

Synthetic biology of minimal living cells: primitive cell models and semi-synthetic cells

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Received: 21 December 2009 / Revised: 26 March 2010 / Accepted: 2 April 2010 / Published online: 10 April 2010
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Abstract This article summarizes a contribution presented at the ESF 2009 Synthetic Biology focused on the concept of the minimal requirement for life and on the issue of constructive (synthetic) approaches in biological research. The attempts to define minimal life within the framework of autopoietic theory are firstly described, and a short report on the development of autopoietic chemical systems based on fatty acid vesicles, which are relevant as primitive cell models is given. These studies can be used as a starting point for the construction of more complex systems, firstly being inspired by possible origins of life scenarios (and therefore by considering primitive functions), then by considering an approach based on modern biomacromolecular-encoded functions. At this aim, semi-synthetic minimal cells are defined as those man-made vesicle-based systems that are composed of the minimal number of genes, proteins, biomolecules and which can be defined as living. Recent achievements on minimal sized semi-synthetic cells are then discussed, and the kind of information obtained is recognized as being distinctively derived by a constructive approach. Synthetic biology is therefore a fundamental tool for gaining basic knowledge about biosystems, and it should not be confined at all to the engineering side.

Keywords Autopoiesis · Semi-synthetic minimal cells · Cell-free protein synthesis · Self-reproduction · Minimal cell size

Introduction

As concisely stated in the first sentence of a recently published review (Liu and Fletcher 2009), “we are much better at taking cells apart than putting them together”. The analytical (taking apart) approach, that has been the common paradigm for biological investigation in the last decades, is not the only way of gaining knowledge about organisms. In recent years, thanks to great developments in biochemical understandings, in molecular and cellular biology, as well as to a growing (re-discovered) scientific “holistic” perception of biological systems, we have witnessed the birth of synthetic biology (SB). SB aims at “designing and constructing biological parts, devices, and systems that do not exist in the natural world and also at the redesign of existing biological systems to perform specific tasks”.

Despite the numerous facets of SB research, what is distinctive in this new biological discipline is its explicit reference to a constructive (synthetic) approach, as complementary to the well known analytical one. This consideration, perhaps not fully perceived by the SB community, represents the truly novel feature of our scientific enterprise for understanding nature and developing (bio)technological advancements. It may be argued that SB is an aspect of a more general bio-inspired paradigm shift that we are going to experience in the next future.

SB projects are interdisciplinary, involving several aspects, such as bioengineering (genetic- and metabolic-engineering), modelling, bio-computing, and the construction of synthetic cells for basic studies (origins of life) or for biotechnological applications (De Lorenzo and Danchin 2008). From the conceptual viewpoint, it is interesting to remark that in the bioengineering perspective there are some typical SB operations *on the biosystem and*

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its parts that can be tentatively classified as additions, eliminations, substitutions, combinations, modifications (change, inversion, minimization, adaptation, etc.). In constructive approaches the concept and the methodology of assembling is also central. The functional and structural integration among the parts is clearly the key factor for the success of these operations, all of which denote the specific attitude of synthetic biologists to work with systems (a discussion on epistemological aspects of SB is provided by Luisi, submitted article).

In this article, I would like to discuss the investigations that are carried out by Luisi's group on a SB approach to study minimal living cells, in particular primitive cell models and semi-synthetic cells. Much of the discussion is therefore not a personal elaboration, but comes from the group work in the last years.

Autopoiesis is firstly introduced, providing the theoretical framework for the developments of our SB strategies. Classical studies on vesicle self-reproduction and their connection with current semi-synthetic constructs are then quickly reviewed. Finally, a recent specific example is specifically commented on. The whole discussion follows the *fil rouge* of constructive aspects in SB and system thinking as ways of making science and of complementing classical approaches.

Autopoiesis and vesicle self-reproduction

The term *autopoiesis* (self-production) refers to a theory that describes the behavior of all biological systems, from cells to organisms. This theory was introduced in the Seventies by the two Chilean biologists Humberto R. Maturana and Francisco J. Varela (Maturana and Varela 1980). Within the context of SB and the construction of synthetic cells, autopoiesis is an extremely powerful conceptual tool to define in general terms what are the structural and functional requirements of a molecular biosystems in order to mimic the basic living features of natural ones. The analysis is particularly simple when we focus on the lowest complexity level, namely the single cell. A minimal autopoietic system can be described by the cartoon shown in Fig. 1. The system (e.g., an autopoietic cell) can be seen as a self-bounded molecular assembly, characterized by the following dynamics. Components from the environment (A, B) are assimilated by the structure (dashed arrows) and transformed into the components of the cell (S, M). Together with these transformations, which are actually anabolic processes, waste material is produced (Y, W) by catabolic processes. It is possible to define two main processes: (1) synthesis of the boundary components (A to S) and self-assembly of S into a semi-permeable membrane; (2) synthesis of all internal

components (M) at the expenses of B (B to M and W). Thanks to these two processes, which are actually coupled, the autopoietic cell: (1) constructs its own boundary; (2) constructs all other internal components, which in turn give rise to processes that produce all components, that in turn give rise to processes ... and so on. The functions of the autopoietic cell are therefore defined recursively according to a circular logic. Notably, the whole system is out-of-equilibrium and thermodynamically open, because it exists only in the act of continuously transforming the nutrients (A, B) into the waste products (Y, W), and by exploiting the free energy of this transformation in order to maintain its internal *organization*. It is important to remark that, despite the ceaseless formation and destruction of the elements (lower hierarchical level) of the autopoietic system, the whole system (at an higher hierarchical level) maintains its own identity. The autopoietic cell is a *self-organizing* entity where its main features (self-producing, self-bounding, homeostasis) are *emergent* properties. Another important aspect of autopoietic systems, is that in their description it is not needed to identify a central control unit, because the property of being autopoietic is not associated with a particular molecule or function, but stems from the coordinated and collective system dynamics, which is also strongly coupled with the environment. Figure 1 also tells us that depending on the relative rate of anabolic and catabolic processes, the autopoietic cell can grow, stays in homeostatic state, or dies, depending on which aspect of metabolism is prevailing in certain conditions.

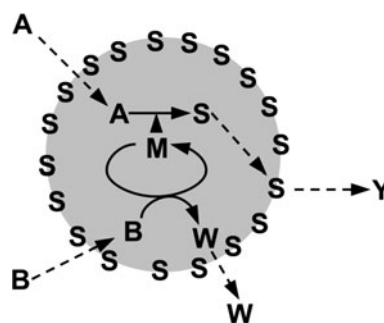


Fig. 1 Schematic drawing of an autopoietic cell. The autopoietic unit (in gray) is composed of a boundary formed by self-assembly of S, the boundary-forming compound. S, in turn, is formed from A, which is available to the autopoietic cell since it is present in the environment. A permeates in the cell, and it is transformed—by means of a metabolic network M, into S. S also decay to a waste product (Y), which does not take part in the autopoietic organization, and it is released into the environment. In order to be fully self-producing, the autopoietic cell must also produce (self-produce) the network M, at the expenses of precursors B and so producing the by products W. Overall, the autopoietic cell uses “nutrients” A and B, release waste products Y and W, and keeps its dynamic continuously out of equilibrium, conserving the organization of the “whole” despite the fact that parts are continuously synthesized and destroyed

In the first formulation, the concept of autopoiesis was associated with the concept of being alive (Maturana and Varela 1980), and autopoiesis was set as necessary and sufficient condition for life. However, successive considerations have suggested that in order to have a complete picture of the living state, the notion autopoiesis needs to be complemented by that of cognition, also developed by Maturana and Varela—namely the selective interaction with the environment (Bitbol and Luisi 2004; Damiano 2009).

If we take the autopoiesis as a starting point, we may ask whether it is possible, and at what extent, to design and build a chemical or biochemical autopoietic system in the laboratory. This question, which was discussed in a seminal paper by Luisi and Varela (1990), is equivalent to saying: can we build synthetic living systems in the laboratory? Today, under the perspective of SB, this question becomes not only timely and fascinating, but also experimentally approachable, and attracts the interest of scientists and of laypersons.

In the last 20 years, several works by Luisi and co-workers have focused on the realization of simple autopoietic chemical systems. The basic ingredient is a synthetic compartment that mimics the cell with respect to size, properties, permeability, and especially boundary self-assembly. Several compartments have been used to construct autopoietic systems that display the dynamics shown in Fig. 1, in particular growth. Here a specification is needed. While autopoiesis means self-production, the nomenclature used in the context of autopoietic models is “self-reproduction”. This is due to the fact that the outcome of an autopoietic growth resulted in being a copy of the original structure (see below). Clearly, this tremendously changed the attitude of looking at the autopoietic approach, which became therefore a theoretical framework for the construction of self-reproducing chemical systems. Reverse micelles, micelles and vesicles have been extensively studied in this respect, and detailed overviews on this work have been recently published (Stano and Luisi 2008; Stano 2009). The main findings of these studies are reported in Fig. 2. The experiments are realized as follows. First, a precursor of the boundary-forming molecule is added externally to pre-existing compartments, typically fatty acid-based compartments. Fatty acids form micelles, reverse micelles and vesicles in different conditions, and moreover have great relevance in origins of life studies, because fatty acid vesicles are the most plausible candidates for primitive cells. As precursors, fatty acid ethyl and methyl esters have been used, as well as fatty acid anhydrides. These precursors can be transformed into the boundary forming components by a simple hydrolysis reaction. During the initial stage, these precursors (P) are taken up at the boundary by the synthetic compartment,

mimicking the nutrients' uptake of cells and of autopoietic model (Fig. 1). Once taken up, the precursor is transformed into the boundary-forming component (S) *at the interface*; in this way the surface of the particle increases. That the transformation occurs mainly at the interface is proved by observing the strong rate enhancement of precursor hydrolysis when the reaction is observed in the absence and in the presence of pre-formed compartments (Bachmann et al. 1992; Walde et al. 1994a, b; Blochliger et al. 1998; Lonchin et al. 1999). As a consequence of surface increase, it is postulated that the resulting particle reaches a structurally unstable state and divides into two or more daughter particles. This is the key event of the “self-reproduction”. Such a conclusion is easily demonstrated for micelles and reverse micelles, whereas it has required special mechanistic investigations for fatty acid vesicles (Berclaz et al. 2001; Rasi et al. 2003; Stano et al. 2006). Studies on the mechanism of vesicle self-reproduction, however, have not yet given a unified view of the phenomenon (Chen and Szostak 2004; Rogerson et al. 2006; Luisi et al. 2008).

Overall, and this is the significant point, the process is equivalent to self-reproduction, since from one structure, two or more similar structures are formed, which are able to reproduce again and again, like living cells. Notice that self-reproduction is not explicitly present in autopoiesis. It is a “by-product” of the autopoietic mechanism, possibly due to the fact that the growing path crosses an unstable state. In fact, as previously mentioned, when an autopoietic mechanism runs by favouring the anabolic (constructive)

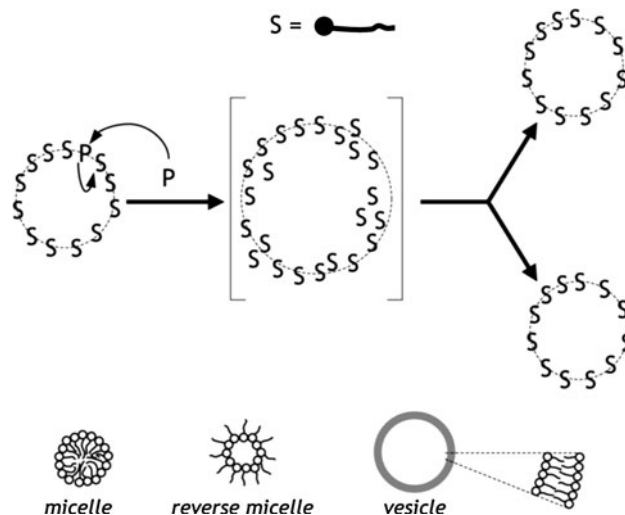


Fig. 2 General schemes for the self-reproduction of supramolecular structures. The uptake of a suitable precursor P by the preformed self-assembled structure brings about the formation of two (or more) self-assembled structures (not necessarily of the same size). This mechanism has been studied for micelles (bottom, left), reverse micelles (bottom, centre), and vesicles (bottom, right; not drawn to scale)

processes, the growth of the particle is expected, and not growth-division.

In the context of synthetic cells, autopoietic self-reproduction can be exploited in order to design and construct compartment-based simple biological systems which exhibit one of the most important feature of living cells, namely growth and offspring generation.

From the very beginning (Bachmann et al. 1992), the relevance of autopoietic self-reproduction for (1) origins of life and (2) for the laboratory construction of cell models was recognized (Walde et al. 1994a, b; Oberholzer et al. 1995; Luisi et al. 1999). In both cases, a kind of *minimal* metabolism should be implemented within the compartment, in order to mimic as much as possible the autopoietic unit displayed in Fig. 1. In the next paragraph, it will be shown what are the strategies for developing these two approaches, namely how to chose “S” and “M” in the (1–2) approaches.

Primitive cell models and semi-synthetic minimal cells

Thanks to autopoiesis, we can clearly define what has to be implemented in a cell model in order to approach the status of living cells in as much as it is possible. First of all, what is required is a self-assembling, self-bounding compartment, and two sets of reactions: the production of the boundary-forming compounds, and the production of “core” compounds, which in turn establish a reaction network able to produce its own compound, as well as the boundary molecules. In modern cells this is clearly integrated in the “metabolism”, the complex set of reactions occurring within the cells, that produce its own components (DNA, RNA, proteins, sugars, ATP, etc.) and phospholipids for building the boundary. The modern cells, however, derive from millions of years of evolution, and therefore it is reasonable to think that early cells were not so complex, yet capable of establishing a kind of autopoiesis. Moreover, when synthetic cells are considered, it is useful to focus on *minimal* cells, namely cells that have the minimal and sufficient number of components. These two concepts are somehow related, because they look at the problem of defining the essential cell model from two different perspectives, namely primitiveness and minimization.

Despite the structural diversity of primitive cells and minimal cells, autopoiesis is a useful theoretical framework for discussing and understanding all such structures, and SB is the operational way of gaining knowledge about these two classes of structures, both unavailable and therefore not approachable in the analytical (disassembling) paradigm. This viewpoint is illustrated by the term “chemical synthetic biology” (Luisi 2007), which defines the application of SB to synthesize biological compounds

and biological systems that do not exist in nature, in order to understand how and why living organisms originate; and for creating living cells in the laboratory, from non living compounds (showing that life is indeed an emergent property). Similarly, in a recent article, Jack W. Szostak discusses the use of the constructive approach for investigating the emergence of cellular life through the synthesis of primitive cell model (Mansy and Szostak 2009).

To study primitive cell models, the current approach focuses on the use of fatty acids as membrane forming compounds (the “S” of Fig. 1). This is due to the commonly agreed hypothesis that membranes of primitive cells were mainly made up of monocarboxylic fatty acids (e.g., with at least 10 carbon atoms), which have been found in carbonaceous meteorites, and which can be also synthesized by Fisher-Tropsch type synthesis (for recent reviews, see Deamer and Dworkin 2005; Walde 2006). Less clear is the choice of elements for the non-membrane components (the “M” components of Fig. 1). Here two schools can be identified as oriented to the RNA world (i.e., self-replicating ribozymes, or ribozymes with peptide ligase or peptide transferase activities, aptamers, etc., see for example Szostak et al. 2001), or toward a metabolism-first scenarios (the establishment of a self-reproducing reaction network of small molecules, and perhaps the first catalytic peptides; for a general discussion, see Shapiro 2007). Both views have pros and cons, but both lack extensive experimental investigations. This is clearly due to the difficulty in defining pre-biotically plausible compounds, intermediates, and conditions like pH, temperature, redox regime, and so on.

Investigation about minimal living cells based on well-known biochemical species shows some differences from the previous approach, from the theoretical as well as experimental viewpoints. This is currently under investigation, due to the availability of compounds to be used in constructing such simplified cells (DNA, RNA, ribosomes, enzymes, etc.). A full discussion of the topic of minimal cells lies outside the scope of this short article, the interested reader can find several reviews on different aspects of this subject (Morowitz 1992; Pohorille and Deamer 2002; Luisi et al. 2002, 2006; Foster and Church 2006; Schwillie and Diez 2009).

From the genetic viewpoint, it is possible to define the minimal cell in terms of minimal genome, i.e., the minimal number of genes required to “specify” the components of the minimal cell. Clearly, the genome does not directly contain the information about the cell assembly and the spatial-topological architecture, at least not explicitly, and therefore there is a kind of conceptual limitation in considering the genome as the minimal and sufficient description of minimal cells. Nevertheless, it is useful to perform such analysis. There is a long tradition in comparative genomics with respect to this problem; one of the latest reports has been

provided by the group of Moya (Gil et al. 2004). It defines the minimal cell in terms of 206 genes, classified as follows: (1) 16 genes for DNA processing, (2) 106 genes for RNA processing and protein synthesis, (3) 15 genes for protein processing, (4) five genes for cell processing, (5) 56 genes for basic metabolism, (6) eight poorly characterized genes. The list of genes of minimal genome has been obtained by comparing the smallest organisms' genomes, namely intracellular parasites and endosymbionts. A functional genomic analysis has provided the number 151 for the number of genes for a minimal cell (Foster and Church 2006). The hypothetical organism characterized by the minimal genome is simple when compared with natural living microorganisms, but still represents a challenge for laboratory construction. Moreover, it can be defined as alive only in very permissive conditions, since in order to simplify the metabolism, several important low-molecular weight compounds have to be provided by the environment.

The current state of the art experimental research on minimal cells exploits the convergence of liposome technology and cell-free technology (Fig. 3). In this scenario, "S" of the Fig. 1 are typically phospholipids, whereas "M" is a sub-set of the cellular genetic and metabolic compounds (DNA, RNA, proteins). A great improvement in synthetic cell construction has been achieved by using a purified kit of 36 enzymes, the PURE system (Shimizu et al. 2001), that synthesize proteins from the corresponding DNA sequences. By this approach, researchers have successfully synthesize model proteins (typically the green fluorescent protein, GFP) inside large or giant liposomes

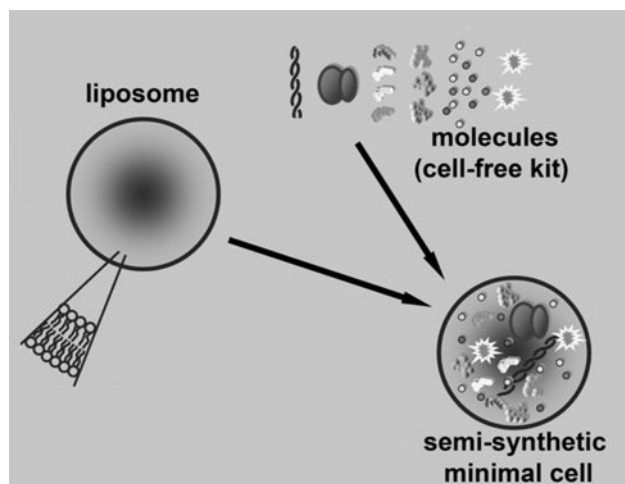


Fig. 3 Semi-synthetic minimal cells are formed by entrapping cell-free kits inside liposomes (e.g., all molecular parts required to perform a certain function as protein expression, such as a gene, RNA polymerase, ribosomes, tRNA, aminoacyl-tRNA-synthase, energy recycling enzymes, and low molecular weight compounds such as amino acids, nucleotides triphosphates, cofactors, etc.). Different methods of liposome formation have been used, and a standard procedure is not yet available

(Oberholzer et al. 1999; Yu et al. 2001; Oberholzer and Luisi 2002; Nomura et al. 2003; Saito et al. 2007; Murtas et al. 2007). This can now be considered a standard achievement. Recent studies have focused on the quantitative analysis and optimization (Hasoda et al. 2008; Saito et al. 2009; Amidi et al. 2009).

It is useful to comment here on four of the most advanced studies in the field of synthetic cells, showing how SB can be used within this context (Table 1). These studies aim at developing a more complex cell model, by focusing not only on the proof-of-principle that a functional protein can be synthesized inside liposomes, but on implementing some simple functions in the synthetic cell, as RNA replication, or pore formation, or lipid synthesis. These functions are required to approach the final goal of an autopoietic cell (core and shell self-production).

The first study (Ishikawa et al. 2004) remarkably shows that a two-step genetic network can be functionally reconstituted inside giant vesicles. In particular, Yomo and coworkers have prepared a plasmid encoding two genes, codifying, respectively, for T7 RNA polymerase and GFP. The first gene is under SP6 promoter, whereas the second under T7 promoter. By co-entrapping a transcription-translation extract—supplied with SP6 RNA polymerase—inside liposomes obtained from the dehydration-rehydration method, it was demonstrated that T7 RNA polymerase is firstly synthesized in functional form, thanks to SP6 RNA polymerase and consequent translation. T7 RNA polymerase—in turn—transcribes the GFP gene into the messenger, that is then successfully translated into the fluorescent protein.

The second study (Noireaux and Libchaber 2004) instead focuses on bypassing the low membrane permeability of synthetic cells. In fact, they were able to show that if alpha-hemolysin is expressed inside giant vesicles obtained by the water-in-oil droplets transfer method (Pautot et al. 2003), a 3 kDa cut-off pore on the membrane is spontaneously formed. Thanks to this pore, low molecular weight compounds, added externally, can enter the synthetic cell and be utilized by the internal machinery in order to produce GFP (from the corresponding gene) for 100 h. The pore may be beneficial in allowing low molecular-weight by-products to escape the cell.

The third case (Kita et al. 2008) is based on the Qbeta replicase activity. This enzyme is a RNA-dependent RNA polymerase, and it is therefore capable of replicating RNA strands. By careful designing RNA, the Yomo group was able to show that RNA can be replicated inside large vesicles obtained by the dehydration-rehydration method by an in situ expressed Qbeta replicase, which was codified by the RNA itself. In other words, RNA codifies for the function (embodied in the enzyme Qbeta replicase) which is required for its own replication. The complementary

Table 1 Remarkable studies on the construction of synthetic cells

Year	Description	Reference
2004	Two-steps cascade synthesis of T7RNA polymerase and GFP inside vesicles	Ishikawa et al. 2004
2004	Synthesis of alpha-hemolysin and GFP inside vesicles. Hemolysin forms a membrane pore that allows external feeding of vesicles (and therefore GFP production) for 100 h	Noireaux and Libchaber 2004
2008	Ribosomal synthesis of Qbeta replicase from an RNA template inside lipid vesicles. Qbeta replicase, in turn, replicates RNA in autocatalytic fashion	Kita et al. 2008
2009	Synthesis of functional glycerol-3-phosphate acyltransferase and lysophosphatidic acid acyltransferase (two membrane-associated enzymes) inside submicrometric liposomes	Kuruma et al. 2009

RNA strand, in turn, codifies for beta galactosidase, whose activity was detected by a fluorescent substrate.

The fourth example (Kuruma et al. 2009) deals with the issue of codified membrane synthesis. We have chosen the first two enzymes of the lipid salvage pathway to synthesize lipids from glycerol-3-phosphate and acyl-CoAs. The difficulty for this study is that the two enzymes required (glycerol-3-phosphate acyltransferase and lysophosphatidic acid acyl transferase) are membrane associated (the first is an integral membrane protein). After careful optimization of the lipid composition of liposome membrane, the two enzymes were successfully synthesized in active form, but the two consecutive reactions which are required to transform glycerol-3-phosphate into phosphatidic acid proceeded with very low yields, and no morphological changes could be observed. Despite this limitation, this work reveals interesting insights into the possibility of expressing functional membrane proteins inside lipid vesicles.

The physically smallest vesicle-based cell model

As a final example of SB approaches to understanding the nature of cells by a constructive strategy our recent investigation on the *minimal size* of synthetic cells is given. This question is of course related to the origins of cells and to the concept of above-mentioned minimal genome. The amount of codifying DNA may be one of the factors that govern the cell size. In fact, cells as *Mycoplasma genitalium*, which have small genomes, have also smaller sizes (ca. 0.02 cubic micron), especially when compared with free-living bacteria (around 2 cubic micron) (Moore 1999). There have been several speculations on the minimal size of cells (Knoll et al. 1999) and the announced existence of nanobacteria (Kajander and Ciftcioglu 1998) appears to be elusive. In other words, the question of the minimal cell size is still a matter of debate.

In order to answer the question whether a very small compartment may sustain cellular life, we have constructed a liposome-based synthetic cell of minimal size (radius

100 nm) and verified the expression of proteins inside it (Souza et al. 2009). Clearly, the synthesis of a model protein is not equivalent to the whole cellular metabolism, however, as also evident by the minimal genome enumeration (Gil et al. 2004), the number of components involved in such key cellular process is very high and represent ca. 50% of it. Moreover, to date—it is the only experimentally practicable way.

In order to construct microcompartments able to host protein synthesis, we form lipid vesicles in situ, by injecting a lipid solution (ethanol) in a solution containing all the molecular components needed to perform the reaction. The total number of physically independent macromolecular components is 82 (see Supplementary Material of Souza et al. 2009). We found that liposomes with a radius of about 100 nm, which is the smallest size ever considered in the literature for protein expression, are still capable of protein expression, and surprisingly, the average yield of fluorescent protein in the liposomes was six times higher than in bulk water.

A careful analysis of the entrapment process has revealed that the probability of co-entrapment of at least one copy of the 82 macromolecular components is negligible. On the other hand, the expression of GFP inside small vesicles demonstrates that a small fraction of liposomes actually contain all the requested components, despite the unfavourable statistics.

This represents a conundrum, that could be explained by assuming that the local concentration of macromolecules in the viable vesicles is at least 20 times higher than the nominal concentration. Further studies are currently devoted to understanding the reasons for this possible over-concentration mechanism, and to identify which biophysical factors could play a role.

Concluding remarks

I have shortly discussed a class of SB approaches for studying primitive cell models and synthetic cells, which

have their root in autopoietic theory and is nowadays developed by several groups. This kind of study has been recently classified as one of the three pillars of SB (De Lorenzo and Danchin 2008), witnessing an increasing interest toward these scientific aspects. Whereas primitive cell models are useful for understanding the origin and development of living cells on Earth, the developments of semi-synthetic cells (Luisi et al. 2006) can provide useful insights into the nature of cellular life as well as possible biotechnological applications (Pohorille and Deamer 2002; Zhang et al. 2008). These can be in advanced drug delivery (artificial cell factories), biosensing, and diagnostics.

There are still several limitations to our ability of constructing cells, and this enterprise is rather challenging. In particular, one of the major achievement would be the demonstration that it is actually possible to create a living cell in the laboratory. This construct could be based on primitive cell models or on semi-synthetic ones, it does not matter. In this context, it is important to specify that the property of being alive must be viewed according to minimal autopoietic requirements, and that even a kind of “limping” life (Luisi et al. 2006) would be satisfactory. Thanks to the rise of interest in minimal cells, more groups are now working on this issue and future interesting advancements appear to be potentially accessible targets.

Acknowledgments This work has been presented at the Second European Conference on Synthetic Biology (ESF ECSB II: Design, Programming and Optimization of Biological Systems, Sant Feliu de Guixol, Spain, 29 March–03 April, 2009); it collects the recent results of Luisi’s group at ETH Zurich and University of RomaTre. I greatly thank Pier Luigi Luisi for inspiring this research and for stimulating discussions. Tereza Souza (University of Jena) and Yutetsu Kuruma (University of Tokyo) are also acknowledged as co-authors of the original papers on minimal cell size and lipid-synthesizing minimal cell. This work has been funded by the SYNTHCELLS project (Approaches to the Bioengineering of Synthetic Minimal Cells, EU Grant #FP6043359); by the Human Frontiers Science Program (RGP0033/2007-C) and by the Italian Space Agency (Grant Nr. I/015/07/0). It is also developed within the COST Systems Chemistry CM0703 Action.

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