From chemical autopoiesis to synthetic biology

Abstract In the early 1990s pioneer experiments on chemical autopoiesis (self-production) led, on one hand, to the discovery of lipidic micro-compartments and their dynamics as useful models for origins-of-life research, and on the other hand, to the adoption of a systemic perspective in experimental research on minimal living cells. Moreover, the underlying idea of constructing cell models by assembling chemical components (the constructive, or synthetic, approach) has provided an operational field now recognized as bottom-up synthetic biology. This article discusses the origin of chemical autopoiesis and recapitulates the very early experiments, then presents examples of current developments that aim at assembling protocells and artificial/synthetic cells both for basic and applied science.

Keywords **Fatty acid vesicles, autopoiesis, protocells, synthetic biology, artificial cell, synthetic cell.**

Résumé **De l'autopoïèse chimique à la biologie synthétique**

Au début des années 1990, des expériences pionnières sur l'autopoïèse chimique (auto-production) ont conduit, d'une part, à la découverte de microcompartiments lipidiques et de leur dynamique comme modèles utiles dans la recherche sur les origines de la vie, et d'autre part, à l'adoption d'une perspective systémique dans la recherche expérimentale sur les cellules vivantes minimales. De plus, l'idée sous-jacente de construire des modèles cellulaires en assemblant des composants chimiques (approche constructive ou synthétique) a fourni un champ opérationnel désormais reconnu : la biologie synthétique ascendante. Cet article discute de l'origine de l'autopoïèse chimique, récapitule les toutes premières expériences, et présente quelques exemples de développements actuels qui visent à assembler des protocellules et des cellules artificielles/synthétiques pour la science fondamentale et appliquée.

Mots-clés **Vésicules d'acides gras, autopoïèse, protocellules, biologie de synthèse, cellule artificielle, cellule synthétique.**

Identifying life in a process, not in a molecule

When the origin of life is discussed, the self-replication of genetic polymers, and in particular of primitive RNA, plays a dominant role. Indeed, this fundamental mechanism is among the most relevant chemical events for explaining the proliferation of molecular sequences, including mutation and selection, and thus molecular evolution. Several factors have contributed to this prominence. At one hand, the mechanism of template-based replication is quite convincing for nucleic acids, which "store" biological information in their sequence. Think, for example, to the well-known mechanisms of DNA duplication that allows cellular proliferation in all organisms, at every level of biological complexity. On the other hand, due to the imperfect duplication, there exist a finite possibility of mutation (inserting a wrong base in the sequence), so that the template-based mechanism also paves the way to explain evolution, when combined with the concept of Darwinian selection.

Based on these considerations, it has been possible to sketch a scenario based on the early molecular evolution of self-replicating RNA populations. Moreover, the discovery of the catalytic role of RNA in the ribosome active site, and thus of the ribozymes (RNA enzymes) suggest that such populations of ancient RNAs could have prompted the relevant chemical transformations required to generate "life". RNA "handles" are still attached to several very relevant biochemical compounds ($NAD^+/NADH_2$; $FAD/FADH_2$; CoA-SH; ATP, etc.), and the centrality of RNAs in protein synthesis (messenger-ribosomal-transfer RNAs) further confirms the very important role of RNA in origins of life. The resulting "RNA-world" hypothesis grounds on these premises [1]. On the other hand, the synthesis of RNA monomers, which are composed by phosphate, ribose, and an aromatic heterocyclic compound in a very precise regio- and stereochemical arrangement, their enzyme-free polymerization in exact (and functional) sequences, as well as the chemical instability of RNA in several conditions are well-known difficulties that still need investigation and clarification.

Then the origin of life is often associated to the origin of self-replicating molecules, and in particular to RNA. In this article, however, we would like to emphasize other equally important aspects of living systems, which are not explicitly considered when life is solely identified with a self-replicating molecule, despite the elegance of the template-based mechanisms. The topic we are dealing with will allow the discussion of a set of fascinating experimental data, and it will also lead to a more general perspective on life and its essence, irrespective of its actual molecular implementation.

The starting point is the critical consideration that identifying the origin of life with the origin and the self-replication of one specific class of molecules (the genetic polymers in particular) does not account for the whole story of what life is, how it works, and how it can be originated. The emergence of complex RNA molecules is crucial, as well as the development of any other relevant metabolic networks. These scenarios, however, do not explicitly include the very key feature of all living organisms – actually a far-reaching one. This is their need of self-bounding and self-production. These two requirements are necessary, respectively, in order to separate themselves from the surroundings, and to "remain themselves" despite the turnover of their molecules. Living organisms are, first of all, objects that we can distinguish and recognize in an environment thanks to a locally different chemical

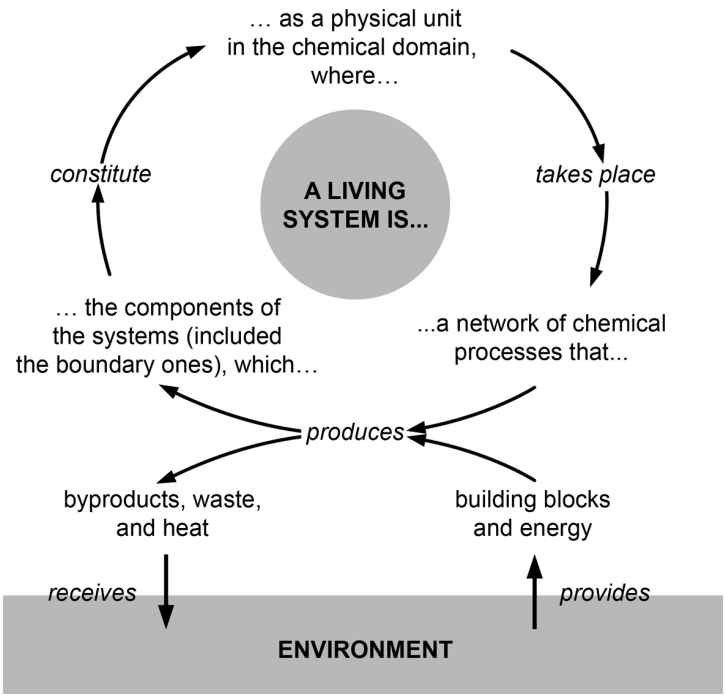

Figure 1 - **The circular logic of autopoiesis**. A living system is autopoietic. As such, it is composed by chemical components that constitute a physical unit where a network of reactions takes places, and as a consequence of those reactions, the components of the system are (re)generated, including the components of the boundary. Moreover, the reactions are fuelled by mass and energy provided from the environment. The latter receives by-products and waste. The ultimate goal of an autopoietic system is its autopoiesis (self-production), thus achieving homeostatic self-maintenance. In particular, despite the turnover of all components, the system – as a whole – maintain its identity in terms of components and relations between the processes of production of components. The autopoietic system is not isolated from the environment, rather it is structurally coupled to it.

composition. We do not identify life with in a space-distributed mixture of components (even if still functioning, like a fresh cellular homogenate). Distinction does not mean isolation. The boundary does not prevent the exchanges of energy and matter with the environment. On the other hand, these exchanges are also essential, because a living organism, despite its self-similarity in time, is not a static entity. All its components are continuously built and destroyed, in an endless molecular "*stirb und werde*".

According to these considerations, which have deep implications in considering living organisms from the viewpoint of *systemic theories* (as it will be clarified later), a living organism – a living system – is ultimately based on a network of chemical reactions that constitutes a physical unity in space (distinct from its environment), and it is limited by a boundary, also generated by the system itself. The whole system operates out-of-equilibrium, consuming energy and matter from the environment. The net result of the reactions occurring within the living systems, however, is just the production of the same molecules that constitute the system (including the boundary molecules), according to what is known as *organizational closure*. Literally, living systems construct themselves from within, and this is their goal, their ultimate function, their Aristotelian final cause. In one word, living systems are *autopoietic*.

Chemical autopoiesis

The theory of autopoiesis (self-production) was put forward in the 1970s by two Chilean biologists, Humberto Maturana and Francisco J. Varela [2], aiming at explaining the phenomenology of living systems from the viewpoint of systemic theories. They defined living systems not according to a list of properties or to the structure of their molecules (e.g., nucleic acids, proteins), but simply and generally as those systems having a particular type of organization, based on precise and peculiar relations between the chemical processes occurring in them: the autopoietic organization (*figure 1*).

The Chilean authors emphasized the need of focusing on the relational aspects of living systems' inner organization, irrespectively of the chemical nature of the components. According to the autopoietic theory, the components of living systems constitute a physical unit wherein they generate, thanks to their reciprocal interactions, a network of transformations that ultimately leads to the production of all components of the living system, at expenses of externally available precursors, and realize, in the physical space, a self-bounded system – distinct from (but coupled with) the environment. The network of an autopoietic organization is not diffused; it is localized thanks to the existence of a self-generated boundary, whose molecules belong to the autopoietic organization too.

All known life forms obey to this autopoietic dynamics, and therefore the theory of autopoiesis provides an operational description of what a living system does in order to be alive, at the level of individual cell. Autopoiesis provides also a recipe, not a blueprint, for obtaining a living organism. It tells us what a chemical network must do in order to become autopoietic, and thus generate the organizational closure typical of all living system. It should be noted, finally, that the equation "autopoiesis = life" it is still under debate, but for the sake of present discussion it is convenient to maintain this view (which is the original one). In particular, the discussion focuses on the question whether autopoiesis is a necessary and sufficient condition for life, or if it is only necessary (interested readers should refer, for example, to [3-4]).

This brief introduction to the autopoietic theory will serve us as a kick-off before describing a scientific path that started about 30 years ago with chemical autopoiesis and that has led to modern synthetic biology projects, the ones focused on the construction of "artificial/synthetic cells". While a large part of this article will deal with the first issue, in the final section we will highlight the principles behind the development of artificial cells, and show, in particular, the existing (or lost) relations with autopoiesis.

Firstly, let us go back to the end of the 1980s, when autopoiesis inspired the discovery of an important physico-chemical mechanism, namely, the self-reproduction of fatty acid vesicles. Francisco J. Varela and Pier Luigi Luisi firstly met in 1983 at a workshop in Alpbach (Austria), soon developing common interest about how to work experimentally on autopoiesis. In the words of Luisi: "[...] *I was leading an experimental research group at the ETHZ* [ETH Zürich], *working with self-organization and biopolymers, and with Francisco, we began to look for experimental systems capable of showing autopoiesis. We spent much time thinking of water structure and its flickering properties, but nothing came out of this. However, something came from my studies on reverse micelles, the small spherical structures formed by surfactants in apolar solvents and having an internal water pool where hydrophilic reactants can be incorporated, and we were able to conceive an autopoietic system based on the idea* [...]" [5]. Over the following few years, Luisi and collaborators began the study of molecular

micro-compartments such as reverse micelles, normal micelles and – importantly – vesicles (*figure 2a*), as simple models displaying autopoietic properties, focusing on the synthesis of their constituent molecules. The seminal paper, co-authored by Luisi and Varela in 1989, illustrates all foundational concepts of the newborn chemical autopoiesis [6].

This research was considered as part of the more general investigation of the relationship between the chemistry of self-organized supramolecular structures and key molecular processes of life, such as self-reproduction. The chemically important notion emerging from these early studies was that lipid micro-compartments such as micelles or vesicles, due to their (spontaneous) capability of self-segregation in form of micro-compartments, easily capture hydrophilic or hydrophobic reactants (or both). The latter assembly can give rise to reactions that, in turn, lead to autopoietic (self-producing) processes. The production of the lipids that form the reverse micelles, the normal micelles or the vesicles is a relevant example, with the very crucial consequence of obtaining a growth-division process (*figure 2b*). It is the combination of autopoiesis and growth-division that really adds to this phenomenology (which, by the way, would have been highly relevant also in absence of the division step).

Here we will shortly summarize the first results on chemical autopoiesis, published in the early 1990s, and show how they impacted on origins-of-life research and how they prompted a branch of current synthetic biology. Without going too much in technical details, the discussion will include some necessary notions about the self-assembly of fatty acids in water and in apolar solvents. Fatty acids, indeed, have a prominent role in this research, not only because they actually lead to the autopoietic growth of micelles and vesicles, but especially because such simple molecules are considered quite pertinent to origins-of-life scenarios.

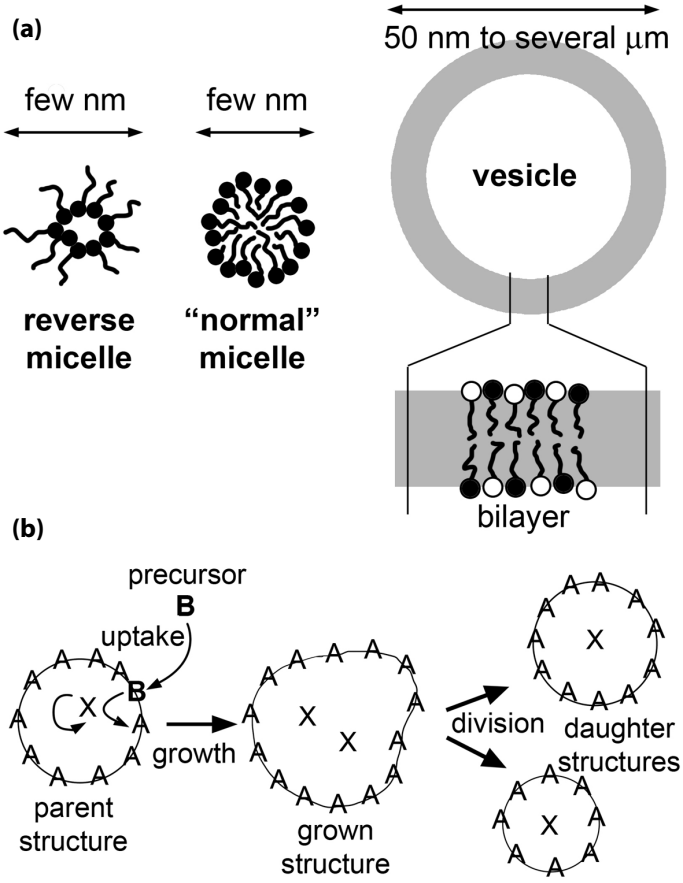

Figure 2 - **Autopoietic structures**. (**a**) The structure of reverse micelle (left), "normal micelle" (centre), vesicle (right; in particular, fatty acid vesicles). These structures form spontaneously by self-assembly processes. Reverse micelles form in apolar solvent when a tiny amount of water is added in the presence of some amphiphilic compounds. The latter self-assemble to expose their hydrophobic tails to the apolar solvent and their hydrophilic head to the water core. "Normal" micelles have the opposite geometry. Amphiphilic molecules self-assemble to exclude the hydrophobic tails from the contact with water, while exposing their hydrophilic head groups. Normal micelles, thus, form in aqueous solutions. Reverse and "normal" micelles are quite small particles (5-20 nm). In contrary, vesicles are large particles formed by a closed spherical shell of amphiphiles that self-assemble as a bilayer. The thickness of the bilayer is few nanometers (e.g., 4 nm), while the vesicle size can vary from 30-50 nm to several micrometers. In the case of fatty acid vesicles, the bilayer is actually composed by approximately equal amounts of dissociated ($R\text{-}COO^-$) and undissociated (R-COOH) molecules, that interact together by hydrogen bonds. Fatty acid vesicles form only in a limited pH range (depending from the nature of the fatty acids; oleic acid/oleate vesicles typically form at pH 8-9). (**b**) General mechanism of autopoietic growth-and-division. A microcompartment, composed of A molecules, uptakes B molecules, which are precursors of A. According to one or more reactions, made possible by X molecules, B can be transformed into A, so that the boundary-forming molecules are produced. The increase of surface leads to physical instability with the consequent division of the grown "parent" particles into two or more "daughter" particles. For a full autopoietic mechanism, X should belong to the autopoietic particle and should be also self-produced by other reactions, which require other components… and so on.

The case of reverse micelles

Fatty acids form reverse micelles in apolar solvents (e.g., in hydrocarbons), in the presence of a minute amount of water. These micelles are made by a fatty acid monolayer around a small aqueous volume. A typical quasi-spherical reverse micelle is shown in *figure 2a* (left). Let us consider the following system: reverse micelles made by sodium octanoate (the sodium salt of octanoic acid) in isooctane. The aqueous core of the reverse micelles contains permanganate ions, which are strong oxidants. If *n*-octanol is added to the system, the following dynamics takes place: *n*-octanol, because of its polar head group (...-CH_2OH), is partly partitioned with the micelle monolayer. The polar head group faces to the micelle lumen, so that an oxidation takes place, with the result of producing new octanoate molecules [7] (*figure 3*). This means that new "boundary molecules" are formed, thanks to a reaction localized in the reverse micelle. In other words, the reverse micelle produces one of its components (octanoate molecules) and thus displays a typical autopoietic reaction. Even more interestingly, as a result of the increase of octanoate concentration, the [water]/[octanoate] ratio decreases, and a competition for the octanoate molecules for water is established. It ultimately leads to a physico-chemical instability causing the splitting of the initial large "mother" reverse micelle into two (or more) small "daughter" reverse micelles (following the general scheme of *figure 2b*). Note that the actual mechanistic details of this process are unknown, but the net effect is the one described above. The process continues till the consumption of permanganate in the micelle core or the consumption of added *n*-octanol. It has been calculated that in some conditions the number of reverse micelles increases ten times.

Permanganate-containing reverse micelles utilize *n*-octanol to form more permanganate-containing reverse micelles. Even if the exact composition of the daughter reverse micelles is different from the composition of the mother ones (due to the consumption of permanganate, the presence of other reaction products, and the different [water]/[permanganate] ratio), the entire process is essentially an autopoietic self-reproduction

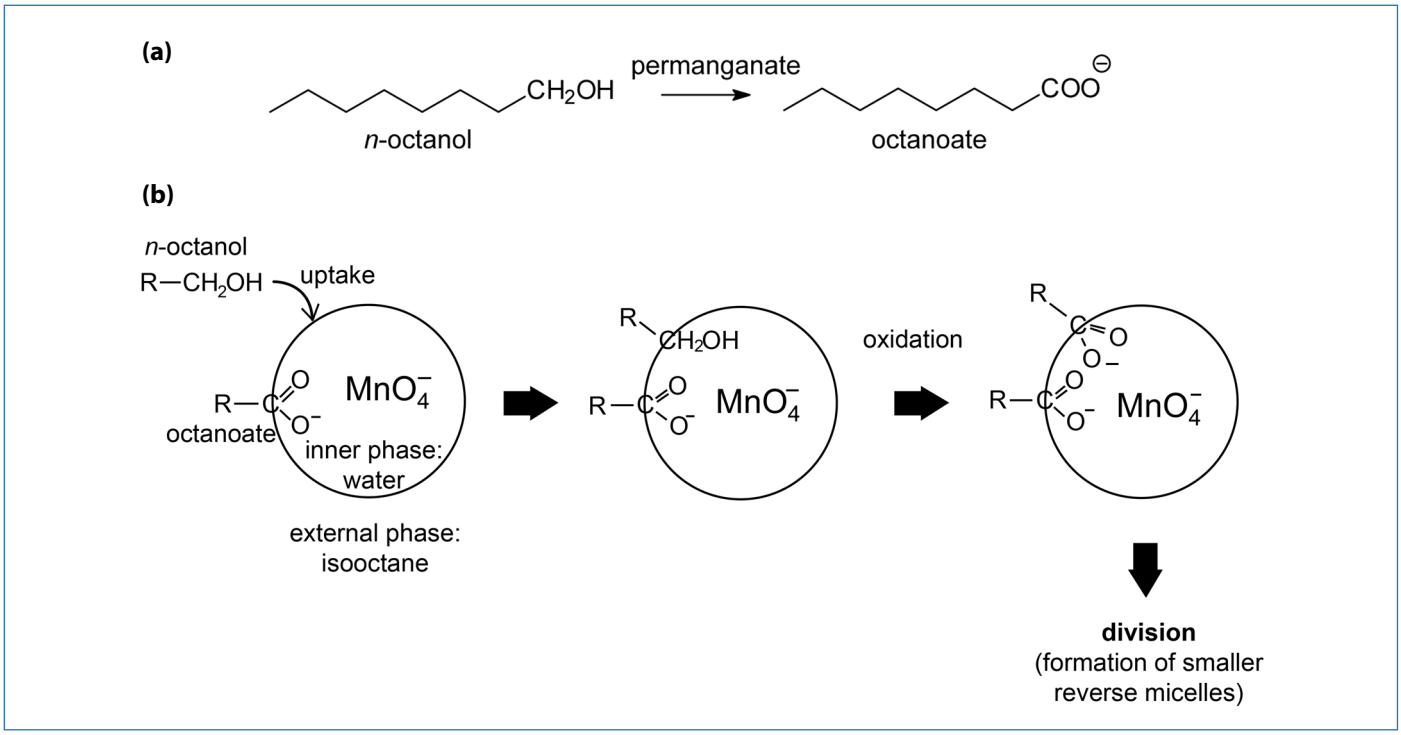

Figure 3 - **Autopoietic self-reproduction of reverse micelles**. (**a**) The chemical reaction occurring in the autopoietic mechanism. (**b**) Octanol (*n*-octanol), which is soluble in isooctane, is added to permanganate-filled reverse micelles made of octanoate. Due to its amphiphilic character, *n*-octanol is adsorbed at the water-isooctane interface and its headgroup (-CH_2OH), which faces toward the reverse micelle aqueous core, is oxidised, forming new octanoate molecules. When a sufficient number of new octanoate molecules are formed, the reverse micelle next divides forming new (smaller) reverse micelles. Note that permanganate and water are not self-produced.

of reverse micelles. Note that, strictly speaking, not all components of the reverse micelles are produced by the autopoietic mechanism, which in this case is very simple and consists just in one reaction. In particular, permanganate is going to be depleted completely after a number of growth-division.

A second relevant aspect of this fascinating chemistry is that two *per se* immiscible reagents (*n*-octanol and permanganate) are able to react with each other due to the interfacial properties of the reverse micelle. Although the self-reproduction of reverse micelles follows mechanisms and routes very different from what happens during the self-reproduction of a biological cell, and reverse micelles are not really the best model of cells, this chemical system was specifically designed to match, even partially, the autopoietic dynamics, and indeed it successfully showed the expected behavior.

The case of "normal" micelles

Fatty acids self-assemble in aqueous solution to form normal micelles (*figure 2a*, center). More precisely, it is not the fatty acid species that undergoes self-assembly, but its sodium salt, generally known as "soap". Normal micelles also undergo autopoietic growth, as reverse micelles do, but mechanistic details are different because of the different conditions of existence (normal micelles exist in aqueous solution, whereas reverse micelles exist in apolar solvents).

Octanoate micelles – this time suspended in an alkaline aqueous solution – are put in contact with a layer of ethyl octanoate, which is lighter than water and not water-soluble, so that it forms an organic layer above the micellar solution (*figure 4*). Part of the ethyl octanoate molecules is absorbed by micelles, because of the low polarity of the micellar core. The aqueous solution contains a base (OH^-) that reacts with ethyl octanoate and produce ethanol and octanoate [8]. Note that this reaction occurs both at the micellar interface and at the interface between the aqueous solution and the layer of ethyl octanoate. The net result of the first process is – again – the production of the micelle component by a reaction, at the expenses of a precursor (ethyl octanoate). In this case the reaction does not occur – strictly speaking – inside the particle undergoing autopoietic growth (as in the case of reverse micelles shown above), but on its external boundary. Due to the increase of the number of molecules constituting the "mother" micelle, the latter becomes unstable and eventually spits into "daughter" micelles (following the general scheme of *figure 2b*). Despite some differences, the overall dynamics is very similar to the previously illustrated case of reverse micelles.

Intriguingly, if ethyl octanoate is stratified over an alkaline solution in absence of pre-formed octanoate micelles, the final product is still a solution of octanoate micelles. To understand how this is possible, we should recall – as mentioned above – that ethyl octanoate hydrolysis also occurs (yet slowly) at the macroscopic interface between the layer of ethyl octanoate and the alkaline aqueous solution. The produced octanoate molecules slowly accumulate in the aqueous solution as monomer, and when it reaches a threshold concentration, the molecules self-assemble as micelles. From that moment, the resulting octanoate micelles catalyze the further consumption of ethyl octanoate, to generate more micelles, which uptake more ethyl octanoate... The entire path follows a two-phase kinetics. In the first "lag" phase (which takes several hours) octanoate molecules slowly accumulate in the water phase; in the second "burst" or "exponential" phase octanoate micelles rapidly and efficiently self-reproduce autocatalytically. Indeed, the plot of the octanoate concentration versus time looks like

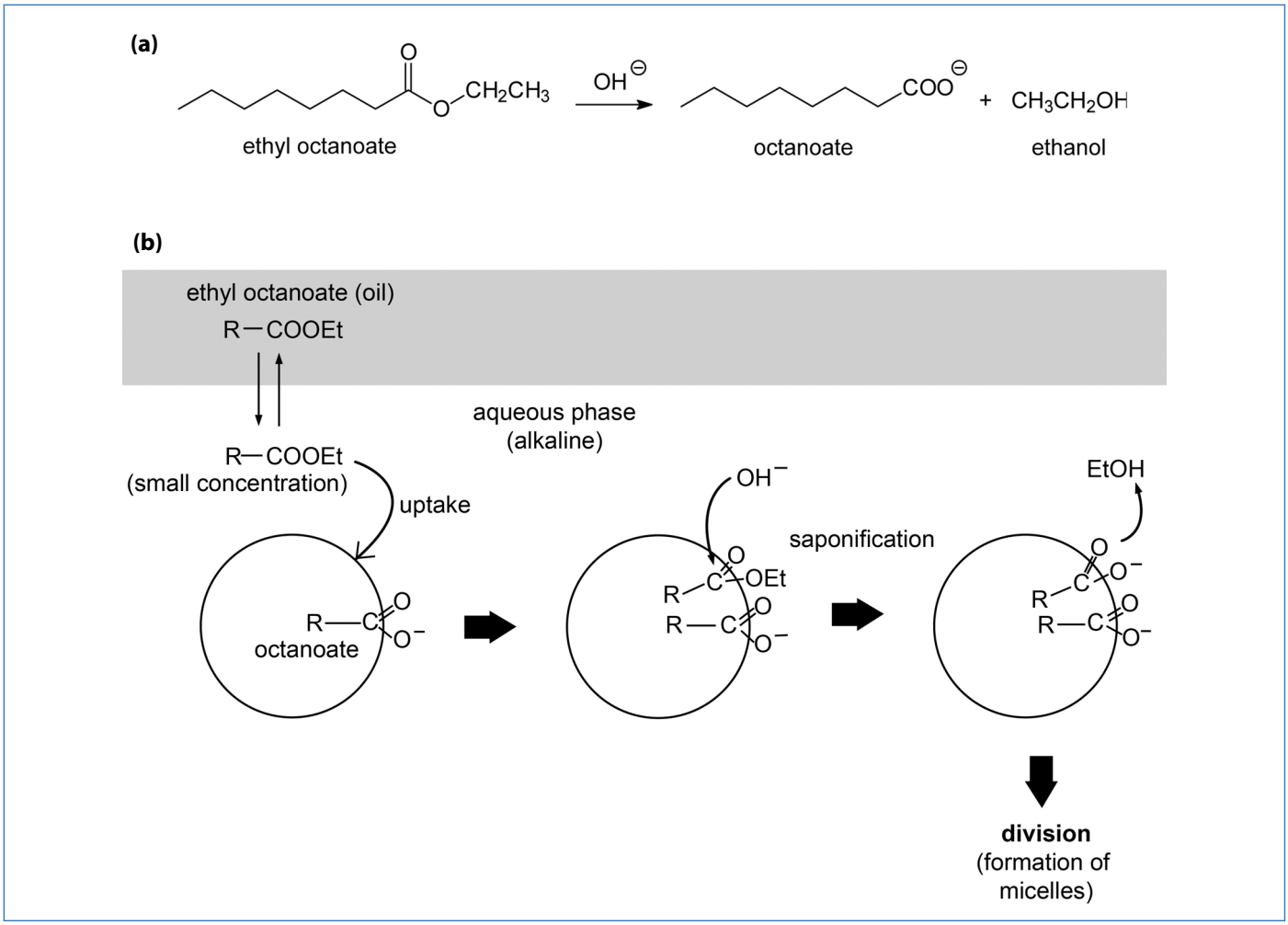

Figure 4 - **Autopoietic self-reproduction of "normal" micelles**. (**a**) The chemical reaction occurring in the autopoietic mechanism. (**b**) Ethyl octanoate, which is not soluble in water, is stratified over an alkaline solution containing octanoate micelles. Due to its amphiphilic character, ethyl octanoate partially dissolves in water, and next it is adsorbed at the micelle interface. Its headgroup (-COOEt) is exposed to water, and can react with hydroxide ions (OH^-). The saponification of the ester takes place, forming octanoate (new micelle-forming compound) and ethanol soluble in water. When a sufficient number of new octanoate molecules are formed, the micelle next divides forming new micelles. Note that hydroxide ion is not a component of the micelle.

a sharp sigmoidal curve, typical of autocatalytic patterns limited only by the resource exhaustion.

It should be recalled at this point the extremely large interfacial area that attends the formation of micelles: for example, if 1 mL of ethylcaprylate added to 1 L of water would be entirely converted into micelles, the total micellar interfacial area would be around 1000 m^2! Thus, the hydrolysis of the water-insoluble ester is accelerated by a very large factor, corresponding to the increase of the available microscopic interface. In essence, therefore, the micelles exert a sort of physical catalysis, providing a "matrix" for absorbing and reacting the otherwise insoluble ethyl octanoate. However, a local acceleration of the ester hydrolysis on the micelle interface – due to physico-chemical effects – could also play a role, although not yet demonstrated.

Fatty acid vesicles

We have seen that reverse micelles and normal micelles both exhibit autopoietic behavior, and thus represent relevant examples of chemical autopoietic systems. But what made this research very exciting was the discovery that fatty acid vesicles, which are well-recognized model of primitive cells, behave exactly in the same manner. Such evidence implies that if a fatty acid-producing reaction takes place inside or on the boundary of fatty acid vesicles, an autopoietic system should be obtained, and moreover, it should lead to a growth-division mechanism. It will mimic in minimal form the key feature of primitive cells, in the sense that self-reproduction is achieved in absence of the complex macromolecular machineries that are present in modern evolved cells.

David Deamer is one of the pioneers of the research on the formation and properties of fatty acid vesicles [9]. It is mostly the pH that determines whether fatty acids, suspended in water, form insoluble "oil droplets" (low pH), vesicles (intermediate pH) or micelles (high pH). In fact, the self-assembly of fatty acids is a function of their degree of deprotonation (*figure 2a*, right). At intermediate pH, which corresponds to about 8.5 in the case of oleic acid, there are the optimal conditions for the formation of vesicles because the fatty acid head group is partially deprotonated (ca. 50%). *Figure 5* shows a cryo-transmission electron micrograph of these vesicles.

The experiments described in the case of normal micelles were easily adapted to vesicles (*figure 6*). Pre-formed oleic acid vesicles can be placed in contact with a suitable oleate precursor, i.e., oleic anhydride. The latter is water-insoluble, but some molecules can be taken up by the vesicles, incorporated into their membrane, and hydrolyzed by the OH^- ions present in the aqueous solution. Because the number of membrane molecules increases, vesicles become unstable and divide as in the case of reverse micelles and normal micelles, generating

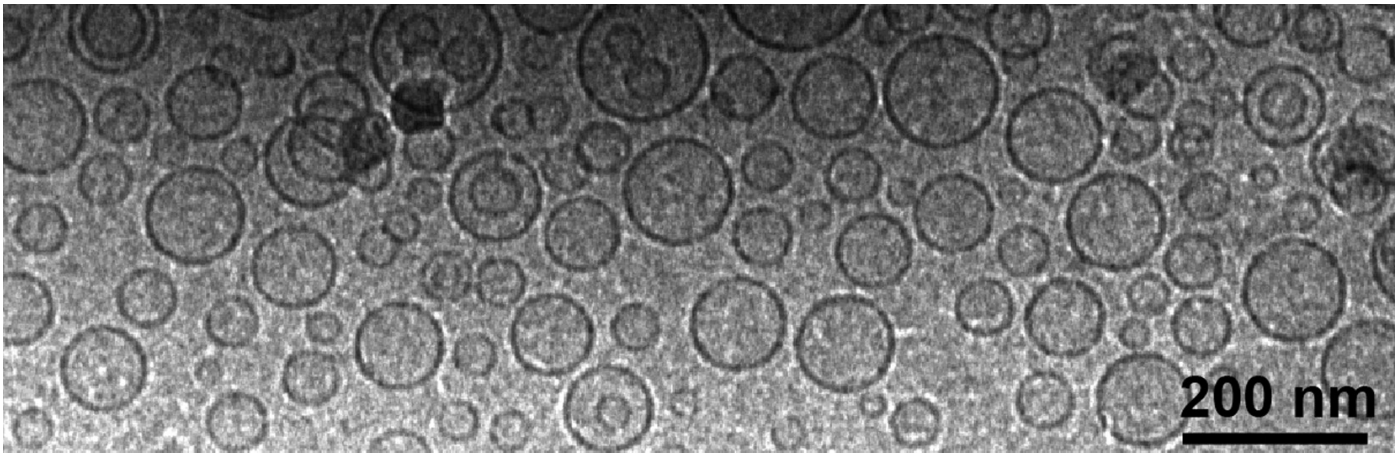

Figure 5 - **Cryo-transmission electron micrograph showing oleic acid/oleate vesicles.**

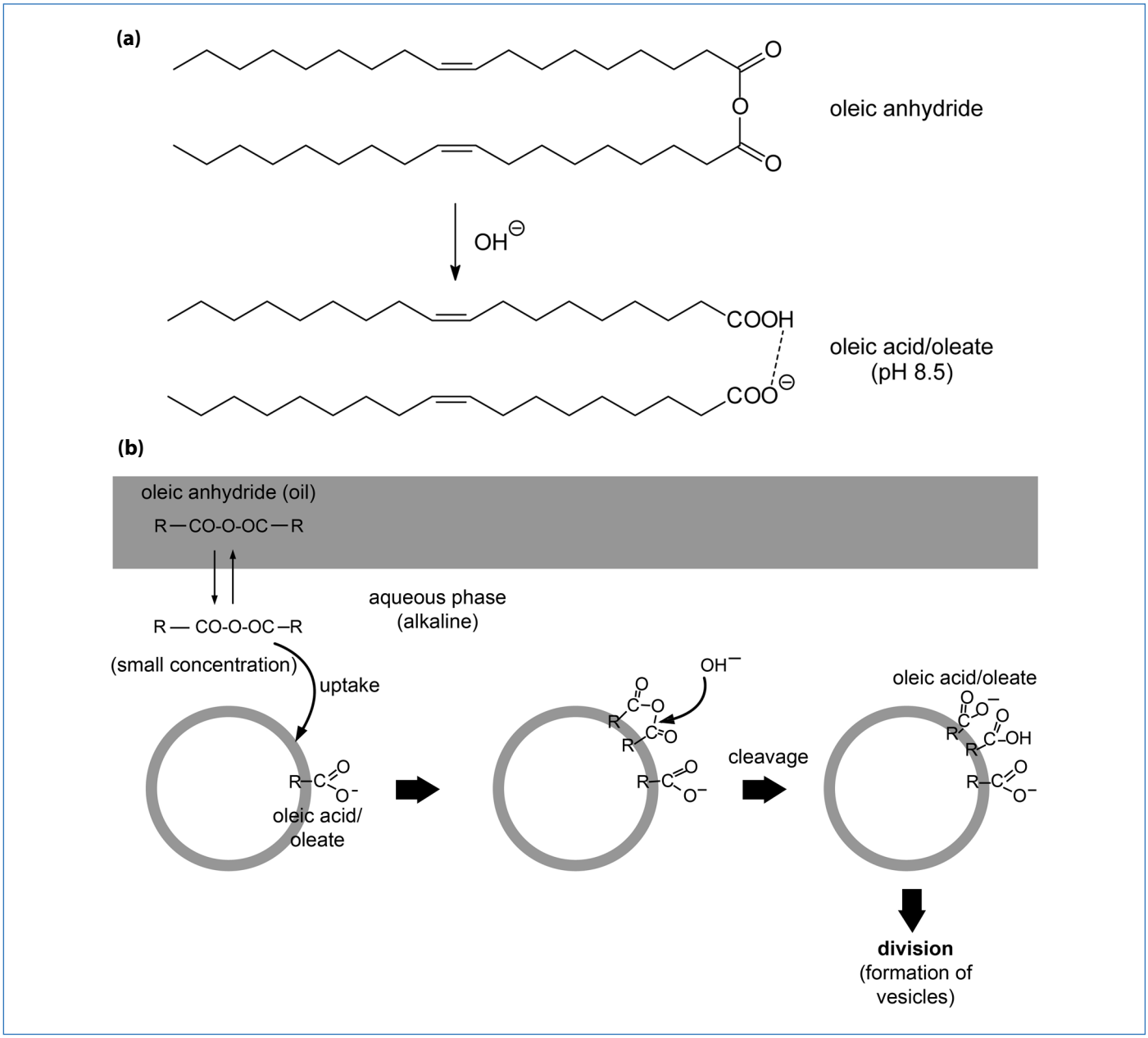

Figure 6 - **Autopoietic self-reproduction of fatty acid vesicles**. (**a**) The chemical reaction occurring in the autopoietic mechanism. (**b**) Oleic anhydride, which is not soluble in water, is stratified over an alkaline solution containing oleic acid/oleate vesicles. Due to its amphiphilic character, oleic anhydride partially dissolves in water, and next it is adsorbed at the vesicle membrane interface. Its headgroup (-COOCO-) is exposed to water, and can react with hydroxide ions (OH^-). The cleavage reaction takes place, forming oleic acid/oleate (new vesicle-forming compound). When a sufficient number of new oleic acid/oleate molecules are formed, the vesicle next divides forming new vesicles – whose size is similar to the size of the parent one ("matrix effect", see [11]). Note that hydroxide ion is not a component of the vesicle.

new "daughter" vesicles from "mother" ones (following the general scheme of *figure 2b*). Vesicles produce other vesicles, autocatalytically and autopoietically [10]. Similar results are obtained when no pre-formed vesicles were present at the beginning, as described for the normal micelles.
Deeper studies on autopoietic self-reproduction of vesicles also revealed another unexpected outcome. Under certain conditions, if pre-formed vesicles of a determined size are used to start the experiment, the final size distribution of the "daughter" vesicles closely resembles the size of the initial "mother" vesicles [11]. The phenomenon – for which still misses an explanation – has been called matrix effect, meaning that the vesicles undergoing growth and division are somehow capable of transferring the information about their size to the progeny vesicles.
Moreover, in addition to the example described in *figure 6*, where oleic anhydride is employed as precursor, an important variant foresees the employment of oleate micelles as precursor. In this second case, the autopoietic reaction is lost, but the entire mechanism may closely model a primitive scenario whereby fresh fatty acids (supposed available from geochemical mechanisms and/or from meteoritic delivery) continuously sustain the growth of fatty acid vesicles (intended as primitive cells).
The take-home message, emerging from these early studies on the autopoietic self-reproduction of reverse micelle, normal micelle, and especially on fatty acid vesicle, is that these micro-compartments, in addition to their well-recognized role of "containment" and "confinement", can display a very intriguing and potentially highly relevant reactivity: their autopoietic growth at the expense of a proper precursor. The combination of this growth with physical instability leads to a growth-division pattern that is equivalent to self-reproduction. These patterns are important because the chemicals that form the structure are produced within the structure itself and – at least in the case of reverse micelles – the reaction is promoted by other components present within the structure. That sort of chemical "machine" does not need external instructions to grow; it does it autonomously.
It should be recalled that in proper conditions, fatty acid vesicles also display *homeostasis*, thanks to the simultaneous synthesis and degradation of fatty acids [12], closely simulating the requirements of minimal autopoietic systems (i.e., a continuous production and degradation of system's components).

Triggering relevant protocell research

The above-mentioned seminal period of chemical autopoiesis was essentially completed in the 1989-1994 period, although several other papers appeared next, revealing more and more mechanistic details. The discovery of autopoietic self-reproduction of lipid micro-compartments was soon recognized as highly relevant for origins-of-life scenarios, generating enthusiasm among the specialists in the field. Perhaps, one can fix the turning point in 2001, when the famous "Synthesizing life" paper, by Jack W. Szostak, David P. Bartel and Pier Luigi Luisi, appeared in *Nature*, harmonizing the concepts of molecular self-replication (typical of the RNA world) and of autopoietic vesicle self-reproduction [13]. The combination and the synchronization of these two mechanisms would indeed generate, according to the autopoietic theory, a cell-like system (in a certain sense, a primitive cell – or protocell – model) that would produce its key component from within, grow and split, generate progeny, and at the same time being capable – at least in principle – of undergoing evolution. Moreover, the resulting structure would recall the *chemoton* (chemical automaton, again related to systems theories) introduced by Tibor Gánti in the 1970s [14].
It must be said that, to date, the type of protocell envisioned by the 2001 paper has not yet been created. However, since the early 2000s until today, the attention paid by numerous groups to the creation of primitive cell models has increased considerably, and a great deal of excellent research has revealed many details about these fascinating systems. Jack W. Szostak, from Harvard University, has significantly contributed to build such a knowledge with several elegant experiments, including the clear-cut demonstration of fatty acid vesicle growth-division by direct visual inspection of giant fatty acid vesicles [15] (in contrary, early studies were carried out with sub-micrometer vesicles, which could not be seen by optical microscopy; the vesicle behavior was indeed deduced from indirect evidences).
There is no space, here, to comment on the details emerged from these investigations. We would like to emphasize, instead, that the first instances of chemical autopoiesis represented a powerful trigger for the birth of a research arena based on the design and the construction of protocells consisting of solute-containing vesicles. The autopoietic theory, indeed, does not only provide an interpretation of living systems dynamics, but it also offers an operative guide – a recipe – for their construction from the bottom-up. The very central idea of exploring cell models at a minimal complexity level (yet endowed with life-like features), together with the input coming from early enzyme-containing vesicles [16-17] lies at the roots of a now-flourishing synthetic biology branch: the one dedicated to the construction of artificial/synthetic "minimal" cells.

From origins of life to synthetic biology

The examples of self-reproduction discussed in the previous sections are very simple cases of self-reproduction. Actually, because the building block synthesized *in situ* was always a boundary molecule, these examples have been referred to as "shell self-reproduction". In order to have a more realistic model of cellular self-reproduction, it is necessary that also the "core" components follow a similar autopoietic dynamics. Ultimately, the goal would be a "core-and-shell self-reproduction" system: a system in which the growth and self-reproduction of the shell occur simultaneously (and synchronized) with the self-reproduction of internal components (which could include, for example, nucleic acids and proteins). The starting consideration is that current knowledge has defined the minimal biological complexity compatible with an autonomous self-standing cell. Comparative genomics has identified the "minimal genome", i.e., the minimal set of genes that correspond one-to-one to the macromolecular components of a hypothetical very simple cell capable of autopoietic growth when placed in a chemically rich environment. The minimal genome is composed of about 200 genes, most of which referring to protein synthesis (ca. 50%), genome replication, minimal metabolism, and few other functions [18]. Surely, this hypothetic minimal cell would not live efficiently as – say – an *Escherichia coli* cell, because it would contain only the essential

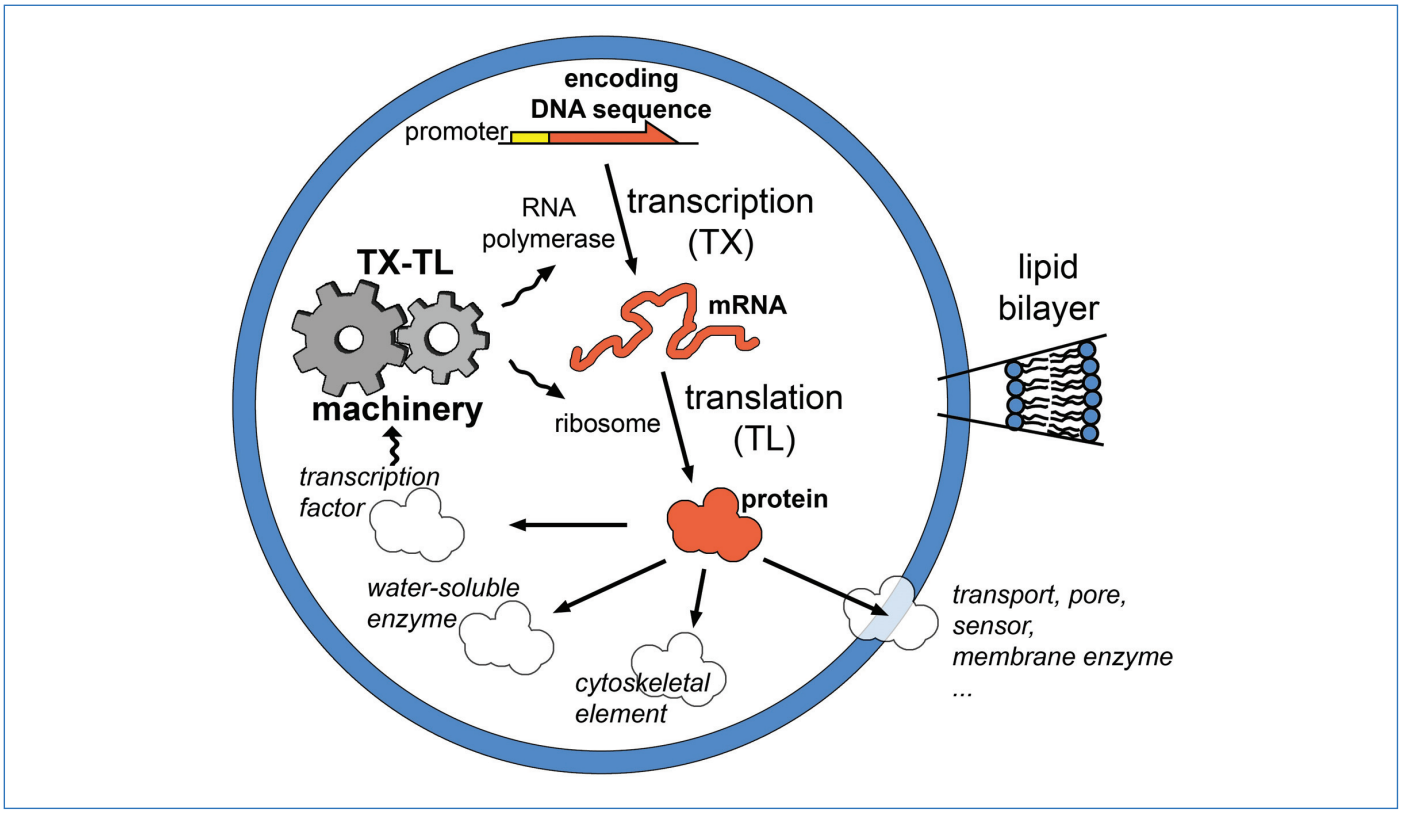

Figure 7 - **Schematic cartoon of a "artificial/synthetic cell" based on the encapsulation of biomolecules inside a lipid vesicle**. Current efforts are devoted to synthesize proteins through gene expression. With this aim, the full transcription-translation macromolecular machinery (generally obtained from *Escherichia coli*) is entrapped inside vesicles together with DNA sequences encoding for the protein(s) of interest. The produced protein(s) can then act as a transporter, pore, sensor, enzyme, cytoskeletal element, or transcription factor. Reproduced from [20] with the permission of Wiley.

"house keeping" genes, and thus it would not be an adaptive system. However, it would be surely recognized as alive (here and now).

The new question becomes: is it possible to build a minimal living cell by a bottom-up approach, imitating what an engineer does when he/she builds a machine from initially separated components? Although the original chemical autopoiesis experiments were carried out employing simple chemical reactions (oxidation, hydrolysis), in order to move toward more complex systems, the focus has to be shifted toward biochemical components, as suggested by the minimal genome concept. As an alternative, it is possible to conceive the construction of a minimal cell displaying core-and-shell reproduction but entirely based on reactions not necessarily matching biochemical ones. It is an open question which is the more challenging of the first and the second strategy. Surely the first one resembles more a reconstruction approach (reconstructing something that already exists, i.e., a biological cell), while the second would produce a minimal form of new life. Of course, *hybrid* approaches are not only possible, but also very interesting, and current research is already moving in this direction.

According to this shift from chemical to biochemical autopoiesis, in the 1990s pioneering research showed that several relevant biochemical transformations could be carried out inside vesicles, and in a few cases inside self-reproducing fatty acid vesicles. In particular, Oberholzer, Walde and Luisi reported the enzymatic polymerization of ADP into poly(A) (a genetic polymer), the enzymatic RNA replication, the polymerase chain reaction, and – importantly – the ribosomal synthesis of poly(Phe) inside vesicles (for a review, see [19]). For example, thanks to the enzyme Qβ-replicase, a template RNA molecule was replicated inside self-reproducing fatty acid vesicles. The system displays strong but unfortunately incomplete autopoietic features. Indeed, while the boundary and the RNA contained in the vesicles were self-produced, the Qβ-replicase was not.

The above-mentioned examples prompted, in the following years (2001-2004), very decisive studies on protein synthesis inside vesicles (*figure 7*), reviewed in [20]. The motivations are the following: protein (enzyme) synthesis is a fundamental "module" of the minimal cell and minimal genome; enzymes, once synthesized inside vesicles, exert a functional role (think about catalysis, pore formation, sensing, expression regulation, structural roles, and so on); the establishment of protein synthesis module is needed as a starting point for functionalizing vesicles that can be also employed in biotechnological context (artificial/synthetic cells, bioreactors).

Perhaps, it is not a coincidence that the pioneering studies on protein synthesis inside vesicles date back to the same period when, in the U.S.A., emerged the concept of "synthetic biology", or the application of an engineering vision to biology aiming at constructing biological parts, devices and systems not existing in nature, for useful application. The traditional "top-down" synthetic biology approach focuses on "rewiring" the metabolism of existing (micro)organisms in order to function as biosensors for a specific target molecule, or a miniaturized factory for producing pharmaceuticals. The construction of minimal artificial cells in the tradition of chemical autopoiesis instead follows a "bottom-up" path. "Top-down" and "bottom-up" synthetic biology share nevertheless a common "constructive" (synthetic) viewpoint, and probably represent the novel scientific frontiers of the 21st century.

The advancements made in the past years have considerably progressed the field of "bottom-up" synthetic biology at an extent that is difficult to summarize here. Protein synthesis inside vesicles is considered a quite standard practice, and a number of water-soluble or membrane-bound proteins have been successfully synthesized from their gene, thanks to transcription-translation molecular machineries. The resulting structures, called artificial/synthetic cells, have been able to display several life-like features like DNA duplication, lipid synthesis, sending/perceiving signals, displaying cooperative or competitive features. Compartments other than vesicles have been also exploited (e.g., coacervates and hydrogels). The current arena of bottom-up synthetic biology is one of the most exciting and challenging directions for research (*figure 8*). Today, after about 20 years from the early reports, the community of synthetic biologists working on the construction of ever-complex cell model is constantly increasing. Very large projects are currently under development in many countries, and an "open-science" initiative was recently started in the U.S.A.[(1)]. There is no doubt, in our opinion, that the current activities will lay the foundations for a radically new biotechnology, to be exploited in future.

However, it is useful to recall what is the connection of current bottom-up synthetic biology with the seminal idea of chemical autopoiesis. Was there a conceptual transformation, a shift in thought, during the transition that led from the first experiments with reverse micelles to the current artificial/synthetic cells? Is there any room for contributing to the origins-of-life question?

As mentioned, the recipe for building a living system simply consists in creating an autopoietic self-bounded chemical network (with the caveat of accepting the equation "autopoiesis = life"). In early studies, systems were based on very simple chemicals and few reactions. The descendants of such pioneer works are instead complex systems made of hundreds of molecules mainly represented by large nucleic acids and proteins. In this latter case, achieving an autopoietic dynamics is very complicated, because the elements of the network are *per se* very complicated molecules. For example, think to an artificial cell based on protein synthesis inside a vesicle. To display a full autopoietic pattern, it is not enough to produce the protein(s) of interest, but also the ribosomes should be equally produced from within. This observation clearly shows that for achieving a true autopoietic system, several sub-systems should be integrated so that their individual activities efficiently coexist in chemically compatible manner. Artificial cells built with biomolecules such as DNA, RNA, proteins are much more performing than the early examples of simple micelles or vesicles in terms of design, capability, programmability, but also have more constraints in terms of the mechanisms required for their autopoiesis. This has led, inevitably, to a shift of interest from the construction of minimal autopoietic systems to the construction of artificial cells not necessarily autopoietic, but capable of doing useful task (e.g., recognize a tumor cell and kill it). Clearly, the long-term goal remains the construction of an autopoietic (and thus living) cell from scratch, but many interesting and useful systems will be originated along the path.

With respect to origins-of-life research, instead, the self-reproduction of fatty acid vesicles still remains a keystone, but other processes have been also studied. Primitive cell models have been built by using allegedly primitive compounds, such as self-replicating RNA, short peptides, mixtures of simple

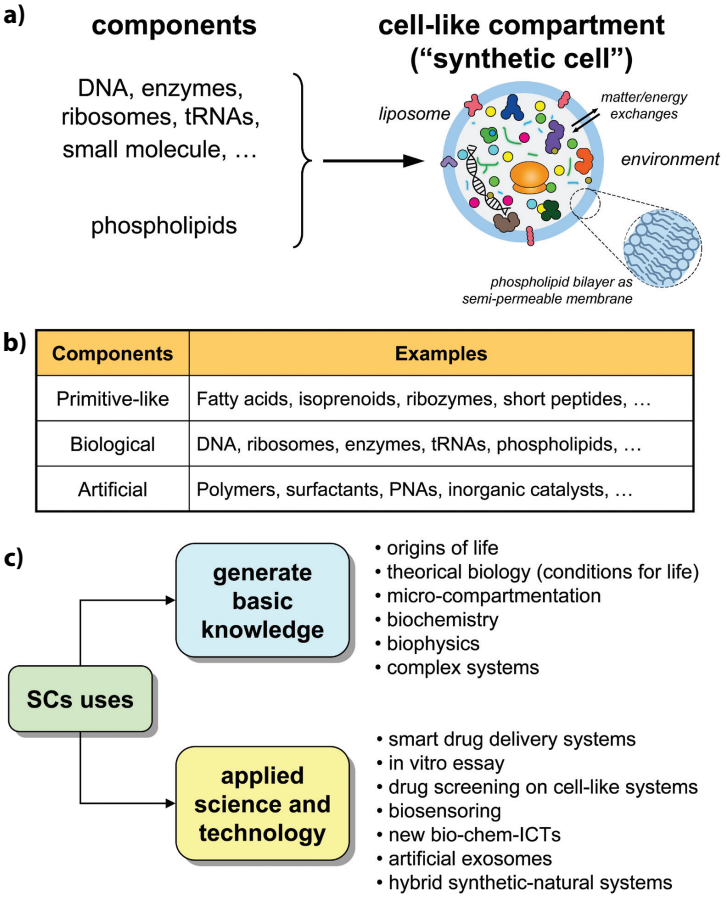

Figure 8 - **Artificial/synthetic cells made by the encapsulation of chemicals inside lipid vesicles (or other artificial compartments)**. (**a**) The case of semi-synthetic cells from biochemical components and liposomes. (**b**) Different types of artificial/synthetic cells can be envisaged, depending on the experimental scope. Hybrid systems are also possible. (**c**) Uses of synthetic cells in basic and applied science. Reproduced from [23], published under CC-BY license.

lipids, etc. As mentioned above, a rewarding goal is still the self-replication of RNA inside a self-reproducing fatty acid vesicle. Accordingly, a minimal protocell should have two RNA species, both are ribozymes. The first is a replicase (to duplicate both RNAs), the second is a lipid synthase (to produce lipids). In a proper environment, it is expected that such a system should display a minimal autopoietic dynamics.

An opportunity for next developments

We have summarized in this article steps and the motivations that prompted the research on chemical autopoiesis, and its long-term influence on modern bottom-up synthetic biology projects, which include artificial/synthetic cells and protocells. Establishing a full autopoietic network is not an easy task. When primitive molecules and simple chemical reactions are employed, problems are the lack of specificity, the need of harsh conditions (sometimes), and the presence of by-products. When biomacromolecules are used, the main problem is the need of other biomacromolecules as catalysts, and thus the system requires the production of proteins, nucleic acids, and especially the production of ribosomes.

The researchers involved in this learn soon that only a systemic view to the phenomenology of life allows novel progress. Surely, the property of being alive does not reside in one or more specific molecules (the RNAs of an RNA world), but in a process. The latter correspond to the very peculiar manner chemical components are organized, and in particular in how their reciprocal relations of production are intertwined. As soon

as one tries to build a living cell, it becomes also evident the crucial role of confinement. At one hand, the boundary should enclose the molecule that must establish the autopoietic network, allowing a distinction between the self and the non-self (the environment), but on the other hand, it serves to connect these two worlds, thanks to its semi-permeability. Moreover, it serves as matrix for reactions, for sensing, and for cell-cell interactions.

At a basic chemical level, the micro-compartmentation that stems from the structure of micelles and vesicles permits reactions that would not be possible in bulk homogeneous milieu, where molecules are diluted. In this respect, an interesting phenomenon should be reported. It has been shown that a transcription-translation mixture of macromolecules can be diluted in order not to produce a protein. If lipid vesicles are allowed to form in such a diluted mixture, it is observed that some of the resulting cell-like particles are instead surprisingly capable of synthesizing proteins, because the macromolecules spontaneously accumulate inside the vesicle in the very moment of vesicle formation [21]. This phenomenon refers only to <1% of the whole vesicle population, but clearly shows an additional (and unexpected) role of lipid compartments: the capacity of concentrating substances in their lumen. The result is particularly relevant for origins-of-life scenarios, because it provides a free thermodynamic ticket to the formation of solute-rich protocells even when the solutes are present at low concentration in the environment. It also demonstrates that experiments initially conceived to build sophisticated artificial cells (based on gene expression) can also reveal patterns relevant in the primitive cell context (and vice versa).

The continuous focus on systemic perspectives and system dynamics in contemporary research has led to another innovative field of inquiry called *systems chemistry* [22]. It is not surprising, then, that the studies on chemical autopoiesis, artificial/synthetic cells, protocells are developed also under this perspective. Systems chemistry can be defined as the chemistry of molecular systems, when seen as a whole, and the chemistry of self-organization, emergence, self-replication, symmetry breaking, out-of-equilibrium, non-linearity, and of all those complex phenomena having roots in chemical networks.

In conclusion, here we have recapitulated the history of a successful marriage, the one between autopoiesis and chemistry, first leading to the birth of micelle/vesicle self-reproduction (chemical autopoiesis), and to several implications in protocell scenarios and more in general, in origins-of-life studies. On the other hand, this sort of *Zeitgeist* decisively contributed to the onset of bottom-up synthetic biology, with the very fecund and long-term project of building artificial/synthetic cells by means of technologies that will revolutionize the science of next generations.

(1) The Build-a-Cell initiative, www.buildacell.org

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