encapsulated red blood cells (left) and thin actin filament bundles with fascin (right). Adapted with permission.<sup>[336]</sup> Copyright 2011, Royal Society of Chemistry.



generate asymmetric vesicles, and implemented active electrophoretic and pinch-flow fractionation to create oil-free, and unilamellar GUVs.

Multiple compartments in GUVs are an interesting and potentially feature-rich scaffold for mimicking prebiotic reactions in protocell models. Microcapillary devices have been arranged to generate vesosomes. For example, two different aqueous phases from separate channels were combined in an oil layer to create droplets which subsequently separate from the oil, transforming into protocells with multiple subcompartments of different contents<sup>[343]</sup> (Figure 9d). Deng et al. developed a variety of microcapillary setups,<sup>[334,344,345]</sup> among them one with coaxial inlets, to load coacervates into double emulsion droplets. We note that coaxial systems require precise micro-positioning of the needles with respect to each other, and are typically more complicated to implement than monolithic chip devices.

Many contemporary research devices are instead fabricated from poly-(dimethyl siloxane) (PDMS) elastomer, and employ w/o/w (double) emulsions for droplet generation.<sup>[343,346]</sup> In one design, a two-phase w/o system was used instead of a double emulsion in conjunction with a flow-focus mechanism (Figure 11a) to generate stable droplets<sup>[139,335]</sup> (Figure 10e). With this design, Deshpande et al.<sup>[335]</sup> used an octanol-assisted system to construct a coacervate-in-liposome system. Krafft et al.<sup>[347]</sup> developed a method to de-wet a vesicle fabricated in a w/o emulsion, using an osmotic gradient composed of sucrose and sodium chloride. Even triple emulsions can be prepared by means of droplet generator microfluidics. Jeyhani et al.<sup>[348]</sup> described a glass capillary-PDMS chip composite that allows creating compartmentalized protocells in an all-water environment.

A very recent report from Staufer et al.<sup>[349]</sup> showed an improvement in the efficiency of cargo loading and encapsulation of biological material in GUVs (viral DNA). The study used a high-throughput flow focusing device. The same group had earlier developed a microfluidic processing method, using a picoinjection tip to load monodisperse GUVs with transmembrane and cytoskeletal proteins.<sup>[350]</sup> Bhattacharya et al. reported a compartmentalized diffusive reactor<sup>[351]</sup> designed to enzymatically synthesize phospholipids de novo by means of a continuous supply of fresh precursors. The vesicles produced by this high-yielding oil-free method have particular potential as functional protocell models. The simplicity of this microfluidic device, which is not droplet based, facilitates implementation by research groups without specialized chip fabrication, assembly, and testing facilities.

The inner and outer leaflets of biological cells are composed of different lipid mixtures, and are organized differently. Experimentally modeling this feature is challenging, but is of interest for protocell studies. A layer-by-layer process was developed by Matosevic and Paegel.<sup>[352]</sup> It utilizes flow focusing, in combination with a trapping architecture (capture cup) in a PDMS chip to produce compositionally complex phospholipid membranes. Lu et al.<sup>[353]</sup> uses two flow focusing regions to replace oil-lipid solutions (while wasting the unused solution) in order to create asymmetric bilayers. Similarly, Arriaga et al. used a device based on triple emulsion droplets, and achieved a degree of asymmetry of 70%.<sup>[354]</sup>

The use of hydrogel shells has been reported as an alternative pathway to model membrane interfaces among the compartments in sub-structured protocells. This method, leading to exceptionally large containers (approximately mm), is based on droplet interface bilayers which are formed by the contact of two monolayer-enveloped aqueous droplets surrounded by a hydrogel scaffold. Devices capable of generating such compartments are based on double coaxial microfluidics,<sup>[355]</sup> or on a hydrogel prototissue matrix.<sup>[356]</sup>

For laboratories that cannot easily establish microfluidic systems, a convenient setup, termed “continuous Droplet Interface Crossing Encapsulation” (cDICE), which combines capillary microfluidics and double emulsion droplet formation in bulk, was developed<sup>[336]</sup> (Figure 11f). The cDICE system generates GUVs by means of a centimeter-scale, low-speed centrifuge. Droplets are fed through a glass capillary into the center of the running centrifuge. The chamber is loaded with two immiscible fluids, typically water and mineral oil, the latter containing a dissolved lipid fraction. While the droplet crosses the interfaces of oil and water, it is step-wise coated with both leaflets of a lipid bilayer, and emerges as a unilamellar vesicle of the size of the initial droplet.<sup>[357]</sup> In 2019, Durre and Bausch<sup>[358]</sup> described a loading variant of cDICE to introduce cholesterol into lipid bilayers, enabling reliable formation of phase-separated vesicles. Another interesting variant was developed by Morita et al.<sup>[359]</sup> It produces cell-sized liposomes of well-controlled lipid composition by means of droplet-shooting and size-filtration in a centrifuge. Water droplets are accelerated from the tip of a glass capillary through a lipid-containing organic layer and are subsequently pushed through a filter layer in an extrusion-like manner to be finally collected in an aqueous reservoir. This simple setup was implemented in an Eppendorf centrifuge tube.

Overall, microfluidics-derived techniques are versatile with a varying degree of technical simplicity. Simple channel devices are typically easy to fabricate from either polymer, silicon, or glass materials. Their features are in the size range of the target structures, allowing in many instances for localized and on-demand preparation of protocells/vesicles, which is only possible in a limited fashion with bulk methods. In the context of prebiotic research, microfluidic technology is well capable of incorporating materials synthesis and catalysis, both in aqueous and nonaqueous environments.<sup>[360]</sup> Ai et al.<sup>[361]</sup> and Trantidou et al.<sup>[362,363]</sup> reviewed several microfluidics-based designs to produce lipid compartments; and Vladislavljiev et al. presented an extensive overview focusing on double emulsions in the form of monocompartmentalized droplets.<sup>[364]</sup> Carugo et al. discussed the advantages and limitations of microfluidic technology for liposome fabrication.<sup>[365]</sup> Supramaniam et al. reviewed microfluidic techniques in the context of synthetic biology.<sup>[366]</sup> This is noteworthy since the combination of protocells and encapsulated chemistry in joint model architectures can be expected to converge in the future.

### 9.3. Miscellaneous Techniques

The following section highlights some technological aspects of vesicle generation by less conventional means. Some of them

are extensions of the methods described above. For example, osmotic shock produced by sequential drying-rehydration cycles of SUVs with water instead of buffer leads to their conversion into GUVs, with the possibility to integrate proteins.<sup>[367]</sup> Repeated freeze and thaw (FT) cycles enhance homogenization of the lipid composition of multilamellar reservoirs, supporting the transformation into unilamellar containers.<sup>[368]</sup> Litschel et al.<sup>[369]</sup> demonstrated that after FT cycles, GUVs can exchange content without fusion and fission, which is of interest for lipid-encapsulated RNA replicators.

GUVs can also be produced by localized heating with IR-B radiation.<sup>[312]</sup> For this technique, spin-coated neutral or charged lipid films were illuminated to generate GUVs ranging from 2 to 10  $\mu\text{m}$  in size (Figure 10g,h). The process was developed further for bulk fabrication.<sup>[370]</sup> The IR-B fabrication method is especially useful for producing small numbers of GUVs locally on demand. In this work,<sup>[312]</sup> and in an earlier study by Akashi et al.,<sup>[371]</sup> the lamellarity of the produced GUVs was investigated. It was determined that 2–4 bilayers were consistently present in fractions of the population. For some protocell studies, this should be taken into consideration, as membrane functions like the incorporation of proteins, pore formation, transport properties, and permeability are affected by the presence of multiple membrane layers. Similarly, the UV-light-triggered formation of micrometer-sized vesicles from lipid aggregates composed of lipids and a synthesized cyclic amphiphile was reported by Shima et al.<sup>[372]</sup> A liquid-phase lipid was found to be necessary to generate liposomes under these conditions.

Vortex trapping, a microfluidic technique designed to produce single GUVs of  $\approx 10 \mu\text{m}$  size, utilizes flow forces to fuse SUVs in a simple chip device with Y-shaped channels.<sup>[373]</sup> The formation of a vortex at the intersecting channels creates shear forces, which fuse the vesicles on a sub-millisecond time scale and hold the newly forming GUV in place.

In another unconventional approach, the supercritical  $\text{CO}_2$ -mediated antisolvent (SAS) method was used to create nano-sized liposomes.  $\text{ScCO}_2$  was first applied to remove the organic solvent from a lipid solution. A uniform lipid layer resulted, similar to the first step of the gentle hydration protocol. This was followed by hydration of the lipid film with aqueous buffer, producing large unilamellar vesicles, which upon return to atmospheric pressure transformed into SUVs.<sup>[374]</sup> Supercritical reverse-phase evaporation (SRPE) is similar to SAS, and involves the addition of an aqueous phase to the solid lipid phase in a sealed cell, followed by  $\text{CO}_2$  exposure. Upon pressure drop inside the cell, and evaporation of the  $\text{ScCO}_2$ , vesicles of homogeneous size distribution remain.<sup>[375]</sup> Even though the supercritical fluid methods create small vesicles, their high encapsulation efficiency and the ability to incorporate both hydrophobic and hydrophilic cargo make them potentially interesting in investigations of prebiotic chemistry in subcompartments.

## 10. Potential Extraterrestrial Habitats for Life

It is an ongoing scientific and philosophical debate whether life on the Earth is unique, or if it was able to develop somewhere else. The increasing number of exoplanets being discovered

is evidence that Earth-like habitats, where life has a chance to develop, can exist even outside of our solar system. Since life, as we know it, is based on water, the search for fingerprints of water is an excellent route to obtain clues if life could have developed elsewhere. This research is currently limited to sources within our reach, e.g. meteorites and space missions. One of the planets which is thought to have had surface water at some early point in time is Mars.<sup>[376]</sup> The exploration vehicles of the earlier concluded Curiosity and the currently active Perseverance missions probed Mars landscapes to identify possibly habitable environments. The search for water and microorganisms was part of the missions. Clark et al. discussed possible origins of life on Mars in a recent article,<sup>[376]</sup> where they present evidence that environmental conditions on the planet show distinct similarities to the early Earth. The resemblance of the Lost City Hydrothermal Field on Earth, with particular reference to low-temperature serpentinization as a process relevant to Earth's habitability, to the olivine-rich region of the Nili Fossae canyons on Mars, which might have undergone similar geochemical processes, are discussed.<sup>[377]</sup>

Attention has been given to over 200 selected moons in our solar system. Jupiter's moon Europa might contain a subsurface ocean which is suitable for life.<sup>[378]</sup> The Galileo spacecraft acquired gravity measurement data of Europa's surface, consisting of an 80–170 km thick ice-liquid water layer.<sup>[379]</sup> Computational models support the findings, and indicate that geothermal and tidal heating maintains liquid water under a 10 km thick ice shell.<sup>[380]</sup> Saturn's moon Enceladus, 40 times smaller than Europa, is assumed to also have a surface structure of liquid water, and an enveloping ice shell.<sup>[381]</sup> Therefore, Europa and Enceladus are highly interesting extraterrestrial bodies in our solar system, which are within reach for possible origin of life research. Phosphine ( $\text{PH}_3$ ) molecules were recently detected in the oxidized atmosphere of Venus.<sup>[382]</sup> Since phosphine is produced by some anaerobic microorganisms on Earth, this finding was perceived as indicative of the presence of similar microbial life on Venus. The publication sparked debate, but was later defended by the authors.<sup>[383]</sup>

Combined evidence from different sources available to us today allows the conclusion that the possible development of life outside our planet is not at all unlikely.

## 11. Future Directions

The elusive transition from the prebiotic to the living world is a complex and fascinating puzzle, to which new interesting pieces are being continuously added. The ultimate goal of origin of life research is to achieve a coherent picture of how this transition occurred. In this context, the successful synthesis of a functional protocellular unit based on a minimal, reasonable set of assumptions regarding the conditions for its creation is an important, if not the most important milestone in this endeavor. A simple cell that is capable of primitive metabolism, has the ability to divide in some way and to pass down its blueprint to the next generation, might be in many ways the expected outcome. The opinions on how to achieve this, and what the most reasonable minimal assumptions are, are diverse, which is evident from the multiple different

“world” hypotheses, the debate on the possible origin of simple biological molecules, and the diverse types of viable protocells together with associated external environments. It is likely that more than one pathway to life exist.

The requirements for this transition appear to be sufficiently well formulated and defined, and provide researchers with inspiration and challenge. The current trends reflected by the scientific literature suggest that sub-compartmentalization, membrane functionality, and coupled chemical reactions are major directions of research, but new aspects are emerging occasionally, and find their way into the community eventually. Examples are newly discovered potential environments for protocell growth, or sources of energy for shape transformations. It also appears that in silico studies, which have gained from the rapid development of computational resources, are increasingly influential for the experimentalists.

We have striven to present an overview of the wonderfully diverse contributions coming from the various scientific branches. The visible diversity in subject and approach is a strong positive factor, leading to novelty and progress. However, it has been repeatedly pointed out that a key limitation for a breakthrough might be the difficulty to approach the challenges in a concerted, interdisciplinary manner. It is reasonable to forecast that inter- and transdisciplinary communication efforts will increase, driven by need and opportunity alike. This is due to an increasing realization of the depth of the problems at hand, the ever-growing portfolio of research techniques in every single discipline, and the limits of what an individual research organization can reasonably pursue. A sturdy increase in boundary-crossing interactions has true potential to advance the origin of life inquiry to a stage where the stack of open questions becomes visibly reduced. We see one particularly promising approach in driving the combination of prebiotic chemistry and chemical reaction networks with protocellular compartments at the systems level. This means that not only different metabolites and their reaction pathways have to interact, but the container itself and its subcompartments should be involved as active contributors. We are well aware of the technical difficulties of introducing chemistry into micro-scale soft matter reactors at the border to complexity. We hope to inspire researchers to approach this problem, starting from their own point of view and expanding into adjacent areas and beyond. To that end, the protocell preparation method overview we compiled may inspire those who are not familiar with the squishy matter to consider combining their own work with a suitable physical model, perhaps in a collaboration or common project.

Stephane Leduc realized at the beginning of the last century that chemistry and biology alone will not be sufficient to answer the questions associated with the spontaneous generation of life. We now have reason to suspect that none of the individual sciences alone can actually achieve this. However, a smart combination may have a chance. The protocell is an excellent proving ground for systems-level transdisciplinary research bound for a breakthrough in the origins of life problem. This view finds support by a most enthusiastic quote by J. Szostak:

“Fortunately, many challenges remain before we will be close to a full understanding of the origin of life, so the future of research in this field is brighter than ever!”<sup>[220]</sup>

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## Conflict of Interest

The authors declare no conflict of interest.

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