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REVIEW

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Approaches to semi-synthetic minimal cells: a review

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Abstract Following is a synthetic review on the minimal living cell, defined as an artificial or a semi-artificial cell having the minimal and sufficient number of components to be considered alive. We describe concepts and experiments based on these constructions, and we point out that an operational definition of minimal cell does not define a single species, but rather a broad family of interrelated cell-like structures. The relevance of these researches, considering that the minimal cell should also correspond to the early simple cell in the origin of life and early evolution, is also explained. In addition, we present detailed data in relation to minimal genome, with observations cited by several authors who agree on setting the theoretical full-fledged minimal genome to a figure between 200 and 300 genes. However, further theoretical assumptions may significantly reduce this number (i.e. by eliminating ribosomal proteins and by limiting DNA and RNA polymerases to only a few, less specific molecular species). Generally, the experimental approach to minimal cells consists in utilizing liposomes as cell models and in filling them with genes/enzymes corresponding to minimal cellular functions. To date, a few research groups have successfully induced the expression of single proteins, such as the green fluorescence protein, inside liposomes. Here, different approaches are described and compared. Present constructs are still rather far from the minimal cell, and experimental as well as theoretical difficulties opposing further reduction of complexity are discussed. While most of these minimal cell constructions may represent relatively poor imitations of a modern full-fledged cell, further studies will begin precisely from these constructs. In conclusion, we give a brief outline of the next possible steps on the road map to the minimal cell.

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The notion of minimal cell

The simplest living cells existing on Earth have several hundred genes, with hundreds of expressed proteins, which, more or less simultaneously, catalyse hundreds of reactions within the same tiny compartment—an amazing enormous complexity.

This picture elicits the question of whether or not such complexity is really essential for life, or whether or not cellular life might be possible with a much smaller number of components. This question is also borne out of considerations on early cells, which could not have been as complex. The enormous complexity of modern cells is probably the result of billions of years of evolution in which a series of defence and security mechanisms, redundancies and metabolic loops (which, in highly permissive conditions, were probably not necessary) was developed. These considerations led to the notion of minimal cell, now broadly defined as a cell having the minimal and sufficient number of components to be considered alive. This automatically precedes the next fundamental, but complex, question, 'What does "alive" mean?' One may choose quite a general definition, defining life at a cellular level as the concomitance of three basic properties: self-maintenance (metabolism), self-reproduction and evolvability (Fig. 1).

Evolvability is a Darwinian notion. As such, it refers to populations rather than individual cells. Consequently, one should take into consideration an entire family of minimal cells in the stream of environmental pressure and corresponding genetic evolution.

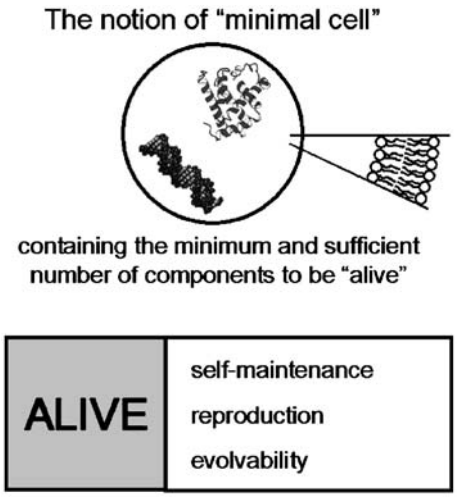

Fig. 1 The notion of minimal cell. As explained in the text, this definition does not identify one particular structure, but is rather a descriptive term for a wide variety of minimal cells

The trilogy defining cellular life may not be perfectly implemented, particularly in synthetic constructs, and several kinds of approximations to cellular life can be envisaged. For example, we may have protocells capable of self-maintenance but not of self-reproduction, or vice versa. Or we might have protocells in which self-reproduction is active for only a few generations, or systems that are not capable of evolvability. In any given type of minimal cell (e.g. one with all three attributes), there may be quite different ways of implementation and sophistication. So, clearly, the term 'minimal cell' depicts large families of possibilities and not simply one particular construct. The idea that the minimal forms of life are not univocally defined, and correspond rather to a large family, is not new in the field of the origin of life and early evolution. However, it is important to keep in mind that we are not simply considering theoretical possibilities, but something new: a synthetic biology approach and the particular methodology of experimental implementation.

The question on minimal cell has been considered for many years. One should recall, in particular, the work of Morowitz [35], who estimated that the size of a minimal cell should be about one tenth smaller than *Mycoplasma genitalium*, based on enzymatic components of primary metabolism. Earlier insights of significance in the field were provided by Jay and Gilbert [19], Woese [67] and Dyson [7]. More recently, reviews by Pohorille and Deamer [50], Luisi [28] and Oberholzer and Luisi [42] have sharpened the question and have brought it to the perspective of modern molecular tools. In fact, the last years have seen a significant revival of interest in the field of the minimal cell. In this article, we wish to review this work, emphasizing experimental aspects. Over the last few years, many theoretical approaches to minimal forms of life have been presented in the literature, but they will be not discussed in this review. This is not out of lack of interest in them, but out of the desire to focus this review on the art of synthetic biology of minimal cells. The idea of writing a review on the subject was also prompted by the rise in interest that the field of minimal cell has been witnessing over the last few years, as documented, for example, by two international meetings on the subject held last year.[1]

To put this work into a more concrete perspective, it is useful to first look at the smallest unicellular organisms on Earth, focusing on the notion of minimal genome.

The minimal genome

Figure 2 compares genome size distributions calculated in a series of assumptions [17] of free-living prokaryotes, obligate parasites, thermophiles and endosymbionts. DNA contents of free-living prokaryotes can vary over a tenfold range, from 1,450 kb for *Halomonas halmophila* to 9,700 kb for *Azospirillum lipoferum* Sp59b. In comparison, consider that *Escherichia coli* K-12 has a genome size of ca. 4,640 kb, and *Bacillus subtilis* has a genome size of 4,200 kb.

Classification of endosymbionts as a separate group shows that their DNA content may be significantly smaller; the smallest sizes are then those of *M. genitalium* and *Buchnera*, with a value that confirms the predictions of Shimkets [55], who states that the minimum genome size for a living organism should approximately be around 600 kb. It is argued that these two organisms have undergone massive gene losses and that their limited encoding capacities are due to their adaptation to highly permissive intracellular environments provided by the hosts [17].

What do these figures mean in terms of minimal gene numbers?

Table 1, also taken from Islas et al. [17], reports the number of coding regions in some small genomes. The table also gives an account of redundant genes, amounting to an average of 6–20% of the whole genome. How can one work with the data of Table 1 to envisage further simplifications of the genome?

Gil et al. [13], in Valencia, asked this question and arrived at the smaller number of 206 genes, basing the figure on their work with *Buchnera* spp. and other organisms. The results are given in Table 2.

Notice that the figures provided by Gil et al. are close to those obtained by other authors based on different considerations, as summarized in Table 3, which reports the most salient data relative to minimal genome calculations and observations. In fact, the question of the minimal genome has been considered, for example, by Mushegian [36], Shimkets [55], Mushegian and Koonin [37], Kolisnychenko et al. [20] and Koonin [21, 22]. In particular, Mushegian and Koonin

[1] The international meetings were the Third COST D27 Workshop held in Crete in October 2004 (http://cost.cordis.lu/src/action_detail.cfm?action=D27) and The International School on Complexity held in Erice, Sicily, in December 2004 (http://www.ccsem.infn.it). See also Szathmáry (2005).

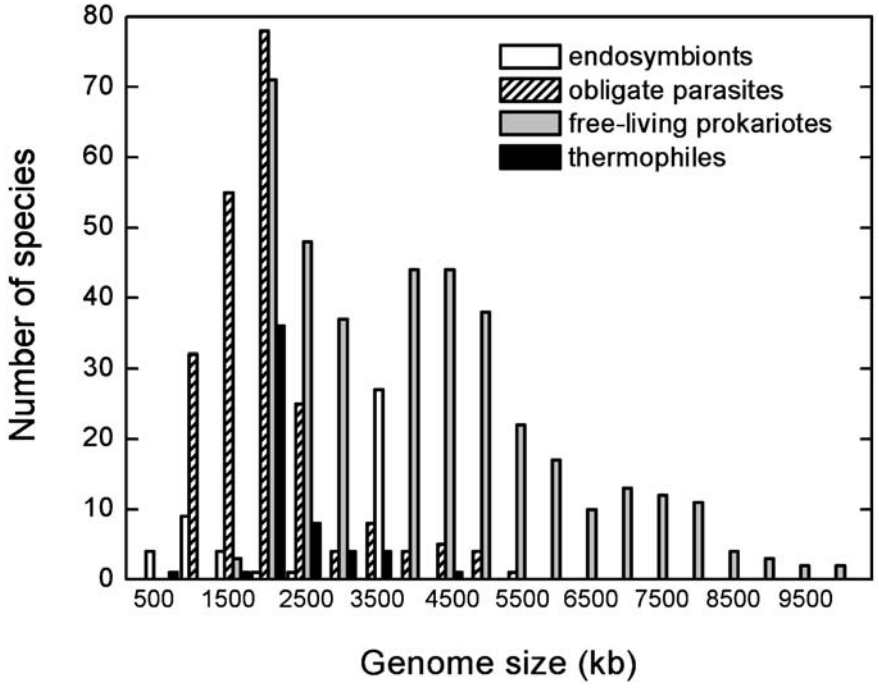

Fig. 2 Prokaryotic genome size distribution (N=641). Genome sizes, complete proteomes and the number of open reading frames were all retrieved from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) (Adapted from Islas et al. [17])

[37] calculated an inventory of 256 genes, which represents the amount of DNA required to sustain a modern type of minimal cell in permissible conditions. This number, as indicated later by Koonin [21, 22], is quite similar to the values of viable minimal genome sizes inferred by site-directed gene disruptions in *B. subtilis* [18] and transposon-mediated mutagenesis knockouts in *M. genitalium* and *Mycoplasma pneumoniae* [15]. Concerning this last work, one may recall that the notion of the 'minimal genome' is approached in quite a different way by Hutchinson et al. In a study carried out at the Institute for Genomic Research in Rockville, MD, Hutchinson et al. knocked out genes from a *M. genitalium* bacterium one by one, and they estimated that of the 480 protein-coding regions, about 265–350 are essential in laboratory growth conditions, including about 100 genes of unknown functions [15].

Taking a step further, the idea was to remove the original genetic material from the bacterium and to insert the

Table 1 Genetic redundancies in small genomes of endosymbionts and obligate parasites[a]

Proteome	Genome size (kb)	Number of ORFs	Number of redundant sequences	Redundancy (%)
Mycoplasma genitalium	580	480	52	10.83
Mycoplasma pneumoniae	816	688	134	19.48
Buchnera sp. APS	640	574	67	11.67
Ureaplasma urealyticum	751	611	105	17.18
Chlamydia trachomatis	1,000	895	60	6.70
Chlamydia muridarum	1,000	920	60	6.52
Chlamydophila pneumoniae J138	1,200	1,070	148	13.83
Rickettsia prowazekii	1,100	834	49	5.88
Rickettsia conorii	1,200	1,366	189	13.84
Treponema pallidum	1,100	1,031	78	7.57

ORFs Open reading frames
[a]Genome sizes, complete proteomes and the number of ORFs were all retrieved from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov)

Table 2 Core of a minimal bacterial gene set[a]

DNA metabolism	16
Basic replication machinery	13
DNA repair, restriction and modification	3
RNA metabolism	106
Basic transcription machinery	8
Translation: aminoacyl-tRNA synthesis	21
Translation: tRNA maturation and modification	6
Translation: ribosomal proteins	50
Translation: ribosome function, maturation and modification	7
Translation factors	12
RNA degradation	2
Protein processing, folding and secretion	15
Protein post-translational modification	2
Protein folding	5
Protein translocation and secretion	5
Protein turnover	3
Cellular processes	5
Energetic and intermediary metabolism	56
Poorly characterized	8
Total	206

[a]Courtesy of Prof. A. Moya (Institut Cavanilles de Biodiversitat i Biologia Evolutiva, Universitat de València)

Table 3 Works on the minimal genome

Description of the system	Main goal and results	References
The complete nucleotide sequence (580,070 bp) of the *M. genitalium* genome has been determined by whole-genome random sequencing and assembly	Only 470 predicted coding regions were identified (genes required for DNA replication, transcription and translation; DNA repair; cellular transport; and energy metabolism)	Fraser et al. [9]
Site-directed gene disruption in *B. subtilis*	Values of viable minimal genome size were inferred	Itaya [18]
The 468 predicted *M. genitalium* protein sequences were compared with 1,703 protein sequences encoded by the other completely sequenced small bacterial genome, that of *Haemophilus influenzae*	A minimal self-sufficient gene set: the 256 genes that are conserved in Gram-positive and Gram-negative bacteria are almost certainly essential for cellular function	Mushegian and Koonin [37]
Computational analysis (quantification of gene content, of gene family expansion and of orthologous gene conservation, as well as their displacement)	A set close to 300 genes was estimated as the minimal set sufficient for cellular life	Mushegian [36]
Global transposon mutagenesis was used to identify non-essential genes in *Mycoplasma* genome	265–350 of the 480 protein-coding genes of *M. genitalium* are essential in laboratory growth conditions, including about 100 genes of unknown function	Hutchinson et al. [15]
Several theoretical and experimental studies are reviewed	The concept of minimal gene set	Koonin [21]
The article focuses on the notion of a DNA minimal cell	The conceptual background of the minimal genome is discussed	Luisi et al. [31]
Full-length poliovirus cDNA was synthesized by assembling oligonucleotides of plus and minus strand polarity	It is possible to create an infectious poliovirus, which is much simpler than a bacterium, by a synthetic approach	Cello et al. [5]
A technique for precise genomic surgery was developed and applied to delete the largest K-islands of *E. coli*, which are identified by comparative genomics as recent horizontal acquisitions to the genome	Twelve K-islands were successfully deleted, resulting in an 8.1% reduction in genome size, a 9.3% reduction of gene count and elimination of 24 of 44 transposable elements of *E. coli*; the goal was to construct a maximally reduced *E. coli* strain to serve as a better model organism	Kolisnychenko et al. [20]
Physical mapping of *Buchnera* genomes obtained from five aphid lineages	They suggest that the *Buchnera* genome still experiences a reductive process towards a minimum set of genes necessary for its symbiotic lifestyle	Gil et al. [12]
Computational and experimental methods on comparative genomics	60 proteins are common to all cellular life; a core of 500–600 genes should represent the gene set of the last universal common ancestor	Koonin [22]
Buchnera and other organism genomes were compared	206 genes were identified as the core of a minimal bacterial gene set	Gil et al. [13]
Comparative genomics	Estimates of the size of minimal gene complement were performed to infer the primary biological functions required for a sustainable, reproducible cell today and throughout evolutionary times	Islas et al. [17]

synthetic one to see whether it works or not [70]. This approach had already been used by Cello et al. [5] at Stony Brook to create an infectious poliovirus that is much simpler than a bacterium.

We have reached the number of 200–300 genes as the minimal genome. This is a considerable simplification of the initial number, but it still corresponds to a formidable complexity, which, once again, induces the question of whether and how it can further go down.

Further speculations

Obviously, only speculations can help us at this point. Imagine a kind of theoretical knock-down of the genome that simultaneously reduces cellular complexity and part of non-essential functions [31].

The first pit stop of this intellectual game is to imagine that a cell without enzymes (then the corresponding genes) needed to synthesize low molecular weight compounds—assuming that low molecular weight compounds, including nucleotides and amino acids, were available in the surrounding medium and were able to permeate into the cell membrane. This would be an entirely permeable minimal cell. Further simplifications [31] finally bring us to a cell that is able to perform protein and lipid biosyntheses through a modern ribosomal system, but is limited to a rather restricted number of enzymes (see Table 4). This cell would have ca. 25 genes for the entire DNA/RNA synthetic machinery, ca. 120 genes for the entire protein synthesis (including RNA synthesis and 55 ribosomal proteins) and 4 genes for membrane synthesis—which brings us to a total of about 150 genes, somewhat less than Gil et al.'s previously introduced figure of 206.

Thanks to the outside supply of substrates, such a cell should be capable of self-maintenance and self-reproduction, including replication of membrane components. However, it would neither synthesize low molecular weight compounds nor have redundancies for its own defence and security (in fact, all self-repair mechanisms are missing). Furthermore, cell division would simply be due to a physically based statistical process.

There is, however, no proof that this theoretical construct would be viable, but this also goes for Gil et al.'s 206 genes. It is nevertheless instructive to take these theoretical knock-down experiments further, with the next victims being ribosomal proteins. Can we take them out? Some indications suggest that ribosomal proteins may not be essential for protein synthesis [69], and there are other suggestions about an ancient and simpler translation system [4, 38].

Of course, this sort of discussion takes us directly into the scenario of early cells at the origin of life; in fact, some claim that the first ribosomes consisted of rRNA associated simply with basic peptides [66]. If we accept this and take out the 55 genes for ribosomal proteins and some other enzymes, we would then have a number of genes around 110.

Further reductions

A large portion of foreseen genes corresponds to RNA and DNA polymerases. A number of data [10, 24, 25, 58] suggest that a simplified replicating enzymatic repertoire, as well as a simplified version of protein synthesis, might be possible. In particular, the idea that a single polymerase could play multiple roles as a DNA polymerase, transcriptase and primase is conceivable in very early cells [31].

The game could go on by assuming that, at the time of early cells, not all 'our' 20 amino acids were involved and that a lower number of amino acids would reduce the number of aminoacyl-tRNA synthetases and tRNA genes.

Table 4 A hypothetical list of gene products, sorted by functional category, that defines the minimal cell according to the definitions used in this paper

Gene product	Number of genes		
	Minimal DNA cell[a]	'Simple ribosome' cell	Extremely reduced cell
DNA/RNA metabolism			
DNA polymerase III	4[b]	4[b]	1
DNA-dependent RNA polymerase	3[c]	3[c]	1
DNA primase	1	1	
DNA ligase	1	1	1
Helicases	2–3	2–3	1
DNA gyrase	2[d]	2[d]	1
Single-stranded DNA-binding proteins	1	1	1
Chromosomal replication initiator	1	1	
DNA topoisomerases I and IV	1+2[d]	1+2[d]	1
ATP-dependent RNA helicase	1	1	
Transcription elongation factor	1	1	
RNase (III, P)	2	2	
DNase (endo/exo)	1	1	
Ribonucleotide reductase	1	1	1
Protein biosynthesis/ translational apparatus			
Ribosomal proteins	51	0	0
Ribosomal RNA	1[e]	1[e]	1[e] (Self-splicing)
Aminoacyl-tRNA synthetases	24	24	14[f]
Protein factors required for biosynthesis and membrane protein synthesis	9–12[g]	9–12[g]	3
tRNA	33	33	16[h]
Lipid metabolism			
Acyltransferase 'plsX'	1	1	1
Acyltransferase 'plsC'	1	1	1
PG synthase	1	1	1
Acyl carrier protein	1	1	1
Total	146–150	105–107	46

[a]Based on *M. genitalium*
[b]Subunits a, b, y and tau
[c]Subunits a, b and b′
[d]Subunits a and b
[e]One operon with three functions (rRNA)
[f]Assuming a reduced code
[g]Including a possible limited potential to synthesize membrane proteins
[h]Assuming the third base to be irrelevant

All these considerations may help to decrease the number of genes down to a number of, say, 45–50 genes (see Table 4 for a living, although certainly limping, minimal cell) [31].

This number is significantly lower than the one proposed by Prof. Moya in Table 2, but is of course based on a higher degree of speculation. Many authors would doubt that a cell with only 45–50 genes would be able to work. But again, the consideration goes on to early cells and to the consideration that the first cells could have not started with dozens of genes from the very beginning in the same compartment. This last consideration permits a logical link with the notion of compartments.

Suppose that these 45–50 macromolecules, or their precursors, developed first in solution (i.e. let us forget for a moment the possibility of compartments). Then, to start cellular life, compartmentation should have come later on, and one would then have to assume a simultaneous entrapment of all these different genes in the same vesicle. This could indeed be regarded as highly improbable; in fact, a scenario in which the complexity of cellular life evolved from within the compartment is more reasonable—a situation where the 45 (or 206) macromolecules were produced and evolved from a much smaller group of components from inside the protocell.

Until now, we have speculated on 'normal' protein/DNA/RNA cells—the ones we know in nature. In a further speculative leap, we could ask the question, 'What about a theoretical RNA cell?' Let us briefly consider this question before proceeding further with the usual cells.

The minimal RNA cell

One of the simplest constructs that responds to the criteria of evolvability, self-maintenance and reproduction is the so-called 'RNA cell' (Fig. 3). This purely theoretical object, developed by Szostak et al. [60], represents a synthesis of RNA and compartment models.

In this case, the combined 'genetic' and catalysing properties of ribozymes play a central role. The RNA cell consists of a vesicle containing two ribozymes: one with replicase activity and the other with catalysing activity for the synthesis of membrane components. The first ribozyme is capable of replicating itself, and the second ribozyme is replicated by the first one. At the same time, a precursor is transformed into a membrane-forming compound, allowing the growth and subsequent division of the parent vesicle. In this way, a concerted core-and-shell reproduction of the entire construct may be obtained.

As mentioned previously, this is a hypothetical scheme based on not-yet-existing ribozymes and a series of additional assumptions (e.g. full permeability of the membrane to precursor A and nucleotides, both present in large excesses in the environment), or the assumption that the cell divides, distributing both kinds of ribozyme to the daughters (so that, in each cell, there are always first- and second-type ribozymes).

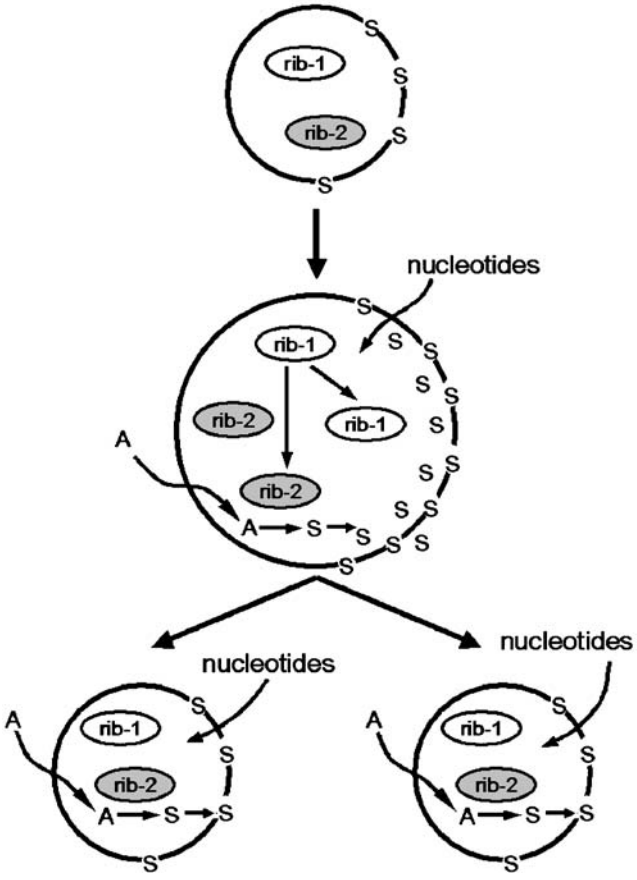

Fig. 3 The RNA cell, containing two ribozymes. Rib-1 is an RNA replicase capable of reproducing itself and making copies of Rib-2. Rib-2 is capable of synthesizing the cell membrane by converting precursor A to membrane-forming S. All necessary low molecular components required for macromolecular synthesis are provided from the surrounding medium and are capable of permeating the membrane (Adapted from Luisi et al. [31]). For the sake of simplicity, an ideal cell division is represented in this figure, where all core components are equally shared between new vesicles

In conclusion, the construct of Fig. 3, although quite exciting for its simplicity, remains a theoretical model, considering that the two ribozymes are still non-existent. In addition, the RNA cell, in a realistic scenario, must eventually evolve into the DNA/protein cell. Despite all these limits, the RNA cell is very interesting for one insight: it shows that, at least theoretically, cellular life can be implemented by a limited number of RNA genes.

Towards the construction of the minimal protein/DNA cell: setting the stage

Going back to the discussion on theoretical and practical backgrounds for the achievement of a minimal protein/DNA cell, it is certainly more complex than a minimal RNA cell, while, at the same time, it is more realistic and accessible from an experimental point of view, since all the ingredients exist. In fact, as already mentioned, self-

replicating ribozymes, although fascinating objects, are not available (and it is questionable whether they ever will be), whereas genes and enzymes of a protein/DNA are available. In particular, the question is whether the construction of the corresponding minimal cell is possible with present laboratory tools.

Traditionally, people working in the area of prebiotic chemistry have been pursuing the so-called bottom–up approach, based on the notion that a continuous and spontaneous increase of molecular complexity transformed inanimate matter into the first self-reproducing cellular entities. For a number of reasons, this approach has not yet been successful, and another approach to the construction of the minimal living cell has been proposed in the last few years (indicated in Fig. 4). We use extant nucleic acids and enzymes and insert them into a vesicle, thus reconstructing the minimal living cell.

While the term 'bottom–up' is recognized and accepted, this alternative route to the minimal cell is less clear and could give rise to different interpretations. The term 'top–down' has been used to indicate the use of extant cellular components (DNA and enzymes) to build simple cellular constructs. However, such terminology could be misunderstood, since, in a way, this is also a bottom–up approach, in the sense that it goes in the direction of increasing complexity (the cell) starting from single components (DNA and enzymes). Moreover, there are different possible interpretations of the terms 'top–down' and 'bottom–up' in the literature, and we believe that, to avoid confusion, the term 'reconstruction' is perhaps more appropriate in minimal cell studies, making it clear that, in this procedure, one does not necessarily reach the construction of an extant cell or something that exists on Earth. Since they do not exist in our biological life, the term 'artificial cell' may be used [50]. This is acceptable; however, since, generally, extant enzymes and genes are utilized, the term 'semi-artificial cell' might be considered more appropriate.

Having clarified this, the next point is to set the stage of the experimental approach. We need a cell-like compartment, with vesicles (liposomes when they are constituted by lipids) being the preferred candidates. Fig. 4 suggests that the incorporation of components into vesicles is the most obvious way to start. In fact, as already well known, several attempts have been carried out in this direction. In all these studies, as we will see, a reaction is supposed to take place in the inner water pool of the vesicles, but to begin with, one must also consider that the membrane surface can also function as a reaction site, particularly if hydrophobic compounds are used. However, this has not been studied in detail yet.

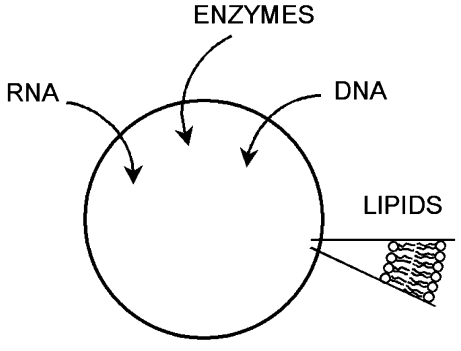

Fig. 4 The semi-synthetic approach to the construction of the minimal cell

This review aims to provide basic information that is limited to experimental approaches—a choice that implies neglecting the many theoretical models of minimal life provided by computer scientists and theoreticians of complexity.

Preliminaries: reactivity in vesicles

Two areas of study are preliminary to the utilization of liposomes as cellular models. The first considers possible analogies between vesicles and cellular membranes in terms of physico-chemical properties, such as stability, permeability and self-reproduction, to see whether (and to what extent) vesicles are close to cellular structures. The second area of inquiry considers the use of vesicles as hosts for complex molecular biological reactions to see if vesicles can indeed support the biochemistry of cellular life.

Concerning the first area of study, it has been shown that vesicles are capable of multiplying themselves at the expense of surfactant precursors [1, 32, 64]; in certain conditions, this may happen with the retention of the original size distribution (the so-called matrix effect; see Bloechliger et al. [3], Lonchin et al. [26] and Rasi et al. [51]). Again, it is not the aim of this article to review all these data, while it is important here to keep in mind that one of the most critical mechanisms of living cells can be simulated by vesicles based only on physical and chemical properties (i.e. without the use of sophisticated biochemical machineries). This consideration is relevant if one focuses on the prebiotic scenario.

Another important preliminary physico-chemical property is membrane permeability to solutes. Here things are more complicated, as vesicles and liposomes offer considerable resistance to the uptake of simple biochemicals in their water pool. This is particularly true with phospholipid membranes, which are commonly used as models for modern membrane bilayers. Note, however, that phospholipids are relatively modern compounds; most likely, the first membranes and vesicles were constituted by surfactants, which could offer higher permeability (although possibly less stability) by virtue of their presumable chemically heterogeneous composition. It is reasonable, in fact, that early cells might have been somehow more 'permissive' in terms of boundary properties and functions.

The use of membrane channels offers a possibility, but has, until now, met only modest success. An exception has been the use of α-hemolysin by Noireaux and Libchaber [39]. This approach has been quite successful. On the other hand, however, one should consider that, in more general terms, α-hemolysin pores are unselective and bidirectional and are therefore characterized by low specificity and are not very efficient due to gradient dissipation. In addition,

α-hemolysin cannot be considered a primeval protein, however precisely these features of low selectivity represent a scenario where the first unspecific protocells were developed. The main problem is how to bring together the high local concentration needed in the water pool of liposomes. This difficulty might be partially circumvented if two or more liposomes, each containing a given substrate, could fuse together to produce liposomes containing all reagents. In fact, fusion of vesicles is becoming an active area of research, and interesting results have been already obtained [33, 47, 57, 61].

Fusion of compartments can also be achieved by utilizing water-in-oil emulsion. Actually, in this way, as we will see further on in detail, protein synthesis could be obtained by mixing compartments containing various ingredients for synthesis.

Concerning the area of biochemical reactions in liposomes, a large amount of experimental work (mainly studies in which liposomes have been used as host systems for molecular biology reactions) has paved the way for significant developments (Table 5).

For example, biosynthesis of poly(A) (a model for RNA) was reported independently by two groups [6, 64]. In both cases, polynucleotide phosphorylase was entrapped in vesicles, and the synthesis of poly(A), which remained in the aqueous core of such vesicles, was observed. In one case [64], internal poly(A) synthesis proceeded simultaneously through the reproduction of vesicle shells due to external addition of a membranogenic precursor (oleic anhydride).

A more suggestive example was provided shortly thereafter [43] with the use of Qβ replicase, an enzyme that replicates RNA template. Also in this case, replication of a core component was coupled with replication of vesicle shell. With an excess of Qβ replicase/RNA template, replication of RNA could proceed for a few generations.

This system, as well as the previous one by Walde et al. [64], is interesting because it represents a case of 'core-and-shell replication' in which both the inside of the core and the shell itself undergo duplication. However, limitations of this analogy should be clear; in fact, a real core-and-shell reproduction should be synchronous, which was not the case.

In particular, even if the RNA template and the vesicle shell replicate, the Qβ replicase is not continuously produced in the process; thus, the system undergoes 'death by dilution'. After a while, new vesicles will not contain either the enzyme or the template; therefore, the construct cannot reproduce itself completely.

Another complex biochemical reaction implemented in liposomes is polymerase chain reaction (PCR) [43]. Liposomes were able to endure the hardships of PCR conditions, with several temperature cycles up to 90°C (liposomes were practically unchanged at the end of the reaction). In addition, nine different chemicals had to be encapsulated in each liposome for the reaction to occur. Depending on liposome formation mechanism and chemical concentration, entrapment efficiency can be different from what is expected on a statistical basis. In particular, it is not obvious that all nine chemicals are simultaneously trapped within one liposome.

Using poly(U) as mRNA, Oberholzer et al. [45] showed the production of poly(Phe), starting from phenylalanine, ribosomes, $tRNA^{Phe}$ and elongation factors entrapped in lecithin vesicles. Compared to the experiment in water without liposomes, the yield was 5%, but the authors argued that the yield was actually surprisingly high,

Table 5 Molecular biology reactions in liposomes

Description of the system	Main goal and results	References
Enzymatic poly(A) synthesis	Polynucleotide phosphorylase producing poly(A) from ADP	Chakrabarti et al. [6]
Enzymatic poly(A) synthesis	Poly(A) is produced inside simultaneously with the (uncoupled) self-reproduction of vesicles	Walde et al. [64, 65]
Oleate vesicles containing the enzyme Qβ replicase, an RNA template and ribonucleotides; the water-insoluble oleic anhydride was added externally	A first approach to a synthetic minimal cell: the replication of an RNA template proceeded simultaneously with the self-replication of the vesicles	Oberholzer et al. [43, 44]
POPC liposomes containing all different reagents necessary to carry out a PCR reaction	DNA amplification by the PCR inside the liposomes; a significant amount of DNA was produced	Oberholzer et al. [43, 44]
POPC liposomes incorporating the ribosomal complex with the other components necessary for protein expression	Ribosomal synthesis of polypeptides can be carried out in liposomes; synthesis of poly(Phe) was monitored by quantification of ^{14}C-labelled products	Oberholzer et al. [45]
T7 DNA within cell-sized giant vesicles formed by natural swelling of phospholipid films	Transcription of DNA and transportation by laser tweezers; vesicles behaved as barriers, preventing the attack of RNase	Tsumoto et al. [62]
DNA template and the enzyme T7 RNA polymerase microinjected into a selected giant vesicle; nucleotide triphosphates added from the external medium	The permeability of giant vesicles increased in an alternating electric field; mRNA synthesis occurred	Fischer et al. [8]

POPC 1-Palmitoyl-2-oleoyl-phosphatidylcholine

considering that the liposomes occupied only a very small fraction of the total volume and that only a very few of them would contain all ingredients by statistical entrapment [45].

The table also reports the work of Fischer et al. [8] on mRNA synthesis inside giant vesicles utilizing DNA template and T7 RNA polymerase and that of Tsumoto et al. [62] on DNA transcription. Further considerations on polymerase activity inside vesicles were reported by Monnard [34].

Protein expression in liposomes

In "Preliminaries: reactivity in vesicles", we have seen the realization and optimization of rather complex biochemical reactions in liposomes. What could one do to approach the construction of the minimal cell? Theoretically, one should increase the complexity of the core of the liposomes so as to reach the limits on the minimal genome, as outlined previously.

This approach has not been used in the literature until now. Researchers have sought to insert conditions for the expression of a single protein in liposomes. For reasons that are easily understood (mostly for detection), the green fluorescence protein (GFP) has been the target protein.

With how many genes? Well, the answer to this question is also not easy to elicit from current data, as generally a calculation of the genes/enzymes involved has not been performed by the authors. Often, commercial kits are used for protein expression, and these are notoriously black boxes where the number of enzymes is not made known (and occasionally entire *E. coli* cellular extracts have been utilized). On the other hand, it is fair to say that, for the expression of one single protein, only a minimal part of the *E. coli* genome is implied.

An overview of the work performed, limited to the expression of proteins in liposomes, is presented in Table 6.

The common strategy is to entrap all the ingredients for in vitro protein expression [i.e. the gene for the GFP (a plasmid), an RNA polymerase, ribosomes and all the low molecular weight components (amino acids and ATP) needed for protein expression] in the aqueous core of liposomes.

Yu et al. [68], for example, have reported the expression of a mutant GFP in lecithin liposomes. Large GFP-expressing vesicles, prepared by the film hydration method, were analysed using flow cytometry as well as confocal laser microscopy.

In the procedure utilized by Oberholzer and Luisi [42], all ingredients were added to a solution in which the vesicles were formed by the ethanol injection method and enhanced GFP (EGFP) production was then evidenced inside the compartments. In this case, the sample was analysed spectroscopically, monitoring the increase of the fluorescent signal of the EGFP. The disadvantage of this procedure is that entrapping efficiency is generally low due to the small internal volume of liposomes obtained with this method. On the other hand, the observation of EGFP production inside the aqueous core of liposomes confirms that the coentrapment of several different solutes was obtained.

A direct observation of protein expression was accomplished by the procedure utilized by Nomura et al. [41] using giant vesicles. The progress of the reaction is observed by laser scanning microscopy, and it is shown that expression of red shifted GFP (rsGFP) takes place with a very high efficiency (the concentration of rsGFP inside the vesicles was greater, in the first hours, than that in the external environment). The authors also show that vesicles can protect gene products from external proteinase K.

More recently, based on the initial report on the expression of functional protein in liposomes [68], Ishikawa et al. [16] were able to design and produce experimentally a two-level cascading protein expression. A plasmid contain-

Table 6 Protein expression in compartments

Description of the system	Main goal and results	References
Liposomes from EggPC, cholesterol and DSPE-PEG5000 used to entrap cell-free protein synthesis	Expression of a mutant GFP, determined with flow cytometric analysis	Yu et al. [68]
Small liposomes prepared by the ethanol injection method	Expression of EGFP evidenced by spectrofluorimetry	Oberholzer and Luisi [42]
Gene expression system within cell-sized lipid vesicles	Encapsulation of a gene expression system; high expression yield of GFP inside giant vesicles	Nomura et al. [41]
A water-in-oil compartment system with water bubbles up to 50 μm	Expression of GFP by mixing different compartments that are able to fuse with each other	Pietrini and Luisi [49]
A two-stage genetic network encapsulated in liposomes	A genetic network in which the protein product of the first stage (T7 RNA polymerase) is required to drive the protein synthesis of the second stage (GFP)	Ishikawa et al. [16]
E. coli cell-free expression system encapsulated in a phospholipid vesicle, which was transferred into a feeding solution containing ribonucleotides and amino acids	The expression of the α-hemolysin inside the vesicle solved the energy and material limitations; the reactor could sustain expression for up to 4 days	Noireaux and Libchaber [39]

ing the T7 RNA polymerase (with SP6 promoter) and a mutant GFP (with T7 promoter) gene was constructed and entrapped in liposomes, together with an in vitro protein expression mixture (of the enzyme SP6 RNA polymerase). In these conditions, SP6 RNA polymerase drives the production of T7 RNA polymerase, which in turn induces the expression of detectable GFP.

Of particular interest is the work by Noireaux and Libchaber [39]. Again, a plasmid encoding for two proteins was used; in particular, the authors introduced EGFP and α-hemolysin genes. In contrast to the cascading network described above, now the second protein (α-hemolysin) does not have a direct role in protein expression, but is involved in a different task. In fact, although α-hemolysin is a water-soluble protein, it is able to self-assemble as a heptamer in the bilayer, generating a pore that is 1.4 nm in diameter (cut-off ~3 kDa). In this way, it was possible to feed the inner aqueous core of the vesicles, realizing a long-lived bioreactor where the expression of the reported EGFP was prolonged up to 4 days. This work certainly represents an important milestone in the road map to the minimal cell because the α-hemolysin pore permitted the uptake of small metabolites from the external medium and thus solved the energy and material limitations typical of impermeable liposomes.

Finally, GFP has also been expressed in another kind of compartment different from vesicles. These are water cavities (aqueous micrometer-sized environments) of water-in-oil emulsion, where it has been shown [49] that a functional protein, representing a tiny volume fraction (~0.5%) of a hydrocarbon sample, can be expressed.

In addition, the desired degree of complexity, intended as the collection of all components required for GFP expression, was obtained by solubilisate exchange and/or fusion between different aqueous compartments, each one carrying a part of the whole biochemical machinery (plasmid; RNA polymerases, ribosomes and cellular extracts; and amino acids).

In summary, in the last few years, a handful of pioneering studies on protein expression within liposomes appeared, and some of these reports evidenced the effect of 'compartmentation' (i.e. a higher yield of protein expression in the vesicles compared to the bulk buffer)—a very interesting phenomenon deserving further investigation.

It is also worth mentioning that, to date, only water-soluble proteins have been expressed, and no attention has been devoted to the expression of membrane-soluble proteins.

It is also important to mention some interesting studies, which, although not directly related to the question of the minimal cell, deal with microtubulation. The combination of giant vesicles, minibeads and molecular motors has been studied by a team at the Institut Curie [52]. The authors show that lipid giant unilamellar vesicles, to which kinesin molecules have been attached, give rise to membrane tubes and complex tubular networks that form an original system emulating intracellular transport. Membrane tube formation from giant vesicles through dynamic association of motor proteins has been also studied by Koster et al. [23], while Glade et al. [14] have shown tubule-mediated collective transport and organization of phospholipid vesicles and other particles.

This kind of work paves the way for the study of intracellular transport and organization at a higher complexity level within semi-artificial cells.

What next?

Keeping in mind the notion of minimal cell, analysis of the data presented in the article reveals what is still needed before we can proceed in this field.

For example, protein expression, as outlined in most salient experiments of Table 6, has been carried out without checking the number of enzymes/genes utilized in the work. We believe that it would be appropriate to carry out protein expression by utilizing known concentrations of the single enzymes/genes instead (and forgetting the commercial kits) to know exactly what is in 'the pot' and to possibly have a hand in the corresponding chemistry. This operation would correspond to the implementation of the minimal genome inside liposomes and may pave the road for the next steps.

Previous discussions and the data reported in Table 6 make clear one other essential element that is still needed before we can reach the ideal case of Fig. 1: self-reproduction. In fact, after having produced GFP, none of the systems of Table 6 has been found capable of reproducing itself and giving rise to a chain of multiplying GFP-producing systems.

In real biological systems, a cell is capable of duplicating and reproducing itself with the same genetic content. This is due to systems of regulation, and this aspect has not been contemplated yet in the experimental set-up of minimal cells. In this context, besides making reference to prokaryotic cell division, the previously cited work on microtubulin might be quite an interesting insight into the problem.

A very interesting case is achieving vesicle self-reproduction by endogenous synthesis of vesicle lipids. Two strategies can, in principle, be pursued: (1) incorporating first the enzymes that synthesize the lipids, or (2) starting from the corresponding genes (i.e. expressing those enzymes within the vesicles).

Early attempts have focused on the enzymatic production of lecithin in lecithin liposomes [54]. The metabolic pathway is the so-called salvage pathway, which converts glycerol-3-phosphate to phosphatidic acid, to diacylglycerol and, finally, to phosphatidylcholine. The four enzymes needed to accomplish these reactions were simultaneously inserted into liposomes by the detergent depletion method, and the synthesis of new phosphatidylcholine (10% yield) was followed by radioactive labelling. Liposome transformation, followed by dynamic light scattering, showed that vesicles changed their size distribution during the process.

This was indeed a complex system, and it was realized later that one could theoretically stop at the synthesis of phosphatidic acid, as this compound also formed stable liposomes. Further studies [27] were oriented to character-

ize the process by means of overexpression in *E. coli* and reconstitution in liposomes of the first two enzymes of the phospholipid salvage pathway to obtain self-reproducing vesicles with only two enzymes.

Production of the cell boundary (as depicted in Fig. 5) from within corresponds to the notion of autopoiesis [29, 30, 32, 63].

The internal synthesis of lecithin in lecithin liposomes would be a significant step forward. In particular, it will be very interesting to see, given a certain excess of the two enzymes, for how many generations cell self-reproduction could go on. However, it is clear that, after a certain number of generations, the system would undergo 'death by dilution'.

Finally, to get closer to the real minimal cell, there is the problem of further reduction of the number of genes. In all systems of Table 6, we still deal with ribosomal protein biosynthesis, and this implies 100–200 genes. We are still far from our ideal picture of a minimal cell, and we can pose, once more, the question of how to devise actual experiments to reduce this complexity.

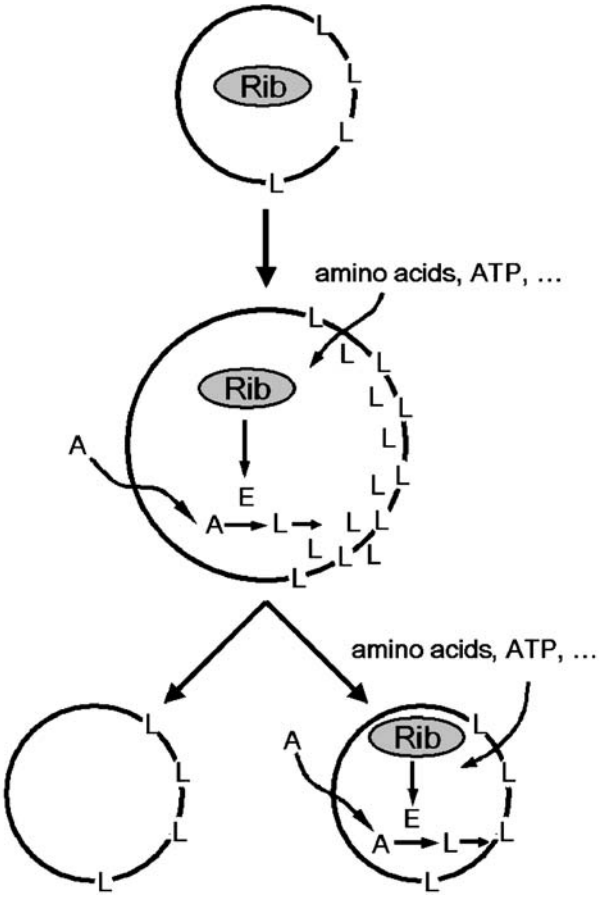

Fig. 5 A cell that makes its own boundary. The complete set of biomacromolecules needed to perform protein synthesis (genes, RNA polymerases and ribosomes) is indicated as *Rib*. The product of this synthesis (indicated as *E*) is the complete set of enzymes for lipid (*L*) synthesis. After growth and division, some of the 'new' vesicles might undergo 'death by dilution'

As a way of thinking, we must resort to conceptual knock-down experiments (e.g. those outlined in the works by Islas et al. [17] and Luisi et al. [31])—a simplification that also corresponds to movement towards early cells. The simplification of ribosomal machinery and of the enzyme battery devoted to RNA and DNA synthesis has been seen as a necessary step.[2]

Is this experimentally feasible? For example, can simple forms of rigid support for reactions (in particular protein biosynthesis) that are operative in vitro as ribosomes be developed? Think of protein-free ribosomal RNA first. Can one operate, at costs of specificity, with only a very few polymerases? Similarly, it might not be necessary, at first, to have all possible specific tRNA, but a few unspecific ones instead. One might even conceive experiments with a limited number of amino acids. Now, all this must be tested experimentally; there is no other way around it.

Concluding remarks

The definition of minimal cell, as given in the beginning of this review, appears simple and is provided with its own elegance. Conversely, experimental implementations of minimal cells may not appear equally satisfactorily and elegant. We have outlined the main difficulties possibly encountered in the construction of an ideal minimal cell, and we have pointed out, for example, that, in the best of hypothesis, death by dilution is one limitation; self-reproduction is one target that has not yet been accomplished.

One problem with the present literature on minimal cells is that the link between the 'minimal genome' and the minimal cell is too weak; in other words, there is no direct correlation. It would, of course, be advisable for researchers working on minimal cells to 'count' the genes that are active in their conditions and to compare the figures with the figures on the minimal genome given by researchers. Even within these limitations, experimental attempts to build a minimal cell are of great value in—but are not limited to—evaluating the specific simplification of the minimal genome. The use of liposomes as a sophisticated 'reaction vessel' is certainly instrumental in the technical realization of the minimal cell, but also has the added value of representing a possible route to the origin of early cells, emphasizing manifold consequences of compartmentation.

Constructs produced in the laboratory still represent poor approximations of a full-fledged biological cell. This dis-

[2] One of the referees, whom we particularly thank for acute comments, suggested that it would be actually useful to define 'a hierarchy of "minimal cells". Some members of this hierarchy might require extensive resources from the environment, such as high-energy compounds. Others might be able to survive in a nutrient-poor environment, presumably more compatible with the "primordial soup". In fact, it would be quite interesting to analyse the differences between different members; they would be quite revealing as far as the nature of life goes'. This proposal may indeed be the basis for future developments of this kind of work on the minimal cell, particularly when experimental data become available on these different classes of artificial protocells.

tance from fully biologically active cells makes it indeed premature to question possible hazards and bioethical issues in the field of minimal cells.

But there is still another very important topic that has not yet been discussed in due light by authors studying the minimal cell: interaction with the environment. Of course, feeding of the minimal cell is somehow taken into consideration, but only as a passive reservoir of nutrients and/or energy. In fact, we believe that the next generation of studies on the minimal cell should more actively incorporate such interactions with the surroundings, questioning, in particular, in which environmental conditions the minimal cell is able to perform its three basic functions.

Yet, these forms of 'limping life', in our opinion, represent a very interesting part of this ongoing research. In fact, these approximations to life are as follows: a cell that produces proteins and does not reproduce itself; or one that does reproduce for a few generations and then dies out of dilution; or a cell that reproduces only parts of itself; and/or one characterized by very poor specificity and metabolic rate.

All these constructs are important because, most probably, similar constructs were intermediates experimented on by nature to arrive at the final goal: a full-fledged biological cell. Thus, the creation of these partially living minimal cells in the laboratory, as well as the historical evolutionary pathway by which this target may have been reached, may be of fundamental importance to understanding the real essence of cellular life. In addition, the construction of semi-synthetic living cells in the laboratory would be a demonstration (if still needed) that life is indeed an emergent property. In fact, in this case, cellular life would be created from non-life, since single genes and or single enzymes are, per se, non-living.

Generally, although the minimal cell can teach us a lot about early cellular life and evolution, it may not necessarily shed light on the origin of life. The reasons for this have been already expressed and lie mostly in the fact that, in our approach to the minimal cell, we start with extant enzymes and genes, where life is already in full expression.

All this is very challenging and, perhaps for this reason, as already mentioned, there has been an abrupt rise of interest in the minimal cell. It appears that one additional reason for this rise of interest lies in a diffused sense of confidence that the minimal cell is indeed an experimentally accessible target.

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