

Toward the Engineering of Minimal Living Cells

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ABSTRACT

The article focuses on the notion of a synthetic or semi-synthetic minimal cell, defined as a system that has the minimal and sufficient structural conditions for cellular life. It is emphasized that two complementary approaches are in principle possible, defined as “bottom-up” and “top-down” approaches. The first one aims at the construction of a minimal cell starting from scratch, and it is argued that a very serious bottle-neck to this pathway lies in the origination of specific macro-molecular sequences, as in nature those were constructed most likely by a particular contingent set of conditions. The top-down approaches utilize extant genes and enzymes, and the work in this case is based on the incorporation of the minimal and sufficient amount of such macromolecules into liposomes, as models for the shell of biological cells. The first phase of this ambitious project foresees the study of conditions under which complex molecular biology reactions takes place in the compartments of liposomes. Examples of these reactions are provided, for example, the production of RNA throughout Q-beta replicase in a self-reproducing vesicle system; or PC Reaction in phospholipid vesicles; or even the incorporation of ribosomes in liposomes, with the production of polypeptide chains. The use of giant vesicles is also illustrated. These systems, due to their large size, offer the advantage that by way of special micro-injection techniques, all sort of biochemical agents can be directly introduced in the compartment; and that the reaction can be followed by optical microscopy. In the final part of the article, the outlook of increasing the complexity of these liposome systems so as to arrive at first semi-synthetic cells is discussed. *Anat Rec* 268:208–214, 2002.

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CONCEPTUAL PREMISES

The idea of constructing artificial living entities, as “minimal” as they are, must be based on a specific definition of life and on the assumption that an object corresponding to such a definition can be constructed in the laboratory. It is therefore necessary to clarify first what the assumptions are in this field of science.

The basic assumption is that life on Earth originated from inanimate matter by prebiotic molecular evolution, i.e., throughout a very long and lengthy series of steps of increasing complexity. Each step is determined by causality laws, and is built on each other in a hierarchy, as shown in Figure 1. This leads up to the formation of self-reproducing protocells that were finally able to display biological autonomy and start the ascent towards biological evolution and the construction of all living en-

tities. The basic idea is namely that life developed by itself- without any transcendental help. It is fair to say that this is a hypothesis, as it has not been demonstrated yet. Our assumption is that this can be demonstrated, and the present article deals with the achievements and with the difficulties encountered in this enterprise. In view of the current controversy over creationism, it perhaps

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should be noted here that science by definition does not invoke miraculous or transcendental principles. Science is a way to explain the phenomenology of the world with the laws of physics and chemistry. One does not need to subscribe to this approach, but if not one is out of the domain of science.

In this article I focus on the notion of the minimal cell, defined as a system that has minimal and sufficient structural conditions for life. In so doing, I primarily review the work and philosophy of my own research group. A more exhaustive analysis of the various approaches to the notion of the minimal cell is given elsewhere (Luisi et al., 2002).

As noted above, the notion of the minimal cell must be based on a definition of cellular life. What is meant by “life” in this context? The definition of life is intrinsically complex (Luisi, 1998). An answer that would satisfy most biochemists is that a cell is living when it simultaneously displays three features: homeostasis, self-reproduction, and evolution. However, this is a final point in the implementation of cellular life, and the real question is whether one can use the term “life” when these three features are not present at one time. On this very point, opinions diverge. For example, for the proponents of autopoiesis (Varela et al., 1974), life is present when the cellular system displays homeostasis, which is the common state of normal cells by normal conditions. Reproduction is a “rather trivial” consequence of the existence of life (Varela et al., 1974).

For several authors who identify themselves with the “RNA world,” the main emphasis in the definition of life is the self-replication and mutation capability of a family of RNA (Joyce and Orgel, 1999)—metabolism being less important for the origin of life. For a comparison between these two views, see Luisi (1998).

The upward escalation toward complexity, as shown in Figure 1, is based on the chemical processes that increase the size and/or complexity of cells, such as polymerization, complex formation, self-assembly, and self-organization. An important idea should be mentioned in this regard: the notion of emergence and emergent properties. This is a significant epistemological enrichment in the apparently arid scenario of prebiotic molecular evolution, as it introduces an important element of “creativity.” Looking at the simple cartoon of Figure 1, it appears that the pathway describing the increase of complexity proceeds via hierarchical levels that are created by the structurally more simple elements of the previous level. Emergence has to do with the observation that the properties of a given level are generally not present in a single constituent’s elements (often expressed as “the whole is more than the sum of the parts”). A typical example given in the literature is water, whose collective properties as a liquid are not present in its two constituent gases, hydrogen and oxygen. In the same way the properties of a gamma globulin are not present in the single amino acids, and the properties of a triangle are not present in the single lines. The notion of emergence appears almost trivial—but only at first glance. A recent review focusing on emergence in chemistry provides a discussion to this point (Luisi, 2002). The connection between the notion of emergence and the argument of this article comes from the fact that life itself can be seen as an emergent property: the molecules that constitute a living cell (DNA, proteins, polysaccharides, lipids, etc.) are not living. The quality “life” arises from the

assembly of these non-living elements, duly arranged in space and time.

TOP-DOWN AND BOTTOM-UP APPROACHES

The construction of a living cell in the laboratory corresponds, therefore, to an exercise in emergence. How should one experimentally approach this construction?

The “simplest” exercise would be to first divide a cell into many constituting parts, show that none of these parts are living, and then reconstitute the living cell by mixing these components together. This kind of experiment has been carried out with viruses and smaller cell constituents, but not, to my knowledge, with an entire cell. A successful experiment of this sort, with a cell family, would provide a nice demonstration of emergence at the level of cellular life.

With this experiment we would be dealing with several hundreds of single components. It is well known that the simplest living cell, such as the parasite *Micrococcus genitalium*, or *Buchnera*, is characterized by 350–450 genes, and contains several hundred macromolecular or low-molecular-weight compounds. A different experiment in the field of emergence of life would be to construct a minimal living cell, namely one that contains, as mentioned earlier, the minimum amount of genes and constituents that is compatible with life. Discussions on the question of the minimal RNA cell (Szostak et al., 2001) and the minimal DNA cell (Luisi et al., 2002) are ongoing, and are a part of an increasingly more active field of inquiry.

The question of the minimal cell is also relevant in the field of the early evolution. It is reasonable to assume that the early protocells were much simpler than modern living cells, being characterized by a minimal number of genes and molecular components. Probably, then, the early protocells were not as efficient as modern cells, and were not characterized by the simultaneous presence of the three above-mentioned features (homeostasis, self-reproduction, and evolution), but rather by one or two at a time. These are also considerations in the study of the emergence of life and the behavior of minimal cells, with the underlying assumption that synthetic minimal cells also serve as models for the early cells.

At this point, the term “synthetic” should be defined. One finds in the literature two main approaches to the synthetic approach, which can be categorized as the bottom-up and the top-down approaches (see Fig. 2).

The bottom-up approach is the experimental rendering of Figure 1. Beginning with very simple molecules, one tries to build more and more complex biological structures with prebiotic reactions. From an historical perspective, the starting point of this approach is the work of Stanley Miller (1953), who produced amino acids by electrical discharge in a gas atmosphere that simulated the prebiotic atmosphere. This work started the whole field of prebiotic chemistry, and we currently believe that almost all biologically important biomonomers (with the notable exception of mononucleotides) can be synthesized in the laboratory by means of reactions that presumably occurred before the advent of life on Earth. Whether things really went this way historically is in the end not very important. The important thing is that such products can be obtained by very simple reactions from very simple prebiotic precursors. The fact that such precursors, and often complex biomolecules, such as α -amino acids and fatty acids, can be found in meteorites or in interstellar space,

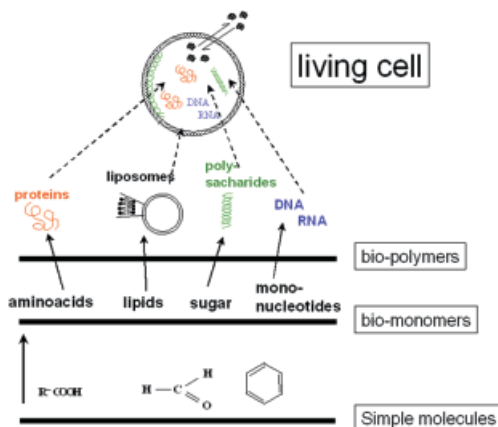


Fig. 1. Schematic and arbitrary representation of prebiotic molecular evolution. This scheme corresponds to a bottom-up approach (see text).

two working directions

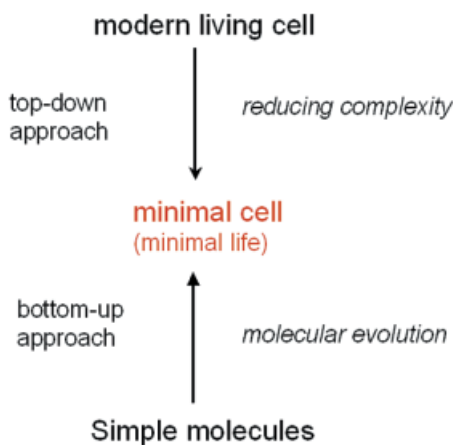


Fig. 2. A scheme representing the two main working directions for the minimal cell project: the top-down and the bottom-up approach.

supports the concept that the very first important step in the latter of Figure 1 can be understood and reconstituted in the laboratory.

Can we then proceed further in the latter of Figure 1? The next step would be the prebiotic synthesis of enzymes and/or nucleic acids. In fact, this step is the bottleneck of the whole bottom-up approach, because we do not know how (experimentally or even conceptually) to make, under prebiotic conditions, chains that are all equal to each other with a specific sequence. In other words, the point is not only the prebiotic synthesis of polymeric co-oligopeptides (and we still do not know how to make these); the point is to construct co-oligopeptides in which all chains are equal to each other, and possibly with a sequence that assumes a folded conformation in solution. A random polymerization of a mixture of amino acids of, say, a 50-residues long peptide would afford an astronomical number of different macromolecules (ca. 20^{50}), in which the probability of having two identical chains would be, in effect, very close to zero.

TOP-DOWN APPROACH TO THE MINIMAL CELL

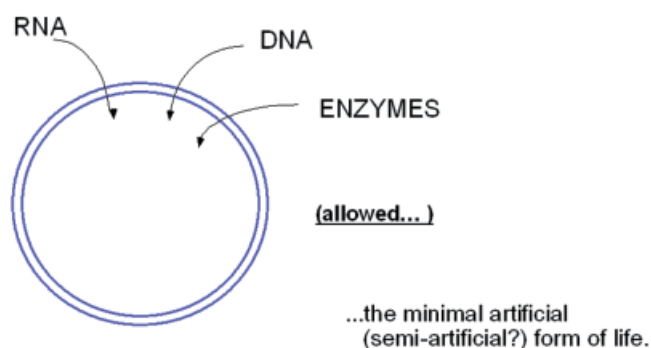


Fig. 3. Implementation of the emergence of life by the top-down approach for the construction of the minimal cell.

The situation is even worse with nucleic acids, because in that case we do not even know how the monomers can be obtained under prebiotic conditions.

One may argue at this point that it is not strictly necessary to have identical sequences of macromolecules—that what is important is functionality. In principle, it is possible to maintain the same function with a library of different macromolecules displaying similar sequences—a kind of “quasi-species.” This may be possible, but we do not know how to produce such families of compounds in the laboratory.

Without enzymes that can catalyze reactions that are under kinetic control, or without self-replicating RNA molecules, it is difficult (if not impossible) to know how to go upward in the pathways of Figure 1 toward a minimal cell. The counter argument is that one does not need to obtain real enzymes at the very beginning, as simple peptides might do some catalysis. However, until now no short peptide with a serious catalytic activity other than hydrolysis has been obtained, and short peptides cannot assume stable folded conformations.

This fact is usually not stressed enough in the literature. The “RNA world” theory concerning the origin of life is based on the assumption that a self-replicating RNA family is already there to begin with. This scenario would allow, at least on paper, the climb up the prebiotic evolution ladder: from self-replicating and mutating RNA, ribozymes would originate that eventually would catalyze the formation of peptide bonds and DNA molecules. Again, this view ignores the problem of sequence, as it is not sufficient to make random long peptides or random long DNA chains. And in fact, the RNA world theory does not describe how the specific sequence can be selected. I believe that the bottom-up approach to the origin of life will not be solved unless the problem of macromolecular sequencing under prebiotic conditions is solved.

Once macromolecular sequences with a given activity are obtained, the question of how to approach the construction of the minimal cell appears, at least on paper, more reasonable. This approach has been termed the “top-down approach,” which involves the use of already extant nucleic acids and enzymes to make a minimal living cell. The procedure is schematically represented in Figure 3.

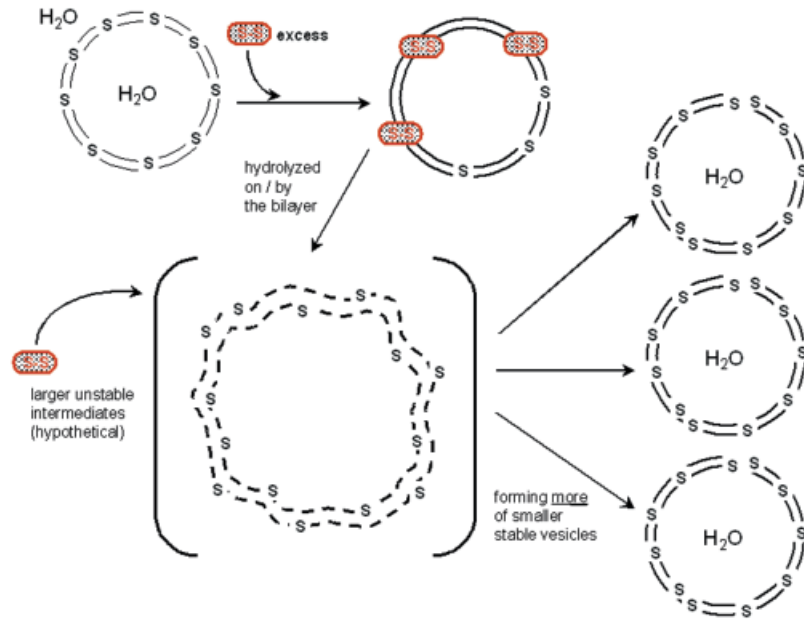


Fig. 4. Schematic representation of the chemical mechanism by which micelles and vesicles undergo an autocatalytic process of self-reproduction.

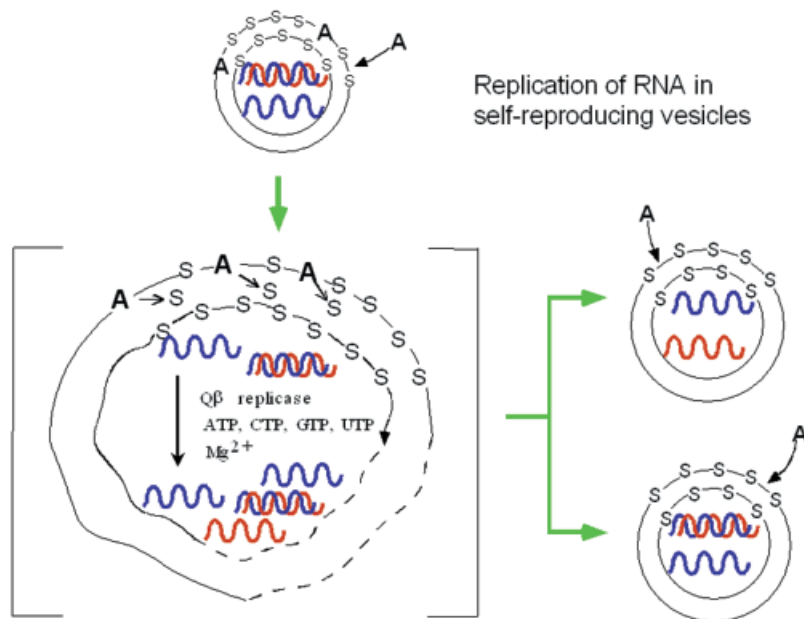


Fig. 5. Self-reproduction of oleate vesicles containing the enzyme Q-beta replicase, which simultaneously replicates an RNA template.

This is a semi-synthetic approach, as the host compartment is a synthetic vesicle or liposome (vesicle formed by lipids). This top-down approach is not a cell reconstitution in the real sense of the word, because such cellular entity does not exist in nature and is therefore artificial, i.e., synthetic. However, it would be confusing to call such a construct “artificial life,” as we usually mean something else by this term—namely a fantasy product, and not a close model to a living cell.

Terminology can be a problem, and one should not be too fussy about it. The term “top-down approach” is also subject to criticism. In fact, the construction of a minimal living cell as depicted in Figure 3 is also a case for upward emergence, i.e., from a mixture of simpler constituents life is formed as an emergent property. The term “top-down” is appropriate if it refers to a normal cell and to the stripping-down process that is required for going from such a complex modern system to the simpler model of Figure 3.

The term “top-down” is also useful to set it apart terminologically from the bottom-up approach shown in Figure 1.

Top-Down Approach

The microcompartment of Figure 3, as mentioned above, is generally a vesicle, or a liposome. Liposomes are generally phospholipid vesicles of 100–500 nm diameter, and special techniques have been developed in the last few years to entrap enzymes and other biological components inside them.

The ultimate goal is to entrap inside a liposome a minimal, but sufficient, number of enzymes and nucleic acids to create an entity capable of cellular life. We have defined this as minimal life, and ideally one would like to have a minimal cellular system capable of displaying simultaneously homeostasis, self-reproduction, and evolution. This is an ambitious and complex project, which can be broken down into a series of simpler tasks, which we can call way-stations along the pathway toward construction of the minimal cell. These way-stations would include: 1) conditions under which biopolymer-containing liposomes display complex molecular biological reactions; 2) a liposome system capable of expressing proteins; and 3) a system capable of self-reproduction.

Currently, the state of the research is such that a few satisfactory examples for the first “way-station” have been described—namely, liposomes carrying out complex molecular biological reactions (Chakrabarti et al., 1994; Oberholzer et al., 1995a,b; Walde et al., 1994b; Nomura et al., 2001; Yu et al., 2001). Also, first preliminary results concerning the protein expression in liposomes have been reported. However, there are no examples in the literature of minimal cells capable of self-reproduction, or minimal cells displaying a satisfactorily metabolism.

Let us examine, then, a couple of examples for the first way-station, which are simply meant to give an illustration of the potential and limits of the semi-synthetic top-down approach using vesicles.

As regards to vesicles, conditions for the self-reproduction of micelles and vesicles from fatty acid vesicles (such as oleate or caprylate) have been described (Bachmann et al., 1992; Walde et al., 1994b). As illustrated in Figure 4, the procedure is based on the binding of a water-insoluble precursor to the outer membrane of the hydrophobic vesicles; hydrolysis of this precursor (which can be an ester or an acid anhydride) yields the surfactant acid, which remains bound *in situ* to the membrane. This brings about a growth process that eventually leads to splicing and multiplication of the vesicles. This is an autocatalytic process: the more vesicles are formed, the more surfactant is produced by hydrolysis on the membrane surface. Evidence for these processes has also been obtained in the case of ferritin-containing mixed oleate vesicles by electron microscopy analysis (Berclaz et al., 2001a, b).

In the case of vesicles, finding conditions suitable for self-reproduction has proved to be easier than finding conditions that could simulate homeostasis. To our knowledge, only one experimental model for a homeostatic system has been described (Zepik et al., 2001). In this case, oleate vesicles are produced by the mechanism of Figure 4, while simultaneously an oxidative reaction destroys the vesicles (by operating on the double bond of the oleate molecule). A balance between these two competitive processes leads to a model of chemical homeostasis in spherical closed compartments (Zepik et al., 2001).

The autocatalytic self-reproduction process has been utilized in conjunction with enzymatic reaction inside the vesicles. The first example was the polymerization of ADP into poly(A) by the enzyme PNPase entrapped in self-reproducing oleate vesicles (Walde et al., 1994a), with the substrate ADP permeating into the vesicles from the outside medium. This reaction has been also studied by the groups of Deamer and Joyce with phospholipid liposomes (Chakrabarti et al., 1994).

A more complex, and in a way more interesting, case is that of the Q-beta replicase, an enzyme that is capable of replicating RNA acting on a template. The experiment that was carried out is illustrated schematically in Figure 5.

In this case also, the vesicles undergo a process of multiplication while the enzyme inside the oleate vesicles produces RNA (Oberholzer et al., 1995b).

Is this a model for a living cell? The fact that the reaction stops as soon as the limiting reagents are used up is no conceptual hurdle, as one can easily devise a continuous reactor system (as described for chemical homeostasis (Zepik et al., 2001)). This is a system in which both the precursor for the oleate vesicle reproduction, and the nucleotide substrates are continuously furnished from the outside. The real conceptual problem of the experiment (Fig. 4) lies in the fact that the enzyme and the template are getting more and more diluted with the increase of generation cycles, i.e., more and more vesicles will be without Q-beta replicase and/or without template, and therefore will be unable to further replicate RNA. In time, the concentration of vesicles containing both enzyme and template would be zero, and the system would not be “living” anymore.

Polymerase chain reaction (PCR) has been made in liposomes (Oberholzer et al., 1995a). This is interesting because the liposomes had to withstand temperature cycles of up to 90°C; furthermore, up to nine different molecular components had to be enclosed inside a single liposome. Furthermore, Oberholzer et al. (1999) have entrapped the entire ribosome system inside conventional phospholipid liposomes, and were able to express a simple polypeptide chain, poly(phe), by using poly(U) as the messenger.

In all of these examples, a considerable problem has been the uptake of substrate nucleotides by the enzyme inside the vesicles. In the case of PNPase, ADP was given in the medium outside, and a modest permeability of the nucleotide into the vesicle compartment was observed. Generally, however, the uptake of substrate from the medium, and its internalization, are not very efficient, and therefore yields are rather modest. Despite this limitation, these experiments have shown that complex molecular biology reactions can be carried out in liposomes, even under extreme temperature conditions.

A few reactions of this type have been carried out using a different type of microcompartments, the so-called giant vesicles (GVs). Those are vesicles whose diameter encompasses several μ (up to 100 μ). Because of their size, these vesicles can be visualized directly under light microscopy, and entire sets of biochemicals can be injected inside them by special microinjection techniques (not dissimilar from those used in cell biochemistry). Fluorescent reactions are usually used to visualize the outcome of reactions. Several groups are presently actively working with these compartments, with the aim of transforming these relatively large microreactors into cell models, using the top-down

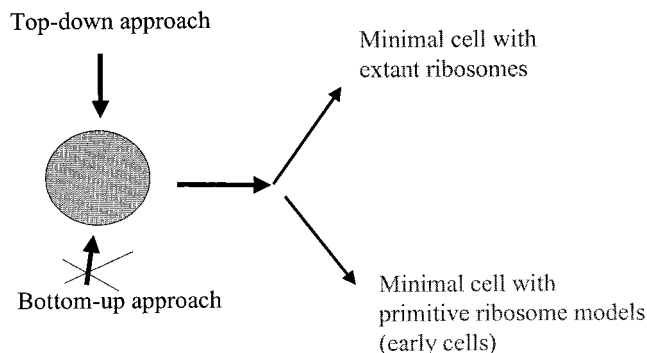


Fig. 6. A schematic of the present situation in the experimental research of the minimal cell.

approach (see Walde and Luisi, 2000, and references therein).

The advantage of using GVs is that everything can be injected directly inside, thereby eliminating the problem of the uptake of material from the outside. On the other hand, injection is not a prebiotic means; furthermore, the chemist is working in this case with only one microcompartment at a time, which greatly reduces the chemical yields and the biotechnological relevance. The work with GVs, however, is becoming more and more popular, owing to the advantage of being able to directly visualize the vesicle, as well as the pathway reaction.

As I mentioned at the beginning, this is mostly a review of our own work. However, a discussion of cell models would not be complete without mentioning the work of the Deamer group. They have shown, among other things (Dworkin et al., 2001), that boundary structures can be formed under plausible prebiotic conditions, which suggests that there is an interesting connection between the bottom-up and top-down approaches. Also, together with Pohorille (Pohorille and Deamer, 2002), they presented a recent review on artificial cells discussing their possible relevance for therapeutic and diagnostic applications.

CONCLUSIONS

What has been accomplished in the field to date? And where do we go from here?

From the conceptual point of view, some clarity has been achieved. We understand better the principles by which life operates at the molecular level, and the notions of minimal life. The notion of emergence, and the top-down and bottom-up approaches have considerably contributed to the preparation of a corresponding experimental program.

As regards the experimental program, it is important to note that minimal life has not yet been achieved in the laboratory. Does that mean that it is in principle not possible? I do not believe so, although as scientists it is always good to have a bit of doubt (perhaps we missed something important in our theoretical analysis).

Generally, it appears at this stage that the bottom-up approach is much more difficult, and that the top-down approach is the one that will eventually arrive first at the goal of making a minimal living cell (see Fig. 6).

One possible difficulty should be mentioned for the top-down approach, which arises from the networking re-

quired of several components inside the microcompartment. In order to achieve this, all components must work in concert while at the same time forming a stable system.

The line of progress in the experimental work is clear: molecular biology reactions in liposomes are now a reality, and are also of a very complex type. This is the prerequisite for increasing in complexity toward the functionality of a protocell. However, even at this first level there are still problems: the most serious is the uptake and permeability of substrates from the outside to the inside of the compartment across the bilayer; the other problem is the poor yield of entrapment and, correspondingly, the poor chemical yields of the reactions.

The next step in complexity in the top-down approach is the use of liposomes for the expression of proteins. First, one will try to utilize the extant ribosome systems and synthetic m-RNA inserted in plasmid vectors. It would be a great success to engineer a liposome system that would allow expression of only one given gene at a time. Clearly, this would also be very important from a biotechnological point of view.

How far away is this goal? The first experiments with the expression of green fluorescent proteins, carried out by different groups, suffered from poor reproducibility and very small yields (Yu et al., 2001; Luisi et al., 2001). However, as is generally the case, optimization screenings should make it possible within a relatively short time to arrive at protein expression, at least in some selective cases. Whether this will permit biotechnological exploitation remains to be seen: the problem of yields may be very serious.

The next step, or way-station as we have termed it, would be the self-reproduction of the ribosome system. Several variations of this project can be envisaged, but it is clear that the reproduction of the ribosome system would impose an extremely high degree of complexity, i.e., a high number of enzymes and components. I believe that a preliminary step for the self-reproduction of the minimal cell would involve chemically simpler model ribosomes. In this respect, one should consider the suggestion that ribosomes can work without their proteins, i.e., simply as RNA material.

The idea of finding simpler ribosome systems is also very important as regards the evolution of early cells, as certainly no complex modern ribosome machinery was present in the first functional protocells. Thus, self-reproducing liposome machinery with a model ribosome would be a major advance in the field. A schematic diagram of these concepts is illustrated in Figure 6.

Up to this point we have considered cell models based on the classic protein/DNA model. It is well known that ribozymes and the RNA world may provide an alternative to this scenario. In fact, it was shown in a recent paper (Szostak et al., 2001) that one can in principle achieve a self-replicating cellular system based on only a couple of ribozymes: one ribozyme that catalyzes the synthesis of the cell membrane, and one that acts as a RNA replicase (for itself and the other ribozyme).

The problem with such ribozymes and RNA replicase lies in the fact that they do not exist—yet. And we cannot foresee when or if they can be prepared in the laboratory.

It is clear from these short concluding remarks that the field of the minimal cell is partly anchored in solid experimental facts, and partly still belongs to the realm of

imagination and not-yet-realized research. But this is a typical realm of frontier science.

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