

Chapter 6

Minimal Cellular Models for Origins-of-Life Studies and Biotechnology

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Abstract Minimal cellular models can be defined as those vesicle-based cell-like constructs that are assembled with the aim of (1) clarifying/understanding unknown aspects in origins-of-life research and hypotheses testing, (2) studying reconstituted biochemical pathways in a simplified system, (3) being exploited for potential biotechnological applications, and (4) developing novel concepts/technologies. These ‘synthetic cells’ are created by the bottom-up approach and within the synthetic/constructive paradigm. Here we shortly review the main ideas behind such novel usage of vesicles, and comment the experimental data collected in the past decades. An intriguing picture emerges, where technical progresses owing to the convergence of liposome, cell-free (and microfluidic) technologies lead to a fecund research area of great potential, which blends fundamental scientific question with the most modern and challenging facets of synthetic biology.

Keywords Protocells • Minimal cells • Synthetic biology • PURE system • Fatty acid vesicles • Synthetic cells • Bottom-up approach • Power law • Micro-compartmentalized reactions • Autopoiesis

6.1 Introduction

Not many scientific questions are so fascinating as the origin of life on Earth. This still unsolved conundrum permeated the history of science of all ages, but only in the twentieth century it became a central question of modern chemical investigation [1, 2], with the emergence of a chemical branch called prebiotic chemistry. Prebiotic chemistry typically focuses on the question “how complex chemical molecules originated from simple and primitively available building blocks?”. Several studies –

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among which the famous Urey-Miller experiment [3] – have shown plausible paths for originating molecules as amino acids, sugars, nucleobases, and so on (for a review, see [4]).

However, the origin of complex chemical molecules is *not* the origin of life. Life does not reside in a particular molecule. Life is a *system* property, deriving from a coherent, cooperative, out-of-equilibrium, and orchestrated dynamics of several molecules which – as far as we know – are spatially and functionally organized as *cells*.

Therefore, in addition to understanding the chemical origin of those molecules, which later we will recognize as the biomolecules responsible for the emergence and propagation of all biological organisms (from unicellular ones to the largest ones), a key and unsolved question refers to the origin, the structure, and the functionality of primitive cells, or similar cell-like systems, and their contributions to the onset of life in our planet (Fig. 6.1).

Several cellular models have been proposed in order to mimic primitive cells. Martin Hanczyc has reviewed these models [5], describing how, in the past decades, scientists have focused on microcompartments of different nature for modeling primitive cells, such as sulphobes, coacervates, autocoils, jeewanu, and

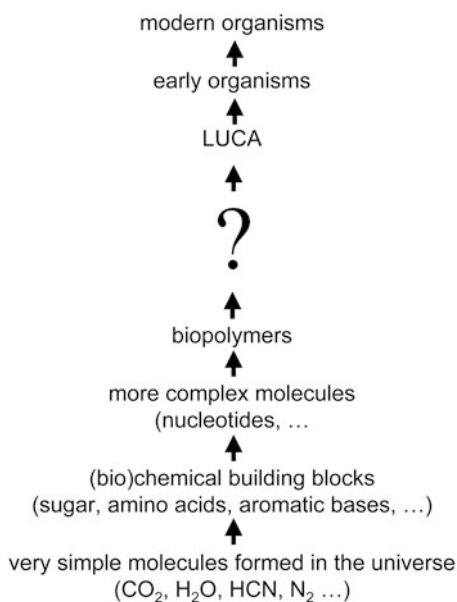


Fig. 6.1 Conceptual map describing the chemical (pre-biotic) and biological evolution, for the smallest molecules to modern biological cells and organisms. The research on primitive cells tries to fill the gap between understandings generated by classical prebiotic chemistry and backward evolutionary considerations ending to LUCA, the last universal common ancestor of all living organisms. The origins of life, according to the vision expressed by the author, should be investigated in the context of primitive cells origin, and not strictly related to the emergence of a particular molecule (e.g., a self-reproducing RNA)

microspheres. Although the interest in coacervates is still relevant, as witnessed by several recent publications [6–8], most of researchers now focus on *vesicles* as main cellular models.

Thus, from when in the 1960s Alec Bangham firstly reported on lipid vesicles (liposomes) [9, 10], the research on these tiny compartments, formed by self-assembly of lipids or other amphiphilic compounds in aqueous solutions, includes, among its several branches, the use of vesicles as cellular models, and in particular as primitive cell models (or protocell models). In particular, such models have been built from simple and primitive amphiphiles, such as fatty acids and some derivatives, but also from phospholipids and other synthetic compounds. The interest toward these models has increased a lot in the past years, till the point that the application of the same principles and methodologies developed for primitive cell models led to the novel perspective of assembling synthetic (or artificial) cells from scratch. In other words, the synthetic – or ‘put-together’ – approach which is so important in origins-of-life studies [11–13], has been extended to modern biotechnology aiming at synthesizing cells with minimal complexity. More specifically, the *bottom-up* construction of synthetic cells is one of the goal of synthetic biology [14], and it will serve advancements for biosensing, in nanomedicine, for understanding cellular mechanisms in a simplified environment, and to design and build novel bionanomaterials [15–20].

Liposome-based cellular models represent, therefore, a promising wide field of inquiry, embracing origins-of-life and biotechnology. In many cases, the same technologies are used, the same analytical methods, the same operational (and sometimes conceptual) approaches. The goal of this chapter is to introduce the readers into this topic, presenting the simplest cases and the most recent reports. Although important steps have been recorded in the past two decades, the field is most at its beginning, and future efforts will certainly lead to exciting discoveries, useful knowledge, and new technological tools.

The chapter is organized in four parts. Firstly, there is a short survey on vesicles types and basic properties with respect to their use as cellular models. Secondly, a short epistemic remark to the “synthetic approach” is made, here intended not only as a methodology but also as a self-standing concept. Next, the use of vesicles as primitive cell model is presented. Finally, we will comment on the synthetic biology operations based on vesicles as artificial cells.

6.2 Types of Vesicles and Their Preparation

Vesicles are microscopic compartments, generally spherical, composed by a closed membrane. When the membrane consists of a lipid bilayer, vesicles are better called ‘lipid vesicles’ or *liposomes*. However, liposomes are not the only type of vesicles. Vesicles have been generated by several types of amphiphilic molecules, like fatty acids [21], terpenoids [22–25], block copolymers [26–29]. These compartments share similar features, like their formation by molecular self-assembly, a typically

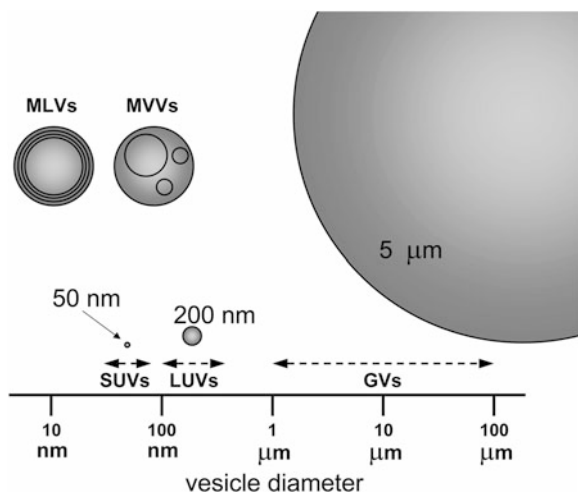


Fig. 6.2 Typical vesicle morphologies. Small Unilamellar Vesicles (SUVs), Large Unilamellar Vesicles (LUVs) and Giant Vesicles (GVs) (sometimes called Giant Unilamellar Vesicles, GUVs, if unilamellar) of diameters 50 nm, 200 nm, and 5 μm are represented approximately to scale. On the top, MultiLamellar Vesicles (MLVs) and Multi-Vesicular Vesicles (MVVs) (also called vesosomes) are schematically represented. MLVs and MVVs do not refer specifically to a determined size, rather to the vesicle morphology

semi-permeable membrane, or their reactivity dominated by surface forces (vesicles, after all, are colloidal particles). However, the specific chemical nature of the building blocks constituting the vesicle membrane is the first aspect that strongly impacts on other properties like stability, interaction with other vesicles or other molecules, methods of preparations, and compatibility with encapsulated material. Vesicles made of the above-mentioned chemicals (phospholipids, fatty acids, terpenoids, and sometimes block-copolymers) have been used in several instances as protocells.

The second aspect to keep into account is vesicle morphology (Fig. 6.2 and Table 6.1). In particular two vesicle types have been widely used, namely, large unilamellar vesicles (LUVs) and giant vesicles (GVs). There are good reasons to focus on these two vesicles types, which are equally important. LUVs (typical diameters: 100–400 nm) are easily produced by well-known and standardized methods. GV (diameter 1–100 μm) are produced by a limited number of methods [30], but have the great advantage of being visible by light microscopy so that their behaviour can be directly assessed by visual inspection. A word of mention should be spent for another vesicle type, which consists in a more complex architecture, namely small vesicles inside a large vesicle. Technically, these vesicles are called multi-vesicular vesicles (MVVs) or vesosomes. Their construction is interesting because they somehow mimic eukaryotic cells with their intracellular organelles. The issue of internal organelle-like vesicles is intriguing, as it is plausible that sub-compartmentalization is a successful strategy for exploiting chemical gradients,

Table 6.1 Types of vesicles (cf. Fig. 6.2)

Abbreviation	Name	Specifications ^a
SUV	Small unilamellar vesicles	Whose diameter is typically < than 100 nm
LUV	Large unilamellar vesicles	Diameter in the ~100–400 nm range
MLV	Multilamellar vesicles	Several concentric vesicles, with variable size
MVV	Multivesicular vesicles ^b	Vesicles containing smaller non-concentric vesicles
GV	Giant vesicles	Diameter in the 1–100 μm range

^a Note that commonly reported diameters are indicated. For example, 100 μm GVs are rare. MLVs and MVVs are terms not strictly related to size, rather to morphology

^b Also called ‘vesosomes’. Mimics of a cell with intracellular organelles

separating potentially interfering chemical paths, and providing at the same time a large surface for solute/membrane interactions. Although vesosomes have not been extensively used, it is foreseeable that future studies on artificial cells will be based on these structures, especially if methods for their systematic production will be optimized.

The third important factor is the method of preparation [30, 31]. Several methods have been employed, depending on the chemical nature of the vesicle building block, on the desired vesicle morphology, and on the compounds that need to be encapsulated or reconstituted in the vesicle. Actually it is not possible to give a general recipe and the choice must be deduced according to experimental restrictions and goal of the research. Such matter should also be tuned on the basis of compatibility between the preparation method and the requirements of lipid/solute system - which in turn should be combined after considering chemical compatibility.

6.2.1 Chemical Nature of Lipids

To be more specific, some general considerations about the chemical nature of lipids are reported below (a detailed discussion can be also found in [32]):

1. Phospholipid and fatty acids (Fig. 6.3a, b) are the two main compounds which have been used to construct protocellular models. Phospholipids, however, owing to their chemical complexity, cannot be considered as very primitive chemicals, and therefore fatty acid vesicles are better suited when the focus is on the membrane structure, behavior, chemical and inter-vesicle reactivity. On the other hand, phospholipid vesicles (liposomes) can still be used as primitive cell models if the focus is on intra-vesicle reactions, or on other aspects, providing that the nature of the vesicle membrane is not a conceptual issue.
2. More in detail, also fatty acids can be differentiated on the basis of primitive plausibility. In this context, short chain saturated fatty acids, such as the decanoic acid/decanoate system (C10:0) [33], probably represents better the nature of

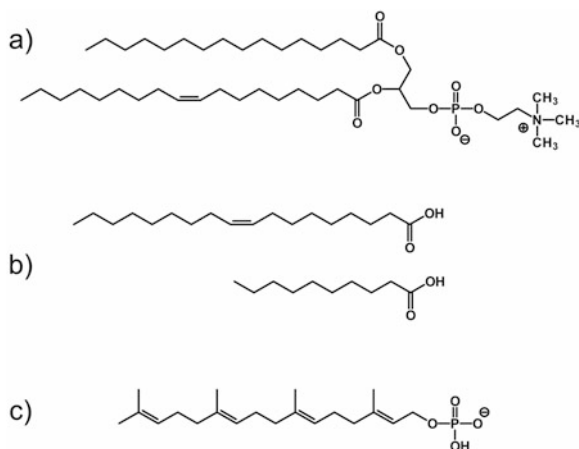


Fig. 6.3 Chemical structures of amphiphiles used for assembling vesicles as cellular models. (a) Phospholipids; here one of the most used lipid is shown, namely a phosphatidylcholine (lecithin) named 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC); (b) fatty acids; in particular the protonated forms of oleic acid (C18:1) and decanoic acid (C10:0) are shown – note that only the second has a realistic primitive relevance; (c) isoprenoids, in particular a partially ionized geranylgeranylphosphate is shown

primitive cells membrane. However, most of the published studies have been carried out with oleic acid/oleate vesicles (C18:1) [34–42] and myristoleic acid/myristoleate (C14:1) [43–46].

- Membranes composed by only one chemical species, on the other hand, are not realistic, and mixtures of diverse amphiphilic molecules better represent primitive membranes [44, 45, 47]. Studies on pure compounds are nevertheless useful to preliminarily decipher the properties of individual compounds, before engaging with the study of mixtures.
- In contrast to fatty acid vesicles, which have been investigated at a considerable extent, isoprenoid compounds (Fig. 6.3c), such as polyprenyl phosphates (alone or as mixtures with polyprenols), have been studied only in a very few cases [22–25, 48, 49]. The ionizable phosphate head group implies a pH-dependence in their self-assembly properties. Linear and branched polyprenyl compounds can form vesicles whose properties are only partially known. This contrasts with the importance of isoprenoids in modern cells. *Archaea* membranes are made of isoprenoid-derivatives monolayers; whereas cholesterol, ergosterol and lanosterol are typically found in *Eukarya* cells.
- Pure fatty acid vesicles, being composed in their ‘stable’ form by about 50% carboxylate (typically as sodium salts) are sensitive to important multivalent cations such as Fe^{2+} , Fe^{3+} , Ca^{2+} and Mg^{2+} [50] (at relatively low concentration) and to monovalent cations (at high concentration) [51]. Note also that H^+ destabilizes fatty acid vesicles by binding to carboxylate ($\text{RCOO}^- + \text{H}^+ \rightleftharpoons \text{RCOOH}$). Indeed, the limited pH-range of existence of pure fatty acid vesicles (generally

between 7–7.5 and 9–9.5) should be always considered. Sensitivity to Mg^{2+} (an important cation due to its interaction with nucleic acids and their precursors) has been improved by mixed systems composed of fatty acids and monoacylglycerols [44, 50–53] or by chelating Mg^{2+} by citrate [54].

6. Most of liposomes-based work has been carried out with the zwitterionic phosphatidylcholine (lecithin), which self-assemble in a very stable membrane and in a wide pH range. The other zwitterionic phospholipid, namely phosphatidylethanolamine does not form generally stable membranes, being characterized by an unfavorable packing parameter [55] ($v/al < 1$; v being the molecule volume, a the effective head group area, l the molecule length). Moreover the positive charge on the head amino group ($-\text{NH}_3^+$) is pH-dependent, whereas the phosphatidylcholine trimethylammonium group is not ($-\text{NMe}_3^+$). Other phospholipids have been generally used as a lipid mixtures (especially the anionic phosphatidylglycerol), with phosphatidylcholine being the main component, possibly also including cholesterol. The simplest phospholipid, namely, phosphatidic acid – which also form vesicles at intermediate pH – has not been deeply investigated in the context of origin of life.
7. Both in the case of fatty acids and phospholipids, care should be taken in order to be aware of the physical state of the hydrocarbon chains, i.e., solid-like or liquid-like. The transition temperature, T_m is an important parameter to consider for designing protocellular systems. The physical state of the membrane will impact on vesicle stability and small-solute permeability. Fatty acids are single-chain charged molecules, and their solubility can be high. Therefore in such systems, the critical aggregation concentration (c.a.c.) is an issue to consider.
8. Polymersomes, which have been occasionally employed to build synthetic cells, have no direct relevance for the origins of life. However, their development might be functional for specific biotechnological applications, in virtue of their great stability.

6.2.2 Vesicle Type (Morphology)

With respect to vesicle type (or morphology, see Fig. 6.2), vesicles are generally classified according to Table 6.1 entries. The most utilized vesicle types in origins of life studies are LUVs and GVs. LUVs have been probably the most common type of vesicles due to several reasons. Some are technical reasons and of-opportunity reasons, and it will be commented below. Another considerations – which might count contrasting opinion among investigators – focus on the idea (on the hypothesis) of how large where primitive cells. To be more detailed, we will see below that the spontaneous hydration of lipids generally brings about quite large vesicles, for example GVs. It is plausible then that in absence of strong shearing forces, large vesicles (in the micrometer range) are better candidates for representing protocells. Contemporary living cells also have similar sizes, from the smallest bacteria (ca. 1 μm) to large unicellular eukaryotes (10–50 μm). However,

one should also consider the stringent conditions for realizing a living dynamics, in terms of number of molecules, local concentration, surface-to-volume requirements. These features are probably better embodied in LUVs (0.1–0.2 μm). An interesting discussion has been developed around the minimal requirements of life, also in terms of dimensions, as reported in the proceedings of a dedicated workshop [56], and experimental studies [57].

As mentioned, LUVs can be produced in highly reproducible way from a wide variety of lipids, thanks to standard procedures (film hydration, freeze-thawing, extrusion, purification by size exclusion chromatography/dialysis). The large part of a vesicle population prepared in this way is spherical and unilamellar. The procedure allows the entrapment of both water-soluble (dissolved in the aqueous buffer used to hydrate the lipid film) and lipid-soluble substances (dried together the lipids). Another advantage is that the resulting dispersion can be manipulated almost as a normal solution, and bulk measurements (absorption spectroscopy, fluorescence, etc.) can be applied. LUVs model small primitive cells, much smaller than bacteria. Their size is instead typical of viruses, and the size distribution is typically narrow (after extrusion). The fact that LUVs can be produced in such reproducible and homogeneous form (uniform with respect to size, shape, lamellarity) make LUVs a quite attractive model, especially if one is interested in average properties (averaged over the whole vesicle population). Fatty acid LUVs and phospholipid LUVs have been extensively used.

GVs, on the other hand, are also widely used. Their main feature is the very large size – in the 1–100 μm range (typically 5–20 μm), which allows their direct visualization by light microscopy in the form of aqueous suspension (whereas LUVs cannot). Importantly, GV has high trapped volume and therefore contain a large number of solutes. GV requires special preparation methods. The two main methods derive from the classical film hydration method that is used to produce LUVs and MLVs. These ‘classical’ methods are the so-called natural swelling method and the electroswelling method. The natural swelling method consists in hydrating thin lipid films without mechanical perturbation. The film, sometimes pre-hydrated by aqueous vapours, is left for a long time (hours) in contact with the aqueous solution without stirring, shaking, etc. Lipid films swell gently, creating GV of various size and morphology, also multi-vesicular GV. Electroswelling is essentially a way to accelerate this process, by application of an alternating electrical field. Lipids are stratified over wires or planar electrodes and alternating current is applied. Swelling occurs in shorter times (less than 1 h). Both methods work well for phosphatidylcholine GV and low ionic strength buffers. This can be a limitation because in many cases physiological-like buffers are necessary. It has been shown that negatively charged GV (e.g., including phosphatidylglycerol in their membrane) can be produced by the natural swelling method in the presence of high ionic strength buffers [58]. Note that fatty acid GV have been produced only by modifications the film hydration/natural swelling method, not by electroswelling, whereas mixed phospholipid/fatty acid vesicles have been produced by the next-discussed droplet transfer method [59].

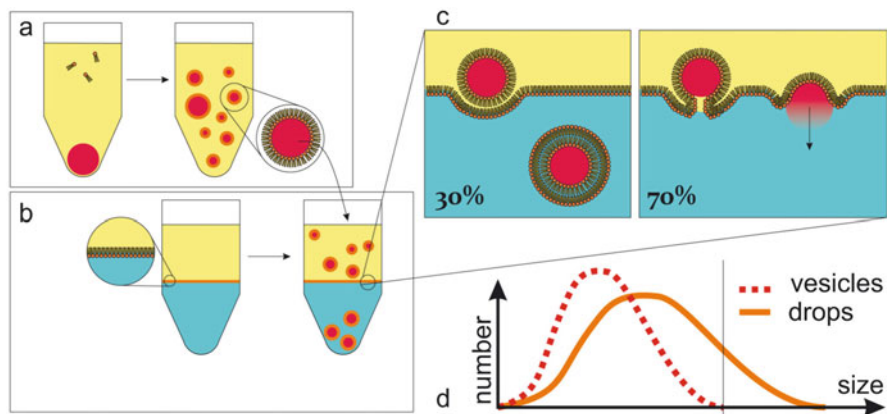


Fig. 6.4 GVs prepared by the droplet transfer method [60]. **(a)** Preparation of a water-in-oil macroemulsion with mineral oil, surfactants (POPC) and the inner-solution. **(b)** Preparation of an oil over outer-solution system with surfactants at the interface. In a second step the macroemulsion droplets are inserted in this system and sink down due to the density difference between the inner- and outer-solution. **(c)** As they wander through the interface they get a second layer of surfactants such that they now have a bilayer of phospholipids, i.e. they are vesicles now, if the conditions are good (according to our measurements, generally in about 30% of all cases) – otherwise they merge with the interface and the inner-solution is released into the outer-solution (which generally happens 70% of all cases). **(d)** This leads to a size distribution of the vesicles which does not allow vesicles greater than a critical size, even though the macroemulsion droplets generated in the first step were greater (Reproduced from [63] with the permission of Springer)

In addition to these two classical methods, which are very useful for studying the properties of lipid membranes, a novel GVs preparation protocol has been introduced recently [60]. This is based on the transformation of water-in-oil (w/o) lipid-stabilized droplets. W/o droplets are centrifuged across a lipid-containing interface and get covered by a second lipid monolayer, so to form GVs (Fig. 6.4). This method forms GVs with traces of the apolar solvent (the ‘oil’) used to prepare the w/o droplets and it is therefore questionable whether or not the resulting GVs can be used for accurate biophysical measurements of membrane properties. On the other hand, the strength of the method relies in its application to encapsulate molecules in the GVs lumen, especially macromolecules [61]. Moreover, asymmetric lipid membranes can be created with this method [62], and mixtures of lipids can be used, provided that the w/o droplets are sufficiently stabilized in the first step of the preparation. Mixed phosphatidylcholine/fatty acids GVs have been successfully prepared by the droplet transfer method [59]. Despite these advantages, the droplet transfer method (as well as the electrosweeling method) cannot be considered of prebiotic relevance. Nevertheless it has been used in several cases to produce solute-filled vesicles which model primitive cells. The focus was therefore not on the mechanism of formation of such vesicles, but on their properties/dynamics/interaction with other vesicles, and so on.

The natural swelling method is therefore the preferred method to simulate the emergence of early cell-like structures from amphiphiles and water. It produces a heterogeneous population of vesicles that realistically represents a sort of primitive ecosystem, where synergies, cooperations, competitions and selections among these coexisting ‘units’ took place. In addition to these important features, especially when the realistic primitive cell modeling is desired, it should be reminded that the GVs prepared by the natural swelling method are characterized by an intrinsic diversity in size, lamellarity, and morphology. This makes difficult to define a sort of ‘average’ behavior. It follows that studies done on more homogeneous samples (LUVs, GVs prepared by the electroswelling method or droplet transfer method) and those done on spontaneously formed GVs by natural swelling complement each other. Moreover, it should be recalled that microfluidics offers a novel technological route for the construction of highly homogeneous GVs [64–71].

6.2.3 Preparation Methods and Solute Entrapment

It is worth to recall the interplay between lipid types, methods of preparation and an essential feature of lipid micro-compartments, namely, their capacity of entrapping water-soluble or lipid-soluble substances. Clearly, this is of vital importance when models of primitive cells are prepared. Water-soluble substances (inorganic salts, sugars, small polar molecules, proteins, nucleic acids, ribosomes, ...) are encapsulated inside vesicles in the moment of their formation (Fig. 6.5). Generally, such molecules have low permeability and their addition after vesicle formation does not bring about their internalization. An exception are small molecules which

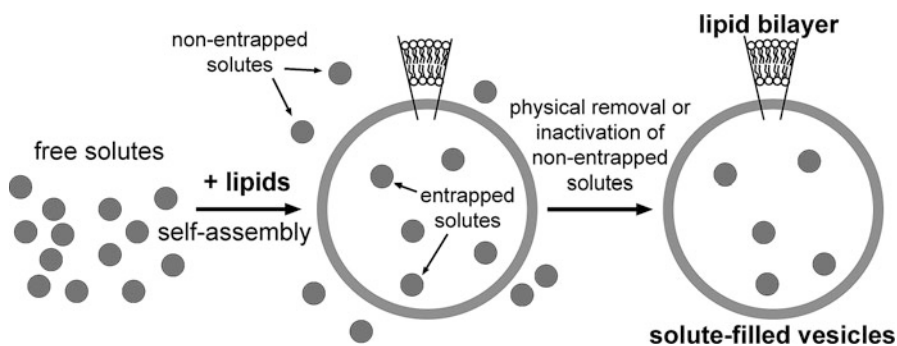


Fig. 6.5 Solute-filled vesicles are simply obtained by letting vesicle formation in a solution of the solute(s) of interest. When vesicle forms, they capture part of the external solution, so that solutes become encapsulated inside. Their permeability is low (as they are water-soluble) and thus they are not released. A purification step generally follows the entrapment, but in some cases, external solutes and their potential reactions are blocked/inhibited by the addition of another (non-permeable) agent

are present in solution in two forms (charged and non-charged), for example in the case of amines (RNH_3^+ and RNH_2), or other compounds with acid/base forms with a similar feature. In some cases, the neutral form can permeate the lipid membrane and be transformed in the charged non-permeable species inside the vesicle, due to a different pH value [72]. This procedure has been applied in the construction of doxorubicine-containing liposomes [73, 74] (for drug delivery) but a similar strategy can work in the case of other molecules of prebiotic interest [75–78].

Lipid vesicles originate, ultimately, from a closure mechanism whereby a curved lipid layer closed on itself capturing a portion of the aqueous solution. In ideal conditions, and in absence of strong solute-lipid interactions (as it could be, for example, when cationic lipids are allowed to form vesicles in the presence of nucleic acids), the entrapment process is equivalent to a random sampling. One can imagine that open lipid bilayers (homogeneously distributed in the solution) capture portions of the solution in random way. Probably the method that more closely matches with this description is the ethanol injection method [79]. In the other methods practical constraints prevent the occurrence of these ideal conditions, but nevertheless it is useful to describe what would be the ideal case and then compare it with the observations.

The fraction of whole volume V captured by a vesicle with volume v is v/V , and this ratio represents the entrapment probability p . The *average* number λ of entrapped solutes will be pN , where N is the total number of solutes in the whole solution. As $N = N_A C_0 V$, it results that $\lambda = pN = v/V \cdot N_A C_0 V = N_A C_0 v$ (where N_A is the Avogadro's number and C_0 the bulk solute concentration). As in all microscopic phenomena, stochastic events related to the randomness of microscopic conditions affect the local solute concentration; consequently the closure of open lipid bilayers produces a population of vesicles where the number n of solute entrapped in a certain vesicle differs from λ , the most probable one. Given a certain vesicle size, a solute occupancy distribution $\wp(n)$ is obtained, that can be typically described by a Poisson distribution (Fig. 6.6):

$$\wp(n) = \frac{\lambda^n e^{-\lambda}}{n!} \quad (6.1)$$

Note that the Poisson distribution becomes similar to the Gaussian distribution when λ is large. This simple analysis reveals that together with vesicles that encapsulate the expected number of solute molecules, there will be always vesicles with n smaller or larger than λ . This means that when vesicle forms, even in the most ideal conditions (negligible solute-solute and solute-lipid interactions, homogeneous and isotropic solutions, homogeneous distribution of lipids in the whole solution), the resulting vesicle population is – by definition – heterogeneous in terms of solute content.

This has two consequences. First, from the technical viewpoint, one has to distinguish among (i) the (very much used) *average* entrapment efficiency (or entrapment yield, or similar values) which considers the overall amount of entrapped

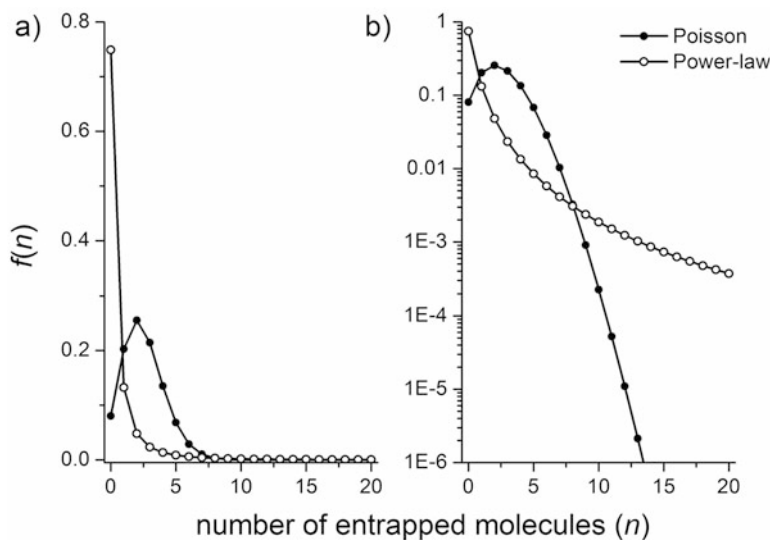


Fig. 6.6 Poisson and power-law distribution. Simulated Poisson and power-law distribution $f(n)$ as function of the number n of encapsulated molecules. The Poisson curve has been generated by using $\lambda = 2.52$, which corresponds to the expected value of encapsulated molecules when vesicles with diameter 200 nm are formed in a $1 \mu\text{M}$ solution. The power-law curve has been generated with $A = 0.75$ and $k = 2.5$ [82]. The plots refer to the same datasets but different y-axis: (a) linear, (b) logarithmic. The latter representation helps understanding that the encapsulation of high number of molecules (say, $n = 20$) when 2.52 are expected is highly improbable according to the Poisson distribution ($\sim 10^{-12}$), but 10^9 times more probable when a power-law is considered (10^{-3})

solute, divided by the number of vesicles (or by the lipid concentration), and (ii) the (less used) *individual* entrapment which instead measures the content of each vesicle, individually. The first measure is obtained by bulk measurements, the second, by techniques that allow the individual analysis of vesicles (e.g., microscopy [80–82], flow cytometry [83, 84]). Second, from the viewpoint of utilizing vesicles as primitive cell model, this fact evidences that populations of primitive cells, being formed by spontaneous lipid self-assembly into vesicles, and spontaneous entrapment of molecules, are ‘diverse’ also in terms of solute filling, in addition to size, lamellarity, morphology [85]. As the internalized solutes play the role of metabolic components, this implies that some protocells would function better than others, and being subjected to a proto-Darwinian selection, in virtue of better function, better reproduction rate, better stability (however, cooperation/synergy should not be neglected [59]). This has a profound implication for depicting realistic origins-of-life scenarios, as it has been indeed done in recent work, although referring to different behaviour [40, 86–88].

According to what has been discussed above, a vesicle population is characterized by a diversity in the inner solute content, which is spread around a certain average value λ . Such average solute number linearly depends on the product of

vesicle volume v and bulk solute concentration C_0 , as expected. Small vesicles display strong stochastic fluctuations in the number of encapsulated molecules. For instance, when LUVs (diameter 100 nm) are formed in the presence of $10\ \mu\text{M}$ solute, $\lambda = 3.15$ meaning that ca. 60% of vesicles have entrapped 2, 3, or 4 solute molecules, whereas the others a quite different number. For example, the probability of finding a vesicle with 10 solute molecules is 0.1%. The ‘long tail’ on the right-side of the solute occupancy distribution mean (λ) is much more important than the left part. Here there are vesicles which are filled with solutes, much more than the expectation. Normally their number is very low, but – as detailed in the ‘*super-filled*’ vesicles Box, there are cases that challenge the Poisson distribution.

‘Super-filled’ vesicles. Although the Poisson distribution is the expected solute-occupancy distribution for an ideal random entrapment process (demonstrated in [84, 89]), recent reports have shown an intriguing divergence from the expectations, as reviewed and discussed in [90]. In particular, a series of paper have revealed that when macromolecules are encapsulated in conventional submicrometer vesicles prepared by different methods, the experimentally observed solute occupancy distribution does not follow the Poisson distribution [82, 91–94]. Rather, it is shaped as a ‘power law’, namely $f(n) = A/(n + 1)^k$, where A and k are positive parameters. This means that most of the vesicles are empty, and few of them are instead solute-filled (Fig. 6.6). However, because the power law function goes to zero slower than the Poisson function, it results that the amount of vesicles with high n is higher for a power law than for a Poisson process. In other words, actually there is a non-zero probability of finding solute-filled vesicles, with n well above the expected average λ . For example, the 0.1% of vesicles prepared in the presence of ferritin (or ribosomes) has an intra-vesicle solute concentration up to 10 times higher than the expected value. Clearly, when vesicles are formed in a mixture of essential macromolecules (essential for the sustainment of a network) some of the vesicles would be capable – against the expectations – of co-entrapping several copies of these solutes. In turn, such ‘super-filled’ vesicles could be very efficient in the realization of an internal reaction network, and so being favoured with respect to the empty or regularly filled one. Experimental evidences about this mechanism have been provided [82, 91, 92, 94]. A mechanism based on the perturbation of open bilayer closure rate has been suggested for accounting the observations (but for a counterexample, see [95, 96]).

The discussion on random encapsulation holds in the case of vesicles prepared by conventional methods, and when the solute encapsulation is the study focus. On the other hand, the above-mentioned droplet transfer method (Fig. 6.4) radically differ from the others and it is best suitable for a ‘directed’ encapsulation of water-soluble

molecules. Due to recent developments, this method has become probably the best method for preparing solute-filled GVs intended as synthetic cells, when there is no intention to study the self-assembly phenomena underlying the spontaneous emergence of solute-filled vesicles (i.e., modeling the origin of primitive cells). The directed entrapment occurs because solutes are firstly emulsified in form of w/o droplet (achieving 100% compartmentalization), and droplets are transformed in vesicles. The remaining source of inter-vesicle variability is now only the solute partition among droplets while they form by coagulation/fragmentation steps [85].

A completely different discussion refers to lipid-soluble molecules. In contrast to water-soluble ones, they have the spontaneous tendency of binding to lipid membranes, and their inclusion in the vesicle structure is easy. The exceptions are membrane proteins. If a protocell model is designed in a way that it includes membrane proteins (enzymes, transport protein, signaling protein) care should be taken to design the lipid type and the vesicle preparation method (and these two things are somehow interlinked). It may seem that the reconstitution of complex membrane proteins in protocellular model is not a fully pertinent exercise. However, it is expected that primordial polypeptides with hydrophobic character could decorate early membranes complexifying their repertoire of functions (e.g., permeability changes, inter-vesicle interactions, vesicle-surface interactions, binding of free floating molecules). In this respect, the inclusion of lipid soluble or lipid-anchoring molecules is certainly relevant.

Moreover, when the protocell model is built to demonstrate the reliability of minimal transformation pathways, or their use for investigating protocell transformations, the reconstitution of modern membrane enzymes can be an essential step. From this perspective, also phospholipid membranes can be employed as primitive membrane models. An example is given by one of the early papers on vesicle self-reproduction. The four enzymes that convert lipid precursors into lipid molecules were reconstituted in lipid vesicles [97]. The goal of the work was to demonstrate that a lipid-producing liposome could grow like a cell, producing from within the building blocks for enlarging the membrane (according to the autopoiesis theory – see below).

In conclusion, when vesicles are intended as cell models, there are several aspects to consider, and these are somehow linked to each other. The type of lipids, the vesicle type, the preparation method and the solute entrapment are all connected, and unfortunately not all combinations are easily accessible or even already explored. Clearly, the choice will depend on the aim of a study. Table 6.2 offers a framework for such discussion. For example, allegedly primitive compounds,

Table 6.2 Different compounds and approaches in vesicles as cell model research

Component type	Scope	Examples
Allegedly primitive	Realistic protocell	Fatty acids, small peptides, ...
Modern biomolecules	Minimal functions	Enzymes, ribosomes, phospholipids, ...
Synthetic	Fully synthetic cells	Polymers, hybrid molecules, ...

as well as components extracted from modern cells (and synthetic molecules as well) have been successfully employed to build cellular models, and vesicles of very different types have been constructed. However, it should be useful, when discussing the choices underlying the constructed models, keep clear what is the aim of the study, and what the model aims at revealing. For example, it is evident that a realistic primitive cell model should be built from allegedly primitive compounds, such as simple membrane-forming lipids (fatty acids, isoprenoids, fatty alcohols, etc.), and molecules which mimic the early catalysts (such as small peptides or ribozymes). When the focus is on minimal cell function, irrespective from the actual molecular species that carry out a certain function, modern molecules have been used (enzymes, DNA, ribosomes, modern lipids, ...). We have to distinguish two possible aims for the second-line approach of Table 6.2. One aim could be the construction of primitive cell models, but using modern molecules to test some hypotheses about minimal function. Another aim could be the construction of a biotechnological tool, a sort of synthetic cell, for some specific applications. Finally, one can imagine also fully-synthetic cell model that show how living-like functions can be achieved in ‘orthogonal’ way – namely – by using compounds not selected in the natural evolutionary pathway (synthetic polymers, ad hoc designed lipids, hybrid materials, and so on). Of course, the distinction between the approaches can become blurred in certain circumstances, and the possibility of hybridization should be considered also positively. Any advancement in this new field carry a scientific and technological potential that should not be necessarily restricted by classifications.

Despite these differences, the approaches indicated in Table 6.2 have a common ground, and this should be emphasized: *assembling a cell-like entity from separated parts*. This operational procedure, whose roots are in the chemical science (building a complex molecule from small parts), has been dubbed as ‘synthetic’ approach. It corresponds to an *operational bottom-up* approach, even when the protocell design might originate from a *conceptual top-down* approach (think, for instance, of designing a protocell by removing unnecessary components from a fully fledged cell, and then construct the protocell by assembling these essential parts). Describing and commenting this approach is the topic of the next section.

6.3 The Synthetic or Bottom-Up Approach

Let us remark better the concepts which lay at the basis of the vesicle usage as cell models. In the introduction it has been quickly said that the ‘synthetic approach’ (also called constructive approach) is typical of origins-of-life research. The philosophy of the synthetic approach can be summarized by the words of Liu and Fletcher [98], saying that “we are much better at taking cells apart than putting them together”, evidencing how in modern science the analytic rather than the synthetic method has been largely applied for gaining knowledge about how biological entities work. Actually the analytical approach has been very successful.

In the field of primitive cell research, however, such an approach cannot be applied, and primitive cells – or better, their models – can be only constructed in the laboratory, by assembling molecular pieces and verifying hypotheses, checking which kind of constraints apply, building minimal metabolic pathways, and so on.

Understanding-by-building can be the motto of this practice. Clearly, difficulties exist due to the ignorance of the exact conditions that allowed the emergence of first cells from non-living molecules, and on the exact order of the steps that accompanied the success of cellular homeostasis and self-reproduction. Nevertheless the synthetic approach is the only one that might give scientific answers to this age-long question, and especially demonstrating that life emerges, as a result of out-of-equilibrium organization, yet according to the laws of physics and chemistry (we will see later that this special type of organization must necessarily be autopoietic).

Understanding-by-building, however, is also a motto of a modern biology branch, synthetic biology (SB). SB is a young discipline born by applying the engineering vision to biology [99]. However it differs from genetic engineering because it focuses on the engineering ('rewiring') of whole cells. SB generally aims at achieving concrete goal, typical of bioengineering, such as creating biosensors, letting biological cells (most often: bacteria) producing fine chemicals, or biofuels, or pharmaceuticals, constructing bacterial strains for bioremediations, and so on. On the other hand, from its very beginning, a special focus was reserved to the construction of *minimal cells*, i.e., living cells with minimal complexity. This goal is important for basic understanding of living cells (what is the minimal non-reducible complexity associated with life) and for biotechnological purposes (eliminating unnecessary circuitry is thought to be equivalent to optimize energy usage in cells).

Mainstream SB research looks at minimal synthetic cells as a product of genomic manipulation (with the already achieved goal of a cell that has been deprived of its native genome and transplanted with a minimal synthetic genome, according to the Venter's team approach [100, 101]). Such a path is now recognized as the 'top-down' approach to synthetic cell (using SB techniques). It is top-down because it starts from a pre-existing organism, and implies a minimization design done by the scientist. An alternative approach to minimal synthetic cells is based on the 'bottom-up' construction, from a minimal set of molecules, not from cells – as it has been described in this review. This second path would lead to the improvement of biological understanding of cellular processes once they are reconstituted from scratch in simplified systems, and when the noise due to other concurrent processes has been eliminated in the novel, minimized, cell-like design.

It is evident that the philosophy, the methods, the synthetic (constructive) approaches for the *bottom-up* construction of minimal cells largely overlap with those which are typical of research on primitive cells. This leads to an unexpected, yet very fertile, common arena which is interesting also from the viewpoint of science epistemology [102, 103]. It is quite intriguing that one of the most

ambitious biology branches shares with the research on the primitive cells its most fundamental conceptual and practical tools.

Bottom-up synthetic minimal cells and models of primitive cells, thus, are two different implementations of the synthetic bottom-up approach. Both aims at assembling cell-like systems from non-living molecular parts, and at understanding the function of cellular systems (understanding by building). Perhaps, the most obvious difference refers to the type of molecules used for building such minimalized cells, as already specified in Table 6.2. It should be noted that as the field is very young, the terminology is not yet crystallized. Different authors uses different terms like primitive cell, protocell, minimal cell, synthetic cell, artificial cell, semi-synthetic cell, semi-synthetic minimal cell. In contrary to their apparent diversity, all vesicle-based cell models built according to the bottom-up synthetic approach show a certain degree of similarity. Very different is the case of synthetic cells built by genetic manipulations of existing cells or by genome implanting, referred, as mentioned, as top-down approach. *Nota bene*: With respect to the bottom-up/top-down dichotomy, it is worth noting that the authentic bottom-up approach, based on non-designed self-organization and emergence is actually not really applied (*stricto sensu*). Rather, the methodological bottom-up approach (assembling a cell-like structure from its parts, based on self-assembly of molecular systems) is often preceded by a design step (deciding what molecules include, foresee patterns, combining parts which function together), which is a typical top-down practice (designing a system with a final goal in mind).

6.4 Modelling Primitive Cells

One of the primary goal of origins-of-life research is creating model of primitive cells, those entities that originated from self-assembly of molecules which are supposed to be available in early times. These very basic structures were very different from the cells as we know them. They were simpler, performed worst than evolved cells, probably they were partially unstable and perhaps ‘limping’ [11]. As it has been recently discussed by us [104], the formation of self-reproducing protocells that are able to display essential features of biological autonomy marks the transition between non-life and life. One generally focuses on the notion of cells with minimal complexity, but what does ‘minimal’ life mean? The definition of life is still an open question that divides scholars [105, 106]. Most would agree on the fact that a living system displays homeostatic self-maintenance, self-reproduction and the capability to evolve. Autopoiesis (self-production) is a theory proposed by two biologists, Humberto Maturana and Francisco Varela [107, 108], that provides an operational definition of life as that process of self-bounded structures that produce their own components own to chemical transformations occurring within the autopoietic organization itself (Fig. 6.7). Autopoiesis does not explain how life originated, but tells us how a living system works – and therefore, how to construct it.

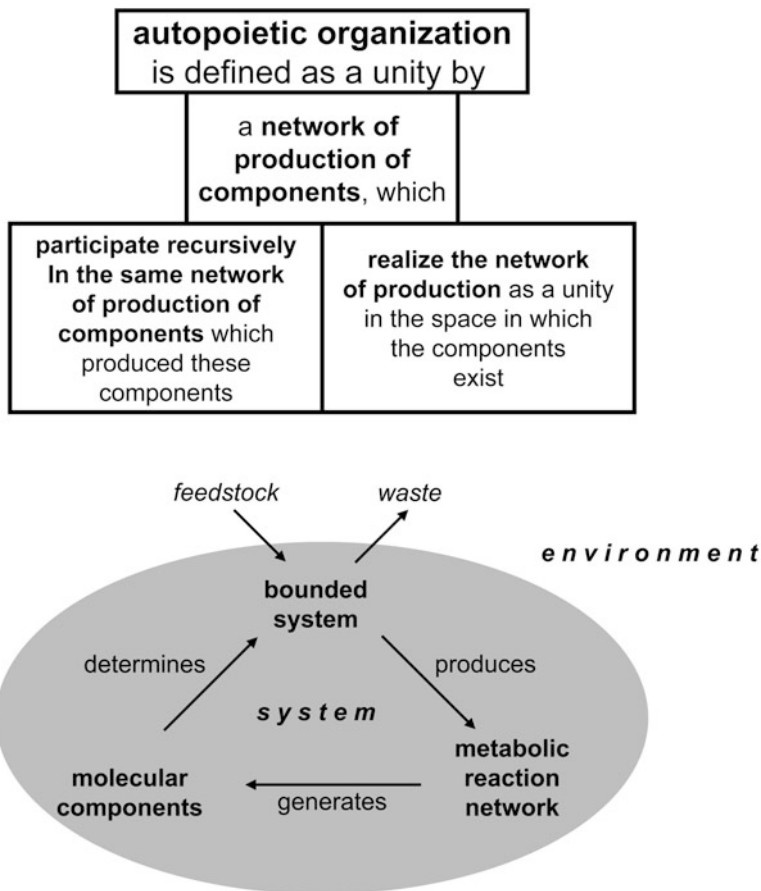


Fig. 6.7 Essentials of autopoiesis theory. *On the top*, the definition of autopoietic organization in terms of a network of production of components [107]; *on the bottom*, application of autopoietic theory to primitive cells, synthetic cells, etc. [109]

Here we present a brief overview of autopoietic theory [109], as recently discussed in our publication [104]. The central feature that characterises all living entities (and in particular unicellular organisms) is their self-maintenance. By self-maintenance, here we mean the self-generation of all components by chemical reactions, occurring within a boundary (e.g., the cell membrane). The boundary is also produced by the internal metabolic system. This self-generation is due to the peculiar form of chemical organization, that is, a dynamical organization typical of autopoietic systems. It is defined as a

(continued)

network of processes of productions of the molecular components, which (1) participate recursively in the network of processes for their own production and (2) occur in a defined region (physical space) delimited by a physical boundary (Fig. 6.7). The physical boundary also belongs to the autopoietic organization. The so-obtained physical entity is an autopoietic unit. Note that the autopoietic organization is a collective, distributed property of the whole system, and does not reside in any particular molecule. In autopoietic theory, the operational closure is often included in the discussion, to mean that the autopoietic unit reacts to environmental changes in order to maintain its own inner autopoietic organization, and that the unit tolerates only changes that can be accommodated within the autopoietic organization.

Based on autopoietic theory, some important experimental achievements have been obtained in the 1990s with fatty acid vesicles, namely, autopoietic self-reproduction of reverse micelles [110], micelles [111] and LUVs [34]. In all cases, fatty acids were used to construct these structures. The self-reproduction of fatty acid GVs has been also reported [42, 112]. Despite the historical and conceptual relevance of reverse micelle and micelle self-reproduction (a programmatic paper co-authored by Luisi and Varela appeared in 1989 [113] where chemical autopoiesis was firstly sketched in the reverse micelle system), in Sect. 6.4.1 only the self-reproduction of vesicles will be commented. Interested readers can find useful a recently published comprehensive review [114]. Next, selected examples showing the occurrence of reactions inside fatty acid vesicles will be presented (Sect. 6.4.3).

6.4.1 Vesicle Self-Reproduction

Autopoietic vesicle self-reproduction has been designed after inspiration to the autopoietic theory. Fatty acid vesicles have been used as model of self-reproducing vesicles. This was a somehow fortunate case, as there are easily accessible experimental routes for realizing a self-reproducing mechanism, *and* fatty acid vesicles are also the most plausible models of primitive cells. Pre-existing fatty acid vesicles (generally oleic acid/oleate vesicles, which are stable at slightly alkaline pH, i.e., 8.5) were provided with feeding material in form of externally added fatty acid precursors (water-insoluble fatty acid anhydride) or directly with free fatty acids in the form of micelles, which are easily deliverable in pseudo-homogeneous phase.

The rationale is the following. According to autopoiesis, an autopoietic cell takes up molecular precursors from its environment and transforms them in the components of its dynamical network (including assembling the physical boundary: the membrane, which limits and define the autopoietic cell against the background). Such a process is central to autopoietic organization, and involves by definition all

components of the autopoietic network. However, the focus on the membrane allows a further advantageous implementation. Autopoiesis also means self-maintenance. Despite the concurrent and ever-present anabolic and catabolic processes, the autopoietic entity maintains its identity. Quantitatively this means that the rate of precursors uptake and components production (V_p) is balanced by the rate of components destruction/release in the environment (V_d), or $V_p = V_d$. No net component production or accumulation takes place. The autopoietic unit self-maintains in a *homeostatic* state. However, if $V_p > V_d$ the autopoietic system can grow, producing new components that can constitute a novel (daughter) system following a growth/division process (Fig. 6.8). Note that the reaction takes place within the boundary of structure/unit. This process, when applied to cells or their vesicle models, has been called autopoietic self-reproduction [35, 115].

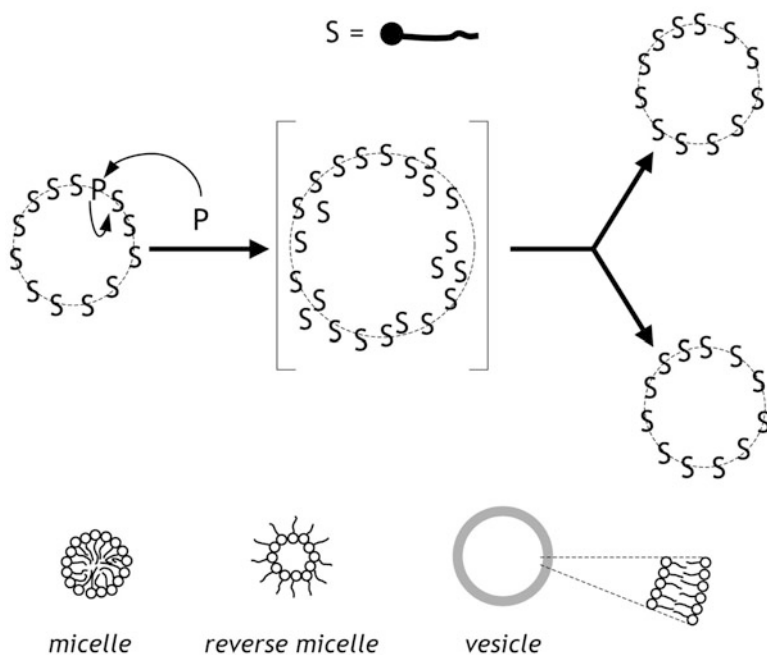


Fig. 6.8 General schemes for the self-reproduction of supramolecular structures. The uptake of a suitable precursor **P** by the preformed self-assembled structure, and its transformation to **S**, the membrane-forming compound, brings about the growth of the structure boundary. The growth induces a destabilization of the structure, which divides in two (or more) similar structures (not necessarily of the same size). The production of **S** proceeds with rate V_p , the destruction/release of **S** (not shown in the drawing) proceeds with rate V_d . If $V_p > V_d$ the structure grows; if $V_p = V_d$ the structure is in a (dynamic) homeostatic state; if $V_p < V_d$ the structure collapses. The autopoietic self-reproduction mechanism has been studied for micelles (*bottom, left*), reverse micelles (*bottom, centre*), and vesicles (*bottom, right*; not drawn to scale) (Reproduced from [116] with the permission of Springer)

6.4.1.1 Premise on Fatty Acids Dispersed in Water

Depending on pH, fatty acids can be found essentially in three different forms when dispersed in an aqueous solution. At low pH (for example, below ca. 7.5) fatty acids are protonated ($R - \text{COOH}$) and separate from the solution. Intramolecular interactions are dominantly hydrophobic. At high pH (for example above ca. 9.5) fatty acids are deprotonated, in the form of soaps ($R - \text{COO}^- \text{Na}^+$) and due to their large hydrated head group they self-assemble into micelles, as expected from the Israelachvili-Mitchell-Ninham generalization [55]. At intermediate pH values, both forms of fatty acids are significantly present, so that hydrogen bonding can occur between the carboxylic form and the carboxylate ($R - \text{COO}^- \dots \text{HOOC}-R$). The pK_a is within this pH range. The average head group area decreases when compared to fully deprotonated molecules, and fatty acids self-assemble as bilayers (and therefore as vesicles). It has been proposed that a dynamic network of hydrogen bonds characterizes fatty acid membrane surface at these intermediate pH values [117]. Note that when compared to short-chain carboxylic acids, the pK_a of fatty acids in their associated form is ca. 3 units higher. This is because the carboxylate charges that originate after deprotonation are near each other in the membrane, making difficult the extraction of further protons.

6.4.1.2 Biphasic System

Oleic anhydride is not soluble in water. When oleic anhydride is stratified over a pH 8.5–9.0 buffer, basic hydrolysis occurs at the macroscopic interface between the oleic anhydride phase (or droplets if the system is dispersed) and the aqueous phase. The number of anhydride molecules hydrolyzed in this way is very small and the hydrolysis occurs very slowly. If oleic acid/oleate vesicles are instead present in the aqueous phase, the oleic anhydride molecules are taken up by the vesicles, which solubilize the anhydride in the membrane (Fig. 6.9a). Anhydride molecules are then easily and rapidly hydrolyzed to form fatty acids, the vesicles' building blocks. The net result is the accretion of the 'parent' vesicles due to in situ synthesis of their membrane components, reaching an unstable state, then split to give rise to 'daughter' vesicles.

Such an approach has been studied in a number of cases [35, 118], which also include intra-vesicle reactions (see Sect. 6.4.3).

6.4.1.3 Homogeneous Systems

At high pH, fatty acids in aqueous solution form micelles. Micelles, in contrary to vesicles, as very small spherical assemblies (the diameter of a small oleate micelles can be estimated to be 3.5–4 nm, the sum of the length of two extended

oleate molecules) (for a discussion on the co-existence of vesicles and micelles, see [119]). Due to the small micelle size, a micelle solution is transparent. Micelles are dynamic systems, whose fatty acid (FA) components are in rapid equilibrium with the monomer form in solution, $FA_n \rightleftharpoons nFA$.

Most experiments have been carried out with the oleic acid/oleate system. When a small aliquot of oleate micelles (high pH) are added to a pH 8.5 buffer (e.g., bicine or borate buffer), the protonation of oleate molecules brings about a structural rearrangement of the micelles which transform into bilayer and then into vesicles. The whole process takes several minutes to occur, as evidenced by turbidity measurements. If, however, pre-existing oleic acid/oleate vesicles are present in the buffered solution, an alternative (and faster) path is available. Pre-existing vesicles uptake oleate molecules from the micelles (or even include the micelles) in their membrane. The membrane area increases due to the insertion of new oleic acid/oleate molecules (Fig. 6.9b). Consequently, the vesicle grow (plausibly in non-spherical way [120]), reach an unstable state, and divide into two daughter vesicles. Many studies have been carried out on this system, especially with oleic acid/oleate

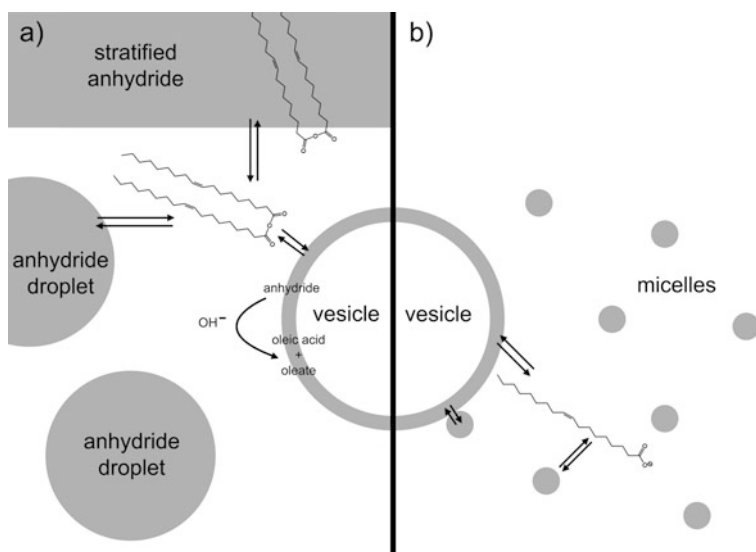


Fig. 6.9 Two different feeding methods for achieving fatty acid vesicles growth and division (cf. Fig. 6.8). **(a)** Oleic acid/oleate vesicles are added to a solution in the presence of oleic anhydride. The anhydride is stratified over the aqueous phase and dispersed in form of oil-in-water droplets under mechanical agitation. Anhydride molecules are taken up by the vesicles, which solubilize the anhydride molecules in their bilayer, where it is hydrolyzed by hydroxy ions to give oleic acid and oleate – the membrane-forming compound. In this way, the parent vesicle increases its membrane area, reach an unstable state (not shown) and divide in two or more ‘daughter’ vesicles (not shown). **(b)** Oleic anhydride can be substituted by oleate micelles, which are added, in a small volume, to an oleic acid/oleate vesicle suspension. Oleate micelles deliver oleate molecules to the vesicle bilayer either via the monomeric form, either after vesicle/micelle collision. As in the case **(a)**, the vesicle growth and division are not shown

LUVs, which has the advantage of being homogeneous (in contrary to the anhydride method, which occurs in a two-phases system), and therefore it can be studied by spectroscopic methods [37, 39–41, 121]. In addition to the increased rate of micelle-to-vesicle transformation due to the uptake-growth-division mechanism, it results that the size distribution of parent and daughter vesicles are approximately similar. This has been called ‘matrix effect’ [36–39]. A possible vesicle intermediate has been visualized by cyro-transmission electronmicroscopy [120]. Moreover, it has been recently extended to oleic acid/oleate GVs. In the latter case, direct observation by light microscopy has shown that GVs uptake oleate micelles, elongate to form tubular vesicles, then fragment into many new vesicles by a pearling/breakage mechanism [42, 122, 123].

6.4.2 Relevance of Vesicle Autopoietic Self-Reproduction

The above-mentioned observations have great relevance in origins-of-life scenario, because they show that the proliferation of cell-like structures is possible also in absence of the complex macromolecular machineries that characterize modern cell growth-division. The only requirement is that adequate precursors are available in the environment of the protocells. Moreover it demonstrates that a quite complex chemical system, hold together solely by non-covalent interactions, can behave in coordinate manner displaying some of the features of biological systems. As we will see in the next section, if a chemical reaction is occurring inside the self-reproducing vesicles, this corresponds to a minimal model of cell. One of the still missing goal is the substitution of the *externally-added* lipids with the *internally-produced* ones. A first attempt was done in 1991, with the incorporation of four lipid-producing enzymes in phosphatidylcholine liposomes [97]. More recent studies have followed this idea [124, 125] but the desired pattern has not been observed yet (mainly because the limited amount of newly-produced lipids). Indeed, the realization of compartmentalized reactions inside self-reproducing vesicles is the closest way for modeling primitive cells.

It is important to emphasize that the vesicle self-reproduction has been discovered by using fatty acids. The ‘substrate’ vesicles have been fatty acid LUVs or GVs, or phosphatidylcholine LUVs [39] (for a study on phosphatidylcholine GVs and fatty acid micelles, see [126]). Studies on other chemical systems have been also reported. In one case, an *ad hoc* designed artificial surfactant was shown to display GVs growth and division [127, 128]. In another case, lipids have been generated by chemical ligation [129] (an interesting case even if self-reproduction was not observed). Whereas fatty acids are prominent candidates for the first membrane-forming compounds [130], terpenoids, which can also have primitive origin [22], have been investigated much less with respect to the dynamical properties of their assemblies. The Murchison meteorite, in addition, also contains a suite of alkyl dicarboxylic acids up to 18 carbons [131, 132], whose self-assembly properties are not well known (C. Thomas and P. L. Luisi, unpublished results).

6.4.3 Reactions and Other Patterns

The second issue for the construction of primitive cellular models from the bottom-up is the realization of reactions inside vesicles. Most of work is done with enzyme-based pathways, which model primitive pathways. The latter are difficult to identify and carry out. Examples of these allegedly primitive pathways are the formose reaction (producing sugars from formaldehyde in basic conditions [133]), or the various enzyme-free paths for oligonucleotide synthesis from activated mononucleotides, or primitive condensation reactions to form oligopeptides, and so on. Enzyme-based pathways, on the other hand, are easy to implement as purified enzymes are available from several sources, or house-made, or synthesized *in situ* by cell-free protein synthesis.

The first two studies (1994/1995) of compartmentalized reactions deserve special mention, as the reactions occurred inside self-reproducing fatty acid vesicles. Both systems focused on the production of nucleic acids inside oleic acid/oleate LUVs: (1) the so-called Oparin reaction (oligomerization of ADP to give poly(A), under catalysis of polynucleotide phosphorylase (PNPase)); and (2) the RNA replication catalyzed by $Q\beta$ -replicase. These two systems are sketched in Fig. 6.10. In the first case, ADP was added to PNPase-containing vesicles. ADP permeates into the vesicles and is polymerized inside the aqueous lumen, with the production of inorganic phosphate [35, 134]. In the second case, a (+)-strand RNA template, nucleotides triphosphate and $Q\beta$ -replicase have being co-entrapped inside vesicles, with the result of producing the complementary RNA (-)-strand [118]. These reactions were simultaneous to vesicle growth and division, so that a simplified model of RNA-producing primitive cell was realized.

Next (1995–1999), the polymerase chain reaction was carried out in phosphatidylcholine LUVs (non self-reproducing) [135] (but see also [136, 137]) and

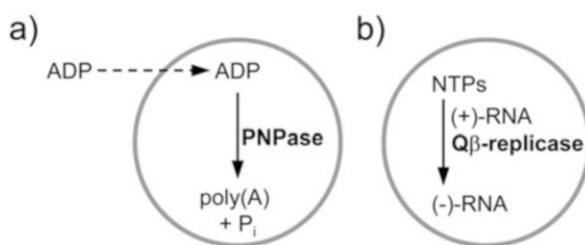


Fig. 6.10 Reactions inside vesicles. (a) The Oparin reaction consists in the oligomerization/polymerization of ADP operated by PNPase. The reaction is interesting from the viewpoint of origins-of-life because it produce a RNA molecule without a template. The Oparin reaction was carried out in DMPC vesicles [134] or in oleic acid/oleate vesicles [35]. In both cases ADP was added externally and ADP firstly diffuses from the environment to the vesicle core. (b) $Q\beta$ -replicase is another interesting enzyme as it is a RNA-dependent RNA polymerase, capable of replicating RNA without need of DNA. All components required for the reaction were co-entrapped inside oleic acid/oleate vesicles [118]. Note that in works [35, 118] autopoietic vesicle self-reproduction occurred simultaneously to internalized reactions

few years later the first production of a long peptide – poly(Phe) – by translation was similarly observed [138]. In particular, the latter example signifies a transition towards an approach that later was recognized as typical of bottom-up synthetic biology (i.e., the synthesis of proteins in the vesicle lumen). This promising and flourishing topic will be specifically discussed in Sect. 6.5. Note, however, that protein synthesis inside fatty acid vesicles has not been reported yet.

A number of studies have been carried out on reactivity inside fatty acid vesicles, in particular for showing the oligomerization of activated nucleotides in the vesicle lumen [44], to the function of encapsulated ribozymes [46, 139], and to face the Mg^{2+} -induced destabilization of fatty acids [54, 88, 140]. Of particular relevance are those studies where ribozymes are encapsulated inside fatty acid vesicles because these systems model quite closely the primitive cell-like structures that played a role in the RNA-world hypothesis [141]. On the other hand, the intra-vesicle synthesis of short peptides, catalyzed by Ser-His [142], has been also reported, and because the reaction product migrates to the vesicle membrane, a mechanism of competition among vesicles emerges [87].

In conclusion, a number of reactions have been reported as occurring inside fatty acid vesicles. However, due to the relatively high solubility of fatty acids (it depends on the chain length), when compared with double-chain phospholipids, they can potentially interfere with the reactions. The field of micro-compartmentalized reaction is certainly richer in examples when phospholipid vesicles (liposomes) are used. We should comment, however, on the necessity of developing experimental cases based on primitive membranes, like fatty acids, terpenoids, or mixtures of different surfactants in order to test hypotheses on primordial compartmentalization and robustness of early chemical pathways.

6.5 Semi-synthetic Minimal Cells

The point of junction between the synthetic approach typical of origins-of-life and the advancing synthetic biology (SB) derives from a common program which focuses on the synthesis of proteins inside vesicles. In origins-of-life perspective, this is a key step that would allow the construction, from a bottom-up approach, of primitive cell models displaying functions for the realization of an autopoietic cell. In synthetic biology, cell-free protein synthesis (CFPS) represents a tool for engineering man-made devices, such as functional synthetic cells for a variety of applications (understanding biochemical paths, for biosensing, as proof-of-concept, etc., up to nanomedicine vehicles).

Such goals can be reached by constructing the so-called ‘*semi-synthetic minimal cells*’ (Fig. 6.11), that can be defined as those cell-like structures built by co-encapsulation of the minimal number of biochemicals (DNA, RNA, enzymes, ...) inside lipid vesicles, in order to achieve a certain function (ultimately, being alive). Here, for brevity, semi-synthetic minimal cells will be referred simply as synthetic cells.

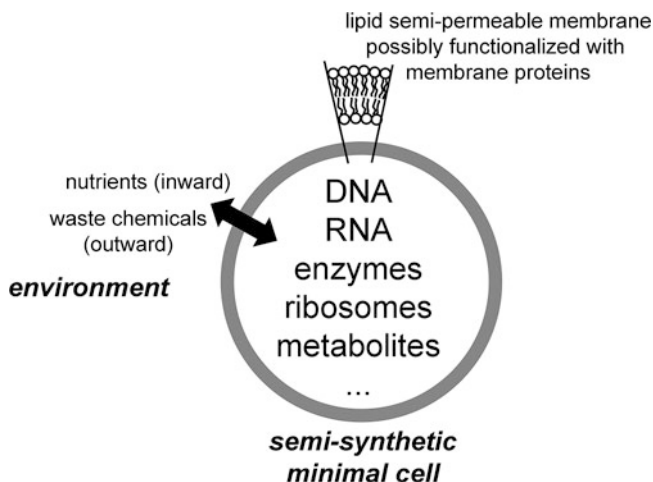


Fig. 6.11 Semi-synthetic minimal cells. The minimal number of genes, enzymes, RNAs, and low molecular-weight compounds are encapsulated into synthetic lipid vesicles. The membrane acts as a boundary to confine the interacting internalized molecules, and thanks to its semi-permeability allows the material exchange between the semi-synthetic minimal cell and its environment (nutrients uptake, waste release). More elaborated model include membrane proteins for transportation, sensing, catalysis

The bottom-up constructive approach perfectly fits with SB philosophy based on the concept of standard biological parts (<http://parts.igem.org>), of ‘biobricks’ and on the idea of letting a structure/function emerge as the result of molecular parts interaction in the form of a molecular system. Moreover, it is well represented by the concepts of orthogonality, modularity, programmability that are typical of SB. However, subtle differences in the epistemology of the research on assembling synthetic cells under the bottom-up SB view and origins-of-life perspectives have been revealed [143].

The bottom-up construction of synthetic ‘cells’ – not necessarily alive – for practical application is an essentially unexplored field where most of concepts, techniques, usages, advantages and limitations have to be discovered. Up to now, such kind of research has been essentially done by scholars more interested in basic science, and especially in using bottom-up synthetic cells as cellular models in search of biochemical/biophysical understanding. Most of researchers working in traditional fields of biochemistry, molecular biology, physiology, biotechnology, and so on can express, especially if working in multidisciplinary teams, a great potential to fully exploit these structures.

The intersection between SB and the practice of assembling synthetic cells has been discussed elsewhere, in most of its conceptual and practical aspects [11, 17, 144, 145]. Here we would like to recall only two of these facets. The

first is the so-called *minimal genome*. The minimal genome can be defined as the minimal set of genes that encode the proteins (enzymes) capable of supporting autopoietic cellular self-maintenance under permissive conditions. By permissive conditions we mean that most of the low molecular weight molecules required for cell metabolism are available in the cell environment and do not require the corresponding intracellular synthetic step. Comparative genomics have revealed that, based on the smallest prokaryotes a minimal gene set could include about 200–300 genes (reviewed in [11]). A study focused on the endosymbiont *Buchnera* set the figure to 206 genes [146], most of which (~50%) are devoted to transcription-translation (TX-TL). As it will become clear in the next section, the goal of creating a bottom-up synthetic cell by including 206 genes is currently beyond the actual possibility. Moreover, this is not the central point in contemporary research, which is instead focused at understanding the interplay between microcompartmentalized reactions and the compartment feature, their interplay in terms of physics and chemistry [17, 85]. The second facet is a technical one, and refers to the development of *microfluidics* for the assembly of synthetic cells – a process that is currently just a potential one, but it can quickly become a realistic procedure. As we have stressed in an early report [144], microfluidic fabrication of solute-filled vesicles is a goal that pave the way to fully programmable systems (Fig. 6.12). Microfluidic devices would reduce both the size heterogeneity and the wide solute occupancy distribution that typically characterize spontaneous vesiculation. By reducing these two stochastic phenomena one achieves vesicle populations (cell models) with uniform and programmable features, that can be very advantageous for practical applications (note that, in contrary, deciphering and exploiting stochastic phenomena is a key value for primitive cell modeling). As mentioned in Sect. 6.2, vesicle-producing microfluidic devices have been increasingly developed in the past years [64–71].

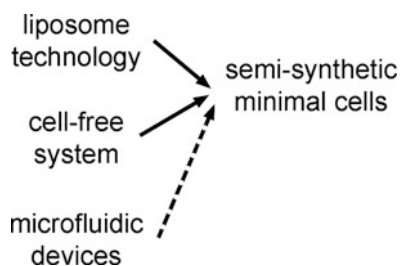


Fig. 6.12 Operative aspects of semi-synthetic minimal cell construction. The techniques currently involved for the production of minimal cells, mainly based on cell-free systems and liposome technology can be possibly improved by the future use of microfluidic devices. Some reports pointing to this direction have been already published

6.5.1 Protein Synthesis Inside Vesicles

As it has been mentioned, the core of current synthetic cell research is the CFPS inside lipid vesicles. After the pioneer report on ribosomal poly(U) translation inside phosphatidylcholine LUVs [138], in 2001 the Yomo group published the first example of a functional protein synthesis, the green fluorescent protein (GFP) in a heterogeneous vesicle population prepared by the natural swelling method [147]. This paper was followed by a 2002 short note on the enhanced-GFP (eGFP) synthesis inside liposomes prepared by the ethanol injection method [148]. From that moment the number of reports on CFPS inside liposomes increased constantly, and a certain number of proteins have been successfully produced in their correct fold, as witnessed by their functionality (reviewed in [144]).

CFPS micro-compartmentalized reactions occurs after co-encapsulating all components (~80) of CFPS ‘kits’, and an encoding DNA (or RNA) sequence, inside vesicle (Fig. 6.13). Typically a DNA plasmid is mixed with a CFPS kit, kept at low temperature to prevent the reaction start, vesicles are formed, and membrane-impermeable killers of the reaction are added externally in order to block the reaction outside vesicles (protease or RNase are often used). The reaction is often started just by increasing the temperature. Therefore, protein synthesis inside vesicles is a combination of liposome technology and CFPS methods. Lipids and CFPS kits must be chemically compatible, capable of forming good vesicles, and matching with the needs of the synthesized protein (think to membrane enzymes). A practical example of how these three constraints have been overcome can be found in [124].

The approach proposed by Noieraux and Libchaber (2004) focused on the expression of α -hemolysin (α HL) in GVs [61]. Thanks to the pore generated by the self-assembly of α HL in the vesicle membrane, it was possible to ‘feed’ the vesicle bioreactor for 4 days, as the energy-rich compounds were added to vesicles and permeate in the vesicles core *via* the α HL pore. Notably, the pore also allowed the release of by-products from the vesicles. After this report, α HL has been often used for this aim.

A quite interesting CFPS kit for the bottom-up SB approach is the PURE system (see the grey-box and Table 6.3).

The PURE system (Protein synthesis Using Recombinant Elements) is a partially recombinant, cell-free, protein-synthesis system reconstituted solely from those essential elements of the *Escherichia coli* translation system [149, 150]. As shown in Table 6.3, it is composed of 36 individually purified His-tagged proteins, purified ribosomes, and tRNAs mix (overall, 83 macromolecular components). It can be considered as a standard chassis for synthetic biology. The PURE system performs CFPS by combining T7-RNA-

(continued)

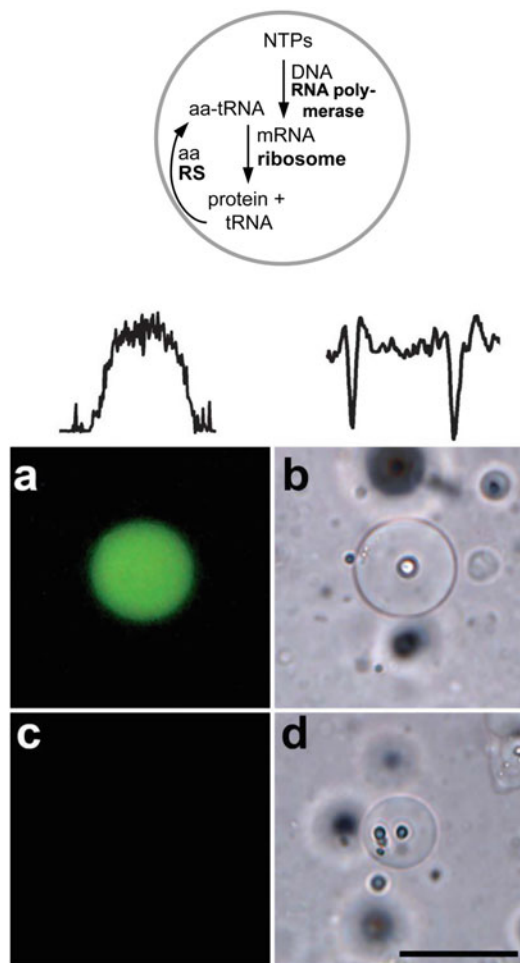


Fig. 6.13 Protein synthesis inside lipid vesicles. *Top*: Reaction scheme. NTPs are polymerized to give mRNA using a DNA template and RNA polymerase. The resulting mRNA is a template for the protein synthesis, fuelled by aa-tRNAs. tRNA are re-charged by aminoacyl-tRNA synthetases (RS) and amino acids. These three modules consume energy (ATP, GTP), which is re-formed inside the vesicle by a fourth module (not shown) at expenses of creatine phosphate. *Bottom*: eGFP synthesis in GV's produced by the droplet transfer method [144]. Panels (a,b): cell-free extracts plus DNA; panels (c,d): cell-free extracts without DNA (negative control); panels (a,c): fluorescence imaging; panels (b,d): bright-field imaging. *On the top*, equatorial profile of micrographs' pixel luminosity (panels a,b). Size bar represents 50 μm (The bottom part (panels a), (b), (c), (d) have been reproduced from Ref. [144] with permission from The Royal Society of Chemistry)

polymerase transcription and *E. coli* translation. With respect to traditional CFPS kits, the PURE system has a lower yield (ca. one third [151]), but its usage adds to the synthetic cell research from the viewpoint of the potential of a full design and modularity. Indeed, one can imagine of modifying the PURE system composition at will, or substitute some of its components with others, and so on.

Table 6.3 Composition of the PURE system [150]

Component	Concentration	Component	Concentration
IF1	2.70 μ M	IF2	0.40 μ M
IF3	3.50 μ M	EF-G	0.26 μ M
EF-Tu	0.92 μ M	EF-Ts	0.96 μ M
RF1	0.25 μ M	RF2	0.24 μ M
RF3	0.17 μ M	RRF	0.50 μ M
AlaRS	1900 U/mL	ArgRS	2500 U/mL
AsnRS	20 mg/mL	AspRS	2500 U/mL
CysRS	630 U/mL	GlnRS	1300 U/mL
GluRS	1900 U/mL	GlyRS	500 U/mL
HisRS	630 U/mL	IleRS	2500 U/mL
LeuRS	3800 U/mL	LysRS	3800 U/mL
MetRS	6300 U/mL	PheRS	1300 U/mL
ProRS	1300 U/mL	SerRS	1900 U/mL
ThrRS	1300 U/mL	TrpRS	630 U/mL
TysRS	630 U/mL	ValRS	3100 U/mL
MTF	4500 U/mL	Ribosomes	1.2 μ M
Creatine kinase	4 μ g/mL	Myokinase	3 μ g/mL
NDP kinase	1.1 μ g/mL	Pyrophosphatase	2.0 U/mL
T7 RNA polymerase	10 μ g/mL	Creatine phosphate	20 mM
ATP, GTP	2.0 mM	CTP, UTP	1 mM
HEPES-KOH (pH 7.6)	50 mM	Potassium glutamate	100 mM
Magnesium acetate	13 mM	Spermidine	2.0 mM
DTT	1.0 mM	10 amino acids	0.3 mM
tRNA mix	56 A260/mL	10-formyl-5,6,7,8-tetrahydrofolic acid	10 mg/mL

The research on protein synthesis inside liposomes greatly advances. A number of proteins have been produced inside vesicles in addition to GFP (or other fluorescent proteins or β -galactosidase and β -glucuronidase as reporter proteins [152, 153]). For example, T7 RNA polymerase to realize a two-steps genetic cascade [154], α HL to create a pore in the membrane [61], lipid-synthesizing enzymes [124, 125], Q β -replicase in order to replicate RNA [152]. More recent applications

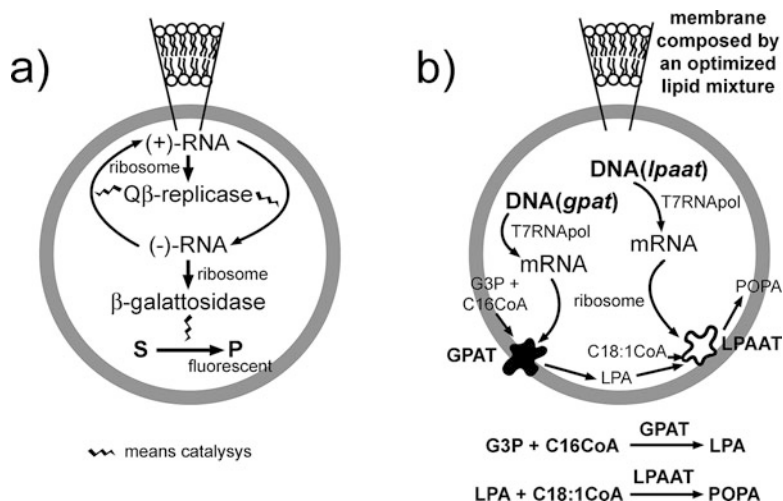


Fig. 6.14 Two remarkable examples of semi-synthetic minimal cell construction. (a) Starting from $Q\beta$ -replicase-encoding (+)-RNA strand and cell-free protein expression system, the production of $Q\beta$ -replicase was carried out inside liposomes, so that the complementary (–)-RNA strand is produced from nucleotides and (+)-RNA template. In turn, (–)-RNA acts as a template for the $Q\beta$ -replicase catalyzed (+)-RNA strand synthesis. The correct production of (–)-RNA is confirmed by the fact that it encodes for β -galactosidase (that successfully catalyzes the formation of a fluorogenic substrate [152]). (b) The two genes encoding for glycerol-3-phosphate acyltransferase (GPAT) and lysophosphatidic acid acyltransferase (LPAAT) need to be co-encapsulated inside lipid vesicles, together with a CFPS kit (e.g., the PURE system). Actually, the two proteins require different redox conditions, so that they were actually synthesized in two different vesicle populations. In two steps, these membrane enzymes produce phosphatidic acid starting from glycerol-3-phosphate and an acyl donor. Note that the vesicle membrane composition is a key factor for realizing a functional system POPC 50.8%, POPE 35.6%, POPG 11.5%, CL 2.1% (POPC 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine, POPE 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylethanolamine, POPG 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylglycerol, CL cardiolipin) [124]

refer to: σ^{70} transcription factor (in order to realize a two-stage genetic cascade) [155]; MreB (bacterial cytoskeleton filaments) [156]; EmrE (a transporter protein) [157, 158]; BmOR1 and BmOrco (olfactory receptors and co-receptors) [159]; Sec translocon (mediator of membrane translocation of single- and multi-span membrane proteins) [160].

For example, Yomo and collaborators [152] assembled a self-encoding replicase system as it follows (Fig. 6.14a). Messenger RNA encoding the RNA-dependent RNA replicase ($Q\beta$ -replicase), was encapsulated inside liposomes together with essential transcription-translation components. The $Q\beta$ -replicase coding sequence was successfully translated and the resultant $Q\beta$ -replicase enzyme was functional. It therefore replicated the RNA gene, producing its complementary strand. In turn, the complementary RNA strand also encoded for β -galactosidase, whose successful translation was detected by measuring the conversion of fluorogenic substrate.

Another instance comes from the attempts of synthesizing phospholipids inside lipid vesicles (Fig. 6.14b). In particular, starting from glycerol-3-phosphate it is in principle possible to synthesize phosphatidic acid by two successive acylation steps (using acyl-CoA as acyl donor). These two reactions are catalyzed by the integral membrane enzyme G3PAT [2.3.1.15] and by the membrane associated enzyme LPAAT [2.3.1.51]. Both enzymes can be synthesized inside liposomes by CFPS (PURE system). Due to some issues about redox conditions the enzymes were produced in two different liposome population to carry out phosphatidic acid synthesis. A more recent version of this approach led to a significant understanding, in several details, of such an approach [125].

Why protein synthesis? As it can be understood by the flourishing research on CFPS inside liposomes, dominating micro-compartmentalized protein synthesis is an essential conceptual and practical goal for the bottom-up assembly of cell-like particles, either intended as primitive cell models or synthetic cells for SB/biotechnology. It is a conceptual advancement because, as we have specified, about 50% of the minimal genome refers to protein synthesis. Currently, TX-TL components, purified from biological organisms, are inserted in liposomes, whereas only a handful of genes is expressed. In future, one can imagine of realizing a PURE system-producing synthetic cells, where all (~80) PURE system components are synthesized *in situ* starting from the corresponding DNA sequences and PURE system. This recursive logic is typical of biological systems. On the other hand, CFPS is important from practical reasons as it paves the way to synthetic cell functionalization with pores, receptor, enzymes, transducers, cytoskeletal elements, and so on. All this will allow a stepwise increase of complexity and a better understanding of biochemical systems by their full reconstitution.

6.5.2 Toward More Complex Functionalization

Some of the most interesting scenarios can be summarized by looking at the contemporary trends, reports, and to the open questions [161].

1. MVV (vesosomes, see Fig. 6.2) are interesting structures consisting of small vesicles contained inside a larger one. This structure is interesting because it models a cell with subcellular organelles. Some reports have shown how to produce such structure, but their use in synthetic cell research, to the best of our knowledge, has not been reported [162–164].
2. The production of biochemical energy (e.g., ATP) is another open issue. A possible way is the conversion of light energy in the form of a chemical gradient (pH gradient), then exploiting such a gradient for fostering ATP synthesis via ATP synthase. Photosynthetic proteins (or bacteriorhodopsin) are membrane proteins, as ATP synthase. Clearly, the synthesis (or the reconstitution) of correctly folded and correctly oriented membrane proteins inside liposomes is

thus very important. We have been recently involved in the reconstitution of the photosynthetic reaction centre in phospholipid GV's [165].

3. Movement is a property that is not strictly necessary for the definition of living beings, but it is intriguing the construction of a synthetic cell capable of autonomous motility. Similarly, cytoskeletal elements, division elements (such as the bacterial Z-ring), and other similar proteins may be accessible targets for the bottom-up approach.
4. From one to many. Most of current research focuses on individual cells, but it is well known that even the simplest organisms often live in community. An attempt to investigate the cooperation aspects of primitive cells has been done by associating GVs in the form of colonies, on a flat support [59]. Intriguingly, the colonies displayed several emerging properties that are not found in individual vesicles. Other approaches based on molecular recognition of DNA-decorated vesicles have been presented [166]. In some cases, GFP was produced in the associated vesicles [167]. Note that such assemblies can be used as tissue models.
5. Mimicking inter-cellular communication is probably one of the most fascinating goals (Fig. 6.15). Inspired by a hypothesis paper on the application of Turing test to artificial cells [168], a first paper appeared in 2009 on liposomes sending a signal molecule to bacteria [133], but the generation of the signal molecule was not under genetic control. The idea was further elaborated within the realm of bottom-up SB approaches (the combination of CFPS and liposome technology), and an experimental plan for the implementation of synthetic-to-synthetic and synthetic-to-natural communication (and vice versa) was devised by Stanó and Rampioni [18] (for other approaches see [169–171]). It was realized also that such an approach would bring about minimal cells as tools for the emerging field of bio-chem information and communication technologies (bio-chem ICTs [172, 173]). From then, important papers appeared on experimental realization of this approach [174–178], that eventually goes in the direction described in the next final point.

6.5.2.1 An Intriguing Perspective: Minimal Cognition in the Chemical Domain

The reference here is to the so-called “embodied approach” to the study of cognition [179], which aims at overcoming the mind-body dichotomy by bringing into focus the fundamental role(s) the biological body plays in cognition. Despite this consideration mainly refers to a high-level cognition, its extrapolation to the minimal terms of unicellular systems can be interesting and perhaps fruitful. Synthetic cell research contributes to the embodied approach to (minimal) cognition thanks to the approaches originally introduced as proto-cybernetics, which are coherent with those explained above (understanding-by-building, constructive/synthetic approach, bottom-up). In particular the construction of artificial systems as models of cognitive and biological processes serves to test and develop scientific theories about the

mechanism underlying the natural processes. This is common to all sciences that study natural processes through artificial models [180], and include software, hardware, and – as a result of origins-of-life/SB bottom-up approaches – also *wetware* models (synthetic cells) [102].

We have seen above that primitive cell models have been built under the paradigm of autopoietic theory [108, 109]. However, according to the proponents of autopoiesis, minimal autopoietic systems are also minimal cognitive systems, by actively maintaining a dynamical coupling with their environment [181], i.e., being capable of perceiving external events and actively reacting to them in a conservative way (generation of an internal meaning to external perturbations).

This theoretical perspective offers to synthetic cell research the possibility of playing a role in the *avant-gard* of artificial intelligence, in form of embodied artificial intelligence. The synthetic cells, in fact, do not process external stimuli under a representational scheme, but being guided by the physico-chemical constraints referring to their intra-cellular dynamical/functional inter- and supra-molecular relationships (embodiment). This new frontier is dense of possible developments [182].

6.6 Concluding Remarks

No questions are more fascinating and difficult to face than unveiling the mechanisms that led to the origin of life on Earth more than 3.5 billions years ago. The scientific search for the physico-chemical basis of such an important event – which *is* within the realm of experimental study, started less than 100 years ago, and it

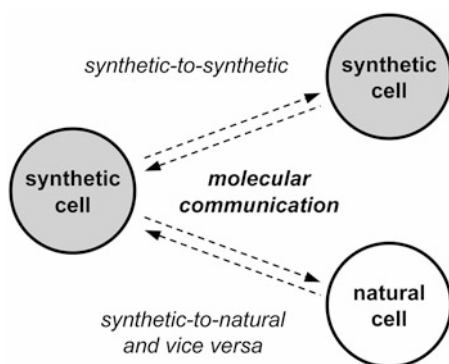


Fig. 6.15 Molecular communication between synthetic cells and between synthetic and natural cells [18]. By a proper design it is possible to engineer synthetic cells for being capable of communicating with other synthetic cells or with biological cells via chemical signaling. This approaches paves the way to nanomedicine – imagine for example an ‘intelligent’ drug delivery vehicle consisting of a minimal cells capable of interacting with biological cells in an organism and synthesize a drug in situ only when necessary

is continuously progressing thanks to technical and conceptual advancements. An important branch of origins-of-life investigations is dedicated to understanding the formation, the structure, the function of primitive cells, filling the gap between pure prebiotic chemistry (the chemistry of molecules) and the last universal common ancestor (LUCA). Is this the realm of the chemistry of molecular systems (*systems chemistry*), where ensembles of molecules behaves and should be considered as a whole.

Cell models based on vesicles are central to this arena, and it is significant that the two faces of this approach look at primitive times, but simultaneously to modern synthetic biology. In this chapter we have presented both directions evidencing the common grounding despite the apparent differences. We are convinced that future developments of these fields will contribute to a *qualitative* jump destined to modify scientific and technological knowledge.

Hopefully, together with enforcing a new and fruitful wave in synthetic biology, cell models will finally demonstrate that living systems emerge from non-living matter without additional requirements. Or, with the words of Eschenmoser and Kisakürek [183]:

The aim of an experimental aetiological chemistry is not primarily to delineate the pathway along which our ('natural') life on earth *could* have originated, but to provide decisive experimental evidence, through the realization of model systems ('artificial chemical life') that life *can* arise as a result of the organization of the organic matter.

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