Chemical Approaches to Synthetic Biology: From Vesicles Self-Reproduction to Semi-Synthetic Minimal Cells

Pasquale Stano[1], and Pier Luigi Luisi[1]

[1]Biology Department, University of RomaTre, Viale G. Marconi 446, 00146 Rome, Italy
stano@uniroma3.it; luisi@mat.ethz.ch

Abstract

The recent advent, success and diffusion of synthetic biology (SB) are mainly related to its application as markedly bioengineering-oriented discipline. In addition to this classical view, SB also means "constructive" biology, and it is aimed to the construction of synthetic (artificial, man-made) biological-like systems, at the aim of understanding basic concepts of living systems and of their parts. In the last years, we have investigated lipid vesicles (liposomes) as cell models, by studying different aspects of their general reactivity, from their self-reproduction to the hosting of simple and complex biochemical reactions. In the attempt of modeling simple autopoietic systems by vesicle populations, it was firstly shown that simple vesicles may grow and divide according to physical laws, also revealing an unexpected pattern recognized as a "matrix effect", consisting in the conservation of the average size in a population of self-reproducing vesicles. Semi-synthetic minimal cells, on the other hand, are defined as liposome-based synthetic cells that contain the minimal and sufficient number of macromolecular components in order to be defined as "alive". Clearly, the design and the construction of minimal living cells require the establishment of the minimal number of life criteria. These have been generally described as self-maintenance, self-reproduction and evolution capability. The current experimental approach to semi-synthetic minimal living cells exploits the combination between cell-free protein expression and liposome technology, and it is conceptually based on autopoietic theory. In the FP6 SYNTHCELL project, we have investigated the expression of functional proteins inside lipid vesicles by using a minimal set of enzymes, t-RNAs and ribosomes (PURESYSTEM) at the aim of constructing functional cell models. In this contribution, we will discuss recent experimental advancements in the field of synthetic cell constructions, giving emphasis to their relevance in synthetic biology, self-organization and biocomplexity, and in origins of life studies.

1. Chemical Approaches to Synthetic Biology

In the last fifty years of biological research we have been "much better at taking cells apart than putting them together'' (Liu and Fletcher, 2009). Recently, however, also thanks to great amount of detailed information gained by the analytic approach, we have the unprecedented opportunity to develop a new kind of biological understandings, namely by the synthetic (constructive) approach. Synthetic biology (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'' (http://syntheticbiology.org). SB is generally seen as a bioengineering discipline, based on design, simulation and construction of novel biological systems, but it also embodies the novel concept, perhaps not fully recognized, of gaining knowledge by constructing biological systems. This attitude is particularly relevant in those cases where the analytical (dissecting) approach cannot be undertaken, as in the case of primitive and minimal living systems.

Classic SB studies deal with the generation of new devices, systems, organisms which are supposed to perform novel "useful" tasks, like the production of fuels, of hydrogen, of a chemical species, for bioremediation, and so on. Notice that in such studies a determined goal is set at the very beginning, and all routes and tools are bent and focused for the purpose of obtaining that goal. Methodologically, SB operations *on* biological systems can be tentatively classified as additions, eliminations, substitutions, combinations, modifications (change, inversion, minimization, adaptation, etc.). They reflect the above-mentioned engineering approach, but are indeed synthetic operations, that define a constructive act and bring about novel systems.

Seen with the eyes of a chemist, SB means the construction of biological systems as in the case of molecules and molecular systems. Molecules react together according to their intrinsic chemical reactivity and environmental conditions, giving rise to complex molecules starting from simpler ones. Supramolecular chemistry describes the self-assembly and self-organization of molecules into structures, kept together by non-covalent interactions. Autocatalytic systems, oscillating reactions, reaction networks, and reactions in micro-compartments are other chemical examples of increasing complexity. The main aim of chemical SB is therefore not the achievement of a specific goal or function, but the study of the properties of a certain construct, which has been built to be tested. Clearly, as in the bioengineering approach to SB, here also the concepts and the methodologies of assembling are central, as well as the functional and structural integration among the parts.

There are several examples of possible applications of chemical synthetic biology, as recently reviewed (Luisi, 2007; Chiarabelli et al., 2009), but in this contribution we would like to focus on the attempts to make minimal living systems, in particular primitive cell models and semi-synthetic cells. Much of the discussion presented here has been published recently in a more extensive form (Luisi et al., 2006; Stano and Luisi, 2010; Stano 2010). We will first introduce the

concept of *autopoiesis*, the theoretical framework that guides the construction of minimal living cells, then we will shortly comment recent results on the self-reproduction of lipid vesicles. Then we shift the focus on more complex constructs, i.e., semi-synthetic minimal cells. Finally, we discuss our latest finding on the assembly of cells from lipids and solutes.

2. Vesicle Self-Reproduction

Studies on vesicles self-reproduction started about 20 years ago in the Luisi's group at the ETH (Zurich), together with other investigations on micelle and reverse micelle self-reproduction. These studies are linked to (and actually inspired by) the theory of autopoiesis, which accounts for the dynamical process at the basis of living entities. The self-reproduction of synthetic compartments, like those listed above, is a pre-requisite for projects aimed to construct synthetic/artificial cells in the laboratory. In fact, since synthetic compartments can grow and divide only due to physical forces, it becomes plausible to design and try to build a minimal living system that self-reproduce thanks to the interplay between chemical transformation and supramolecular reactivity, as shown in the case of micelles and vesicles. Ultimately, projects as the Minimal Cell, Synthcells, Los Alamos Bug, and similar ones are related to such reactive pattern.

2.1 Autopoiesis

The term *autopoiesis* (self-production) refers to the description of the behavior of all biological systems, and especially cells, the simplest 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 a powerful conceptual tool for defining 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 simplest autopoietic dynamics is shown schematically in Figure 1 (Luisi, 2003). The autopoietic unit is a self-bounded material structure, where boundary components (**L**) are formed by internal chemical transformations mediated by the network **E**. In such way, the precursor(s) **P** enter the autopoietic unit and are then transformed into **L**. Eventually **L** decays to a waste product **W**. At the same time, the chemical network **E**, which can be composed by few or several components (not shown) is not static, but also continuously destroyed and reconstructed at the expenses of building blocks **Q** (giving the by-products **Z**). Overall, the autopoietic unit stays out of equilibrium but maintains its identity despite the continuous transformation of its components. Its existence relies on environmental conditions, due to the need of assimilation of components from outside. For this reasons the autopoietic cells establish a sort of minimal cognitive relationships with its environment.

Notice that the "shell" (the boundary formed by **L** molecules) as well as the "core" components (the **E** sub-system) are simultaneously produced by the internal autopoietic dynamic, i.e. the autopoietic system actually produces its own compounds and its own processes.

Living cells are autopoietic units, but the contrary is not necessarily true (for a discussion, see Bitbol and Luisi, 2004).

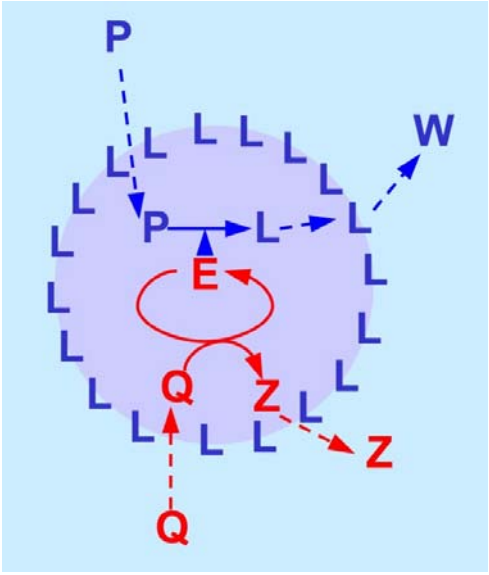

Figure 1. Schematic drawing of an autopoietic cell.

Clearly, in living cells **L** molecules are the lipids and the proteins of cell membranes, whereas **E** is the genetic/metabolic network. **P** and **Q** are the basic nutrients for cell growth, and **W**, **Z** the waste materials. Is it possible to build a (minimal) autopoietic cell in the laboratory? To answer this question, we firstly have to conceptually simplify the structure shown in Figure 1 by reducing the complexity of the elements involved in the autopoietic dynamics (reducing their number, and simplifying their structure/function).

One first answer to this question has been provided in terms of vesicle self-reproduction, which consists in an autopoietic growth (and division) based on the scheme indicated in Figure 1. In particular, it has been demonstrated that a supramolecular assembly of **L** molecules (a vesicle, but also a micelle or a reverse micelle) can grow at the expenses of a precursor **P**, without any internal metabolism (without the red sub-system shown in Figure 1).

We will see later how synthetic cells are now designed in order to display a similar autopoietic mechanism, based on a minimal DNA/RNA/enzyme genetic/metabolic network (**E** in Figure 1).

2.2 Recent advancements in vesicles self-reproduction

We have recently reviewed the whole field of vesicles self-reproduction, from the historical and scientific viewpoints (Stano and Luisi, 2010). The mechanism underlying vesicle self-reproduction is based on the following points: (1) existence of a proper precursor **P**, that can be chemically converted into the membrane-forming compound (**L**) by hydrolysis, oxydation, deprotonation, and other simple transformations; (2) uptake of **P** by existing vesicles, and transformed into **L** therein; (3) the vesicle growth must

proceed in a way that an unstable physical state is soon reached, which precedes the division into two or more daughter vesicles. It has been shown long ago that fatty acid vesicles can grow and self-reproduced at the expenses of fatty acid anhydride (Walde et al., 1994), and fatty acid micelles (Bloechliger et al., 1998). Oleic acid systems are typically used in this context. In these systems, the above-mentioned conditions (1-3) are satisfied. In particular, condition 3 is thought to derive from unbalanced surface-to-volume growth, which brings about to vesicle instability (Fiordemondo e Stano, 2007; Luisi et al., 2008). One of the most intriguing results from such studies is known as the "matrix effect" (Bloechliger et al., 1998; Lonchin et al. 1999; Berclaz et al., 2001; Rasi et al., 2003). During the investigation of vesicles self-reproduction it was discovered that the size of pre-existing vesicles was somehow conserved in the next vesicle generation. In particular, it was shown that the size distribution of vesicles (formed after addition of **P** to a pre-existing vesicles population) was very similar to the size distribution of pre-existing vesicles, as if the vesicle size acts as a "template". The mechanism of matrix effect is not yet understood, but a recent investigation brings about evidences on possible intermediates. Freeze-fracture electron-micrographs suggest the transitory existence of elongated "twin" vesicles (Stano et al., 2006) resembling bacteria during binary division. Previous results obtained with ferritin-containing vesicles (Berclaz et al., 2001) indicate that in some conditions the solute molecules are redistributed among daughter vesicles. An interesting report on self-reproduction of *giant* fatty acid vesicles has been recently provided by Szostak and coworkers (Zhu and Szostak, 2009), who demonstrated that elongated tubular vesicles, derived from micelle uptake, can divide into into several smaller vesicles. Interestingly, experiments done with a permeable buffer indicate that vesicle pure-growth or vesicle growth/division is indeed governed by the surface-to-volume growth ratio. Experiments from Sugawara's group (Kurihara et al., 2010) with synthetic surfactants show that self-reproduction can also occurs by a translocation mechanism, i.e., a new vesicle, born inside the mother one, comes out *via* a not well understood physical translocation through the parent membrane.

3. Minimal Cells

As noticed before, although the details of vesicle self-reproduction are yet unknown, such studies prompted the development of more complex models of minimal self-reproducing systems, namely the construction of vesicle–based cell–like systems, with the final aim of creating living cells in the laboratory. These constructs, which are called protocells, artificial cells, minimal cells, synthetic cells or semi-synthetic cells, are the subject of flourishing research into the origins of life and synthetic biology communities. Among the most active groups in the field, we must recall David Deamer at the University of California, Jack Szostak at Harvard, Tetsuya Yomo at the Osaka University, Steen Rasmussen at the FLinT (Southern Denmark University).

We limit ourselves to the discussion of our current approach, known as the semi-synthetic one (Luisi et al., 2006). Such approach (Figure 2) consists in using lipid vesicle as cellular model, and implement a sort of minimal metabolism based on DNA/RNA/enzyme components. The philosophy behind minimal cells lies again in the autopoietic theory. In particular, emphasis is placed on the need for a cellular system of minimal complexity.

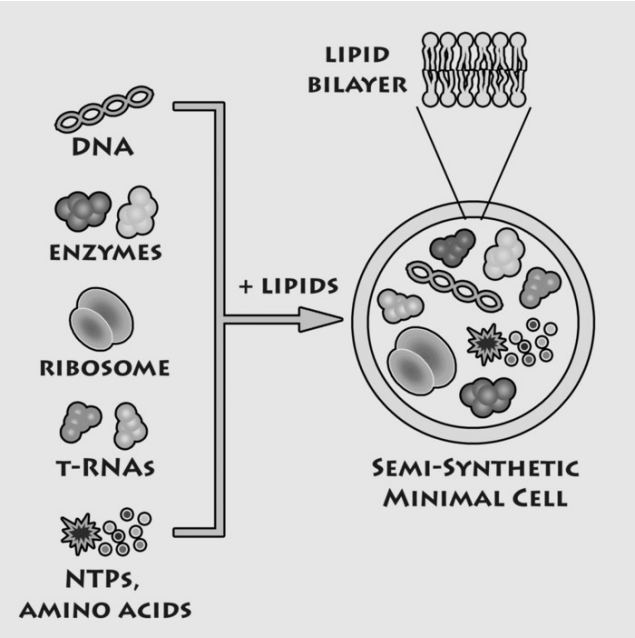

Figure 2. Semi-synthetic approach. Reproduced with permission from Elsevier from Chiarabelli et al. (2009).

Minimal cells are thus composed of the minimal number of genes, enzymes, ribosomes, tRNAs and low molecular weight compounds that are encapsulated within a synthetic compartment as in the case of lipid vesicles. The resulting construct, which is similar to a living cells and displays minimal living properties (self-maintenance, self-reproduction and possibility to evolve) is generally designed on the basis of the minimal number of functions required and on the minimal complexity of the biochemical elements needed for its construction.

Conceptually, therefore, semi-synthetic minimal cells come from one of the operations mentioned as typical of SB approaches (elimination of unnecessary elements in a system). The result of such simplification resembles very much the biological notion of *minimal genome*, i.e., the minimal number of genes requested to make a living organism. Classical studies based on comparative genomics (reviewed by Luisi et al., 2002, 2006) suggest that such number lies between 200-300 genes, and the figure of 204 genes has been proposed by Moya and coworkers on the basis of a recent study (Gil et al., 2004). A similar result (151 genes) has been obtained by Forster and Church (2006) by reasoning on the minimal biochemical requirements of a minimal cell.

In principle, therefore, it would be possible to build a synthetic cell by inserting a minimal genome inside liposomes, as well as all the macromolecules and low molecular-weight compound required for decoding the genome. This has not been done yet, and although several

advancements have been recorded in the recent years, this goal appears to be not easily reachable. We describe below some key milestones along the road-map to minimal cells, according to the semi-synthetic approach. We then conclude this contribution by giving a summary of most recent results from our group, and a survey of some general aspects and modern trends of minimal cell studies.

3.1 Pioneering studies

The first report dates back to 1999, and describes the first proved ribosomal polypeptide synthesis (poly(Phe) from poly(U)) inside liposomes (Oberholzer et al., 1999). The demonstration that ribosomal protein synthesis can occurs inside vesicles actually allows the design of more complex systems, based on DNA transcription into messenger RNA and translation of the latter into protein (therefore developing a *function*). Semi-synthetic minimal cells approaches are based on this idea. From the experimental viewpoint they consist into a convergence of *in vitro* biochemical systems and liposome technology. By using cell extracts or – more recently – reconstituted transcription/translation kits, as the PURE System introduced by Ueda and coworkers (Shimizu et al., 2001), functional proteins can be expressed inside vesicles. The basic idea is the following. Firstly, the protein expression cover about 50% of the minimal genome; second, it has a sufficient complexity to be used as a (partial) model of a whole cell metabolism; third synthesizing *functional* proteins inside liposomes, e.g. enzymes, structural proteins and so on, paves the way to implementing minimal cellular functions, like genomic replication, lipid synthesis, environment sensing, membrane functionalization, active transportation of nutrients inside, motion, etc.

Since the report from Yomo's group in 2001 (Yu et al., 2001) there have been several reports on the synthesis of a functional soluble protein (GFP, green fluorescent protein) inside lipid vesicles (reviewed in Luisi et al., 2006, Chiarabelli et al. 2009; Stano 2010). This can be considered a standard achievement. Recent investigations are instead devoted to more quantitative studies (Hasoda et al. 2008; Saito et al. 2009; Amidi et al. 2010; Sunami et al., 2010).

3.2 Recent advancements

It is useful to mention here two of the most recent results, that differ technically and conceptually from the standard achievement described in the previous paragraph. The first is our report on the synthesis of transmembrane protein inside lipid vesicles, without the help of specialized proteins, but simply exploiting the self-assembly properties of the protein and lipid membrane (Kuruma et al., 2009). The work aimed to construct a minimal cell capable of synthesizing lipid molecules from inside, as shown in Figure 1. The underlying biochemistry is the two-steps transformation of glycerol-3-phosphate into phosphatidic acid, a membrane-forming compound. In order to carry out these transformations, two active enzymes need to be synthesized inside a lipid vesicle, namely the glycerol-3-phosphate acyltransferase (G3PAT, a transmembrane enzyme) and the lysophosphatidic acid acyltransferase (LPAAT, a membrane-associated enzyme) (Figure 3).

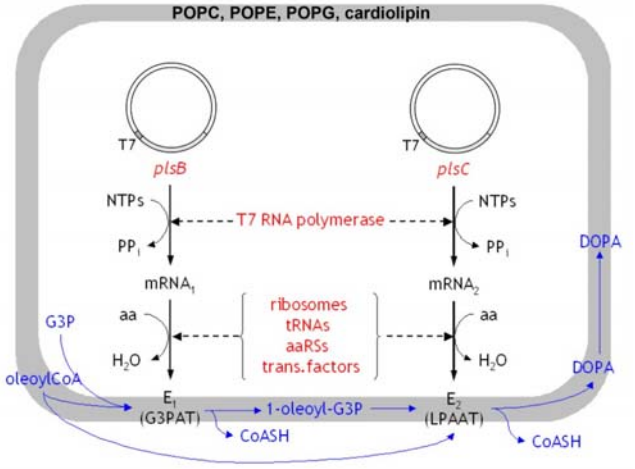

Figure 3. Lipid-synthesizing minimal cell. All translational factors are encapsulated inside liposome, which is composed by four kinds of phospholipids. The composition of lipid membrane is a key factor for obtaining simultaneously a good entrapment of molecules inside liposomes, high yield of protein synthesis, and functional forms (correct folding, insertion) of the target enzymes (G3PAT and LPAAT).

The desired two-steps reaction could be achieved only by changing the redox conditions, and unfortunately the amount of produced phosphatidic acid was too low to observe a macroscopic change on vesicles. This study represents, however, an important advancement along the roadmap to minimal self-reproducing cells.

The second most recent result deals instead with the attempt of synthesizing a functional protein (GFP) inside small vesicles (diameter 200 nm) (Souza et al., 2009). This study was intended as an experimental investigation on the *minimal size* of cells, an old debated question in biology. By using the protein synthesis as a paradigm of the whole cellular metabolism, we have indeed successfully demonstrated that 200 nm vesicles (plausible models for small ancient cells) actually support a complex metabolism as the transcription/translation one. Interestingly, a careful analysis of the statistics of co-entrapment of all macromolecular components (ca. 80) involved in the protein synthesis revealed a surprising conclusion. In fact, according to the classical description of solute entrapment, the Poisson probability of co-encapsulating the ca. 80 different molecules (0.1-1 μM each) inside 200 nm (diam.) vesicles is practically zero (10^{-26}). Nevertheless, the protein was synthesized in some compartments, and therefore the apparent contrast between observed and predicted behavior represents a conundrum. In order to explain the observations, we made the hypothesis that local (internal) solute concentration was ca. 20 times higher than the nominal (bulk) one. We have recently investigated

this phenomenon by entrapping ferritin inside liposomes, and analyzing the occupancy frequency in each liposome by means of cryo-TEM visualization (Luisi et al. submitted), see below for a short comment on such study.

3.3 On the entrapment of solutes

Projects on the construction of minimal cells foresee, as basic assembly step, the formation of solute-containing lipid vesicles. It is interesting to notice that such important process has not been studied in great detail. It is clearly recognized that the entrapment process depends on the mechanism of vesicle formation, on the nature of lipids and solutes, and by the concentrations used in the experiment. The general hypothesis is that the average number of entrapped molecules (N_0) depends on the concentration of solutes (C_0) used and on the vesicle volume (V), i.e. $N_0 = C_0V$. Deviations from the expected average number are typically modeled by a Poisson distribution. In our recent investigation on the encapsulation of ferritin inside lipid vesicles – a study that was triggered by the conundrum of simultaneous multiple entrapment of several components inside liposomes, see above – we discovered that the description of entrapment phenomena is not well described by the standard model (Luisi et al., submitted). When vesicles are allowed to form spontaneously in the presence of solutes, the surprising result is that the classical description fails (at least for submicrometric vesicles) with respect to: (*i*) the average number of solute per vesicle, (*ii*) the expected occupancy distribution.

In particular, we have observed that a small fraction of vesicles are filled by several solute molecules, confirming our working hypothesis of high internal solute concentration, and that the occupancy profile does not follow the Poisson distribution, being aligned instead as in a long-tail distribution. Experiments are currently in progress to fully characterize the vesicle system.

This result indicates that SB studies on the construction of synthetic or semi-synthetic cells actually drives also advancements in basic science. In fact, thanks to such approach it becomes evident that our simple model of vesicle formation needs a revision, since there are suggestions that membrane closure into a vesicle is not a passive event, but might bring about solute recruitment with the consequent formation of high internal solute concentration, which is a pre-requisite for the spontaneous formation of functional cells.

3.4 Next developments and conclusions

In conclusion, there has been a big progress in the ability of constructing minimal cells by the semi-synthetic approach. The state of the art is represented by the synthesis of water-soluble as well as membrane proteins. This will allow the realization of more complex systems that are capable of implementing additional function, especially in the direction of constructing a minimal autonomous cell, and a self-reproducing cell. As evident in Figure 1, the final goal will be the simultaneous and possibly functionally coupled core-and-shell reproduction.

In order to discuss next development, we have to distinguish among conceptual advancements and technical ones. Moreover, it is also useful to discuss the general aspects of semi-synthetic approach, within SB and with respect to other research lines.

New directions in minimal cell research, as anticipated, should focus on the self-reproduction of the genetic/metabolic molecules as well as a more efficient lipid synthesis, the so-called *core-and-shell reproduction*. Such goal can be reached by duplicating DNA and by implementing the *in situ* ribosome synthesis. The other two set of key macromolecules, tRNAs and aa-tRNA synthase need also to be synthesized inside vesicles. Lipid synthesis is particularly relevant, and together with phospholipid synthesis, fatty acid synthesis should be considered (for a preliminary report, see Murtas 2009). The study on the cell-free synthesis of transcription factors (Asahara and Chong, 2010), and on a short biosynthetic pathway (UDP-*N*-acetylglucosamine pathway, by Zhou et al., 2010), point toward the realization of more complex systems by the *in vitro* gene expression approach. Another interesting direction has been pioneered by Davis and coworkers, who let synthetic cells send a chemical message (ribose-borate complex, synthesized inside the synthetic cell via the formose reaction) to a bacteria population, stimulating a quorum sensing response (Gardner et al., 2009). It is expected that further development may concern a two-way communication between synthetic and natural cells (for a discussion, see also Cronin et al. 2006, for a potential application as drug delivery systems, see Zhang et al. 2008). Further studies might be devoted to the explicit investigation of stochastic effects within synthetic cells (such concept has been only marginally discussed in Tsuji and Yoshikawa, 2010; Saito et al., 2009; Yamaji et al., 2009; Carrara et al. 2009, Sun and Chiu, 2005; Dominak and Keating, 2007; Lohse et al., 2008), as well as an explicit approach that take into account the whole vesicle population instead of focusing on single vesicles (competition and selection, see Stano, 2007; Chen and Szostak, 2004; Cheng and Luisi, 2003; and cooperation). From the technical viewpoint, it is remarkable the use and the possible future developments of microfluidic devices for producing and filling giant vesicles (Ota et al. 2009).

A more general discussion, on the other hand, must focus on the relevance of semi-synthetic cells as primitive cell models. Clearly, the compounds used to build a semi-synthetic cell are not primitive, and the resulting semi-synthetic cell is "minimal" in the sense of minimal number of functions. In other words, simplicity of minimal cell does not necessarily translate into primitiveness. In other words, one has to also point to simpler cellular models, highlighting chemical and physical aspects of minimal cells, which are still not completely clear. Some efforts have been done in this direction by the group of Szostak, who recently reviewed the main results of his research and the issue of constructive approach (Mansy and Szostak, 2009; Schrum et al., 2010). In order to build more primitive cell models it is necessary to complement the notion of minimal cells with more basic models, and several strategies can be tested. For instance, one

could focus on the synthesis of very simple polypeptides, or by implementing some small metabolic network, or exploiting the catalytic properties of small peptides (such as Ser-His, see Li et al., 2000; Gorlero et al., 2008), peptide-membrane interaction, and the reduction of ribosome complexity. For example, Chris Thomas, a former PhD student of Luisi's group, and Erica D'Aguanno (graduate student), studied the interaction of rRNA with poly-L-arginine, showing that stable complexes, in definite molar ratio, form rapidly and spontaneously by simple mixing the two components. The resulting complexes show a compact structure as evident by cryo-TEM imaging and dynamic light scattering, and have similar dimension and gross form of ribosomes. This may suggest a simple origin for ribosome particles as ribonucleic acid/basic peptide complexes.

In summary, research on synthetic cells is now flourishing after a long "incubation" stage. Although limited, the number of groups interested in such research is increasing, and the issue of creating compartment-based cell model is approached from the experimental as well as modeling (Solé et al., 2007; Rasmussen et al., 2009) viewpoints. We are confident that synthetic cell studies will impact on basic biological knowledge, especially in revealing physico-chemical and dynamic aspects of cell-like functions, as well as by becoming important tools in biotechnology and drug delivery.

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References

Amidi, M., de Raad, M., de Graauw, H., van Ditmarsch, D., Hennink, W. E., Crommelin, D. J. A., and Mastrobattista, E. (2010). Optimization and quantification of protein synthesis inside liposomes. *Journal of Liposome Research*, 20:73-83.

Asahara, H., and Chong, S. (2010). In vitro genetic reconstruction of bacterial transcription initiation by coupled synthesis and detection of RNA polymerase holoenzyme, *Nucleic Acid Research*, doi:10.1093/nar/gkq377.

Berclaz, N., Bloechliger, E., Mueller, M., and Luisi, P. L. (2001). Matrix effect of vesicle formation as investigated by cryo-transmission electron microscopy. *Journal of Physical Chemistry B*, 105:1065-1071.

Bitbol, M., and Luisi, P. L. (2004). Autopoiesis with or without cognition: defining life at its edge. *Journal of the Royal Society Interface*, 1:99-107.

Blochliger, E., Blocher, M., Walde, P., and Luisi, P. L. (1998). Matrix effect in the size distribution of fatty acid vesicles. *Journal of Physical Chemistry*, 102:10383-10390.

Carrara, P., Stano, P., and Luisi, P. L. (2009). Giant vesicles and w/o emulsions as biochemical reactors. *Origins of Life and Evolution of Biospheres*, 39:308-308.

Chen, I. A., Roberts, R. W., and Szostak, J. W. (2004). The emergence of competition between model protocells. *Science*, 305:1474-1476.

Cheng, Z., and Luisi, P. L. (2003). Coexistence and mutual competition of vesicles with different size distributions. *Journal of Physical Chemistry B*, 107:10940-10945.

Chiarabelli, C., Stano, P., and Luisi, P. L. (2009). Chemical approaches to synthetic biology. *Current Opinion in Biotechnology*, 20:492-497.

Cronin, L., Krasnogor, N., Davis, B. G., Alexander, C., Robertson, N., Steinke, J. H., Schroeder, S. L., Khlobystov, A. N., Cooper, G., Gardner, P. M., Siepmann, P., Whitaker, B. J., and Marsh, D. (2006). The imitation game – A computational chemical approach to recognizing life. *Nature Biotechnology*, 24:1203-1206.

Dominak, L. M., and Keating, C. D. (2007). Polymer encapsulation within giant lipid vesicles. *Langmuir*, 23:7148-7154.

Fiordemondo, D., and Stano, P. (2007). Lecithin-based water-in-oil compartments as dividing bioreactors. *ChemBioChem*, 8:1965-1973.

Forster, A. C., and Church, G. M. (2006). Towards synthesis of a minimal cell. *Molecular Systems Biology*, 2:45.

Gardner, P. M., Winzer, K., and Davis, B. G. (2009). Sugar synthesis in a protocellular model leads to a cell signalling response in bacteria, *Nature Chemistry*, 1:377-383.

Gil, R., Silva, F. J., Peretó, J., and Moya, A. (2004). Determination of the core of a minimal bacteria gene set. *Microbiology Molecular Biology Reviews*, 68:518-537.

Gorlero, M., Wieczorek, R., Adamala, K., Giorgi, A., Schininà, M. E., Stano, P., and Luisi, P. L. (2008). Ser-His catalyses the formation of peptides and PNAs. *FEBS Letters*, 583:153-156.

Hasoda, K., Sunami, T., Kazuta, Y., Matsuura, T., Suzuki, H., and Yomo, T. (2008). Quantitative study of the structure of multilamellar giant liposomes as a container of protein synthesis reaction. *Langmuir*, 24:13540–13548.

Kurihara, K., Takakura, K., Suzuki, K., Toyota, T., and Sugawara, T. (2010). Cell-sorting of robust self-reproducing giant vesicles tolerant to a highly ionic medium. *Soft Matter*, 6:1888-1891.

Kuruma, Y., Stano, P., Ueda, T., and Luisi, P. L. (2009). A synthetic biology approach to the construction of membrane proteins in semi-synthetic minimal cells. *Biochimica et Biophysica Acta*, 1788:567–574.

Li, Y., Zhao, Y., Hatfield, S., Wan, R., Zhu, Q., Li, X., McMills, M., Ma, Y., Li, J., Brown, K.L., He, C., Liu, F. and Chen, X. (2000). Dipeptide Ser-His and related oligopeptides cleave DNA, proteins and a carboxyl ester. *Bioorganic Medical Chemistry*, 8:2675–2680.

Liu, A. P., and Fletcher, D. A. (2009). Biology under construction: in vitro reconstruction of cellular function. *Nature Reviews*, 10:644–650.

Lohse, B., Bolinger, P.-Y., and Stamou, D. (2008). Encapsulation efficiency measured on single small unilamellar vesicles. *Journal of the American Chemical Society*, 130:14372–14373.

Lonchin, S., Luisi, P. L., Walde, P., and Robinson, B. H. (1999). A matrix effect in mixed phospholipid/fatty acid vesicle formation. *Journal of Physical Chemistry B*, 103:10910-10916.

Luisi, P. L. (2003). Autopoiesis: a review and a reappraisal. *Naturwissenschaften*, 90:49-59.

Luisi, P. L. (2007). Chemical aspects of synthetic biology. *Chemistry and Biodiversity*, 4:603-621.

Luisi, P. L., Allegreti, M., Souza, T., Steiniger, F., Fahr, A., and Stano, P. (submitted) Spontaneous protein crowding in liposomes: A new vista for the origin of cellular metabolism.

Luisi, P. L., Ferri, F., and Stano, P. (2006). Approaches to semi-synthetic minimal cells: a review. *Naturwissenschaften*, 93:1-13.

Luisi, P. L., Oberholzer, T., and Lazcano A. (2002). The notion of a DNA minimal cell: A general discourse and some guidelines for an experimental approach. *Helvetica Chimica Acta*, 85:1759-1777.

Luisi, P. L., Souza, T., and Stano, P. (2008). Vesicle behavior: In search of explanations. *Journal of Physical Chemistry B*, 112:14655-14664.

Mansy, S. S., and Szostak J.W. (2009). Reconstructing the emergence of cellular life through the synthesis of model protocells. *Cold Spring Harbor Symposia on Quantitative Biology*, doi: 10.1101/sqb.2009.74.014

Maturana, H. R, and Varela, F. J. (1980). *Autopoiesis and cognition: the realization of the living*. Reidel, Dordrecht

Murtas, G. (2009). Internal lipid synthesis and vesicle growth as a step toward self-reproduction of the minimal cell. *Systems Synthetic Biology*, doi: 10.1007/s11693-009-9048-1.

Oberholzer, T., Nierhaus. K. H., and Luisi, P. L. (1999). Protein expression in liposomes. *Biochemical Biophysical Research Communications*, 261:238-241.

Ota, S., Yoshizawa, S., and Takeuchi, S. (2009). Microfluidic formation of monodisperse, cell-sized, and unilamellar vesicles. *Angewantde Chemie International Edition English*, 48:6533-6537.

Rasi, S., Mavelli, F., and Luisi, P. L. (2003). Cooperative micelle binding and matrix effect in oleate vesicle formation. *Journal of Physical Chemistry B*, 107:14068-14076.

Rasmussen, S., Bedau, M. A., Chen, L., Deamer, D., Krakauer, D. C., Packard, N. H., Stadler, P. F. editors (2009). *Protocells: Bridging Nonliving and Living Matter*, MIT Press, Cambridge, Massachusetts.

Saito, H., Kato. Y., Le Berre, M., Yamada, A., Inoue, T., Yoshikawa, K., and Baigl, D. (2009). Time-resolved tracking of a minimum gene expression system reconstituted in giant liposomes. *ChemBioChem*, 10:1640–1643.

Schrum, J. P., Zhu, T. F., and Szostak, J. W. (2010). The origins of cellular life. *Cold Spring Harbor Perspectives Biology*, doi: 10.1101/cshperspect.a002212.

Shimizu, Y., Inoue, A., Tomari, Y., Suzuki, T., Yokogawa, T., Nishikawa, K., and Ueda, T. (2001). Cell-free translation reconstituted with purified components. *Nature Biotechnology*, 19:751-755.

Solé, R. V., Rasmussen, S., and Bedau, M. editors (2007). *Towards the artificial cell. (Vol 362) Philosophical Transaction of the Royal Society B.*

Souza, T., Stano, P., and Luisi, P. L. (2009). The minimal size of liposome based model cells brings about a remarkably enhanced entrapment and protein synthesis. *ChemBioChem*, 10:1056–1063.

Stano, P. (2007). Question 7: New aspects of interactions among vesicles. *Origins of Life and Evolution of Biospheres*, 37:439-444.

Stano, P. (2010). Synthetic biology of minimal living cells: primitive cell models and semi-synthetic cells. *Systems and Synthetic Biology*, doi: 10.1007/s11693-010-9054-3.

Stano, P., and Luisi, P. L. (2010). Achievements and open questions in the self-reproduction of vesicles and synthetic minimal cells. *ChemComm*, 46:3639-3653.

Stano, P., Wehrli, E., and Luisi, P. L. (2006). Insights on the oleate vesicles self-reproduction. *Journal of Physics: Condensed Matter*, 18:S2231-S2238.

Sun, B., and Chiu, D. (2005). Determination of the encapsulation efficiency of individual vesicles using single-vesicle photolysis and confocal single-molecule detection. *Analytical Chemistry*, 77:2770-2776.

Tsuji, A., and Yoshikawa, K. (2010). Real-time monitoring of RNA synthesis in a phospholipid-coated microdroplet as a live-cell model. *Chembiochem*, 11:351-357.

Walde, P., Wick, R., Fresta, A., Mangone, A, and Luisi, P. L. (1994) Autopoietic self-reproduction of fatty acid vesicles. *Journal of the American Chemical Society* 116:11649-11654.

Yamaji, K., Kanai, T., Nomura, S. M., Akiyoshi, K., Negishi, M., Chen, Y., Atomi, H., Yoshikawa, K., and Imanaka, T. (2009). Protein synthesis in giant liposomes using the in vitro translation system of Thermococcus kodakaraensis. *IEEE Transactions on Nanobioscience*, 8:325-331.

Yu, W., Sato, K., Wakabayashi, M., Nakatshi, T., Ko-Mitamura, E. P., Shima, Y., Urabe, I., and Yomo, T. (2001). Synthesis of functional protein in liposome. *Journal of Bioscience and Bioengineering*, 92:590–593.

Zhang, Y., Ruder, W. C., and LeDuc, P. R. (2008). Artificial cells: building bioinspired systems using small-scale biology. *TRENDS in Biotechnology*, 26:14–20.

Zhou, J., Huang, L., Lian, J., Sheng, J., Cai, J., and Xu, Z. (2010). Reconstruction of the UDP-*N*-acetylglucosamine biosynthetic pathway in cell-free system. *Biotechnology Letters*, doi: 10.1007/s10529-010-0315-8.

Zhu, T. F., and Szostak, J. W. (2009). Coupled growth and division of model protocell membranes. *Journal of the American Chemical Society*, 131:5705–5713.