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Integration of biological parts toward the synthesis of a minimal cell

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Various approaches are taken to construct synthetic cells in the laboratory, a challenging goal that became experimentally imaginable over the past two decades. The construction of protocells, which explores scenarios of the origin of life, has been the original motivations for such projects. With the advent of the synthetic biology era, bottom-up engineering approaches to synthetic cells are now conceivable. The modular design emerges as the most robust framework to construct a minimal cell from natural molecular components. Although significant advances have been made for each piece making this complex puzzle, the integration of the three fundamental parts, information–metabolism–self-organization, into cell-sized liposomes capable of sustained reproduction has failed so far. Our inability to connect these three elements is also a major limitation in this research area. New methods, such as machine learning coupled to high-throughput techniques, should be exploited to accelerate the cell-free synthesis of complex biochemical systems.

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Introduction

The bottom-up synthesis of a minimal cell represents a challenging but conceivable goal for the synthetic biology community [1]. The design of a minimal biological cell from scratch deals with the creation of an out-of-equilibrium system capable of self-reproduction and open-ended evolution [2,3]. Such project consists typically of the assembly of molecular components toward a gradual increase of complexity under the fundamental laws of thermodynamic [4]. The construction of reduced cell analogs in the laboratory increases basic knowledge of unicellular life, its primary goal, but also provides novel platforms for biotechnological and biomedical applications [5–10]. The construction of predictable biochemical systems programmed with genetic information is one of the other major objectives of such ambitious projects. As first stated by Virchow [11], a cell originates from another cell. As a consequence, the logic of self-reproduction, one of the most fundamental features of biological systems, is difficult to break down. An approach to this problem consists of synthesizing cell analogs from its basic natural molecular components.

The molecular synthesis of living entities relies on three features: information, metabolism, self-organization. Each of these parts is made of molecular machineries, each of these parts is indispensable to construct synthetic compartments capable of sustained self-reproduction and evolution [12,13]. Self-organization is needed for the formation of the compartment and for protein macromolecular assemblies; metabolism is needed for the self-maintenance of the system, nutrients synthesis and waste recycling; information is essential for evolution and regulation of cellular functions. Taken separately, considerable work has been done on each of these three topics. However, the integration and the coordination of self-organization, metabolism and information into cell-sized compartments have failed so far.

Many definitions of life have been proposed that could direct or help the construction of a minimal cell in the laboratory. Although certainly useful in defining a context, capturing life in concepts or lists of properties is not enough. The definition of life stays elusive and many different definitions can be written [14] that would satisfy biologists, chemists, physicists and philosophers. The Autopoiesis theory, one of the first conceptual efforts to define cellular life, is a formulation of chemical self-reproduction and self-maintenance [15]. The first synthetic cells genetically programmed to sustain self-reproduction will certainly arise in ideal environmental conditions far from real conditions to be considered as really alive. This is why the definition of unicellular life remains volatile: beyond their biochemical and biophysical attributes, the first biological cell-analogs will be also defined by their external synthesis medium, which can have a wide range of conditions (type of primary source of energy to be exploited, osmotic pressure for mechanical robustness, ionic strength for molecular interactions, among many others aspects).

Most of the credibility in this research area has been provided by the origin of life approach to synthetic cells. The origin of life is still one of the major motivations for the construction of cells from the bottom-up. However,

with the era of synthetic biology and the considerable heritage of soft matter, purely constructive approaches to minimal cells are conceivable. High-throughout methods, lab automation and machine learning algorithms are powerful tools to accelerate the prototyping of cell analogs in the laboratory [16–19]. Whether fully predictable and controllable DNA-programmed synthetic cells can be obtained is a question that cannot be answered yet. The top-down creation of a bacterium with a reduced synthetic genome also supports the construction of a cell from its molecular components, although both projects address different questions [20,21].

On the construction of minimal complex biological systems

The construction of biochemical systems *in vitro* is not just an exercise, it is a forward engineering approach necessary to understand the emergence of complexity in genetically encoded systems, to capture, in isolation, the cooperative link between the molecular machineries making living systems, and to characterize the molecular repertoire and networks found in biology. The purpose of cell-free biology is also to be quantitative and to expand the capabilities of natural systems [22].

Arguably one of the most challenging goals of cell-free synthetic biology is the bottom-up construction of minimal cell systems. It is a multidisciplinary research area, a problem of biology, chemistry and physics. Such projects have only recently become conceivable, but several approaches to assembling self-reproducing minimal cells using the basic molecules of life have been advanced [23–29]. The vocabulary used is often confusing: artificial cell, minimal cell, protocell, semi-synthetic or synthetic cell, reduced cell, coacervates, cell mimicry, partial cell, cell imitation and other jargons reduce the visibility of the work done in this research area.

The *protocell* approach explores the origin of life through the construction of cells from prebiotic components [30]. The most basic protocells do not contain information-carrying molecules, and are solely based on self-assembly and metabolism [13]. Sophisticated protocells, also deprived of complex molecular machineries, use peptides or RNA for information and fatty acids for membranes. The goal is to develop molecular scenarios for the emergence of cellular life on Earth in prebiotic conditions, from the formation of cell-sized compartments to their autonomous growth and reproduction [31,32]. The *artificial cell* approach consists of merging natural and synthetic chemical components to engineer chemical carriers or genetically programmable systems with predictable behaviors and to expand the capabilities of biological systems [33]. Such approach may lead to the design and construction of orthogonal-life. Polymersomes, mechanically more robust than natural membranes, are an example of artificial cells [34]. The synthesis of self-reproducing entities using molecules of real cells, often refers as the *minimal cell* approach, seeks to advance knowledge of biological self-reproduction through the assembly of synthetic cells made of natural components [29,35]. The bottom-up construction of minimal cells seeks to understand the cooperative link between the major molecular machineries and mechanisms making real self-reproducing cells. Although the minimal cell approach is well established in the community, there are potentially as many possible minimal cells as laboratory conditions.

All these approaches revolve around the same challenges: how to integrate and boot up information, metabolism and self-organization to create sustained self-reproduction of a container. Emulsions droplets have appeared as valuable intermediate synthetic cell systems. In particular, water-in-oil emulsions offer an easy way to create cell-sized compartments useful for proto-, artificial and minimal cells work [36•,37].

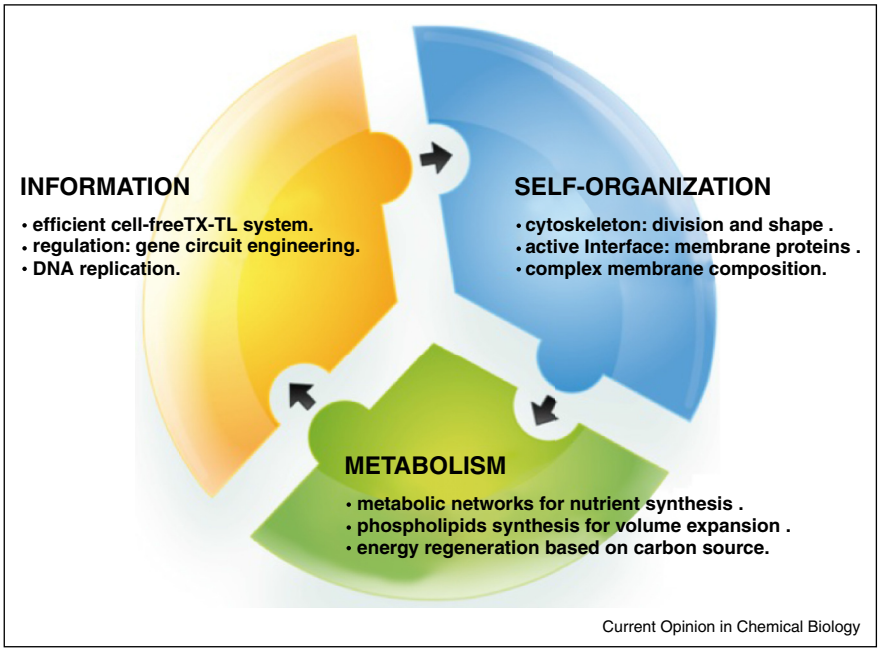
The bottom-up modular design of minimal cells: container–metabolism–information

A minimal cell would incorporate the machineries necessary for the execution of a small synthetic DNA genome based on bacterial regulation into a liposome supplemented with nutrients and a basic chemical energy regeneration system. The modular design [12], which consists of integrating and connecting three molecular modules (Figure 1), arises as the main strategy.

The three indispensable parts for the bottom-up construction of a biological minimal cell are: self-organization (the physical boundary that makes the container and other self-assembly processes), metabolism (energy processing and regeneration) and information (DNA program) [12,13]. This tenet is also common to the protocell and artificial cell approaches to some extent. Many pieces of this puzzle have been experimentally realized, but they have never been put together successfully, not even closely.

The container makes the linkage genotype-phenotype [28]. The physical boundary of a minimal cell is a closed phospholipids bilayer, namely a liposome. Soft matter and medical research have provided numerous methods to create cell-sized liposomes [38•,39–43]. Despite this considerable heritage, the creation of stable phospholipids compartments is challenging because of the complex reactions that must be encapsulated. All of these methods work well for diluted aqueous solutions. Just adding salts at physiological conditions (≈100 mM) and macromolecules at low concentrations (proteins, ribosomes, DNA, or RNA) dramatically decreases the yield of liposomes formation and their stability. The encapsulation efficiency varies from a method to another, and depends on the composition of the inner and outer solutions. One

Figure 1



Information, self-organization and metabolism are the three indispensable pieces of the puzzle to synthesize a minimal cell. Integration of the three parts in a functional whole has failed so far. Some of the major challenges are highlighted for each part. For information: the development of more powerful cell-free TX-TL system is a permanent quest. Considerable efforts have to be spent to engineer predictable synthetic gene circuits, and DNA replication has to be implemented. For self-organization: complex cytoskeletal structures have to be expressed and assembled for division and shape (creation of asymmetries). Efficient secretion of integral membrane proteins is still a major problem, while the encapsulation of cell-free TX-TL reaction into liposomes with complex phospholipids composition is still lacking. Metabolism: although significant progress have been made for energy regeneration, more sophisticated metabolisms have to be developed to construct minimal cell robust energetically. Finally, phospholipids synthesis has to be carried out for volume expansion. The ultimate objective is self-reproduction of cell-sized container. Another essential objective is that all the functions to achieve self-reproduction are encoded in the DNA.

of the most serious bottlenecks for the container is the development of the interface as an active membrane capable of sensing the environment and exploiting the external environment for nutrients. Although some progresses have been made [44,45], the insertion of cell-free synthesized integral membrane proteins into phospholipids bilayer is still limited. Simple lipidic membranes have also a limited mechanical robustness, which necessitates a precise balance of the osmotic pressure. However, this aspect, as well as the formation of membrane domains, could be beneficial for the production of membrane instabilities and primitive division mechanisms without cytoskeleton [46,47]. Molecular crowding is an essential physical aspects that favors the self-assembly of supramolecular structures, such as cytoskeleton, as well as gene expression [48]. Another challenge to minimal cell systems is to emulate such crowding to perform functional self-assemblies. The development of highly concentrated cell-free TX-TL systems is a possible answer to this problem [49].

The metabolism is a complicated issue because in real living cells, nutrients synthesis and recycling are performed by complex sets of enzymes and pathways, which are often coupled. Bottom-up minimal cells have to be under perfusion of pure energy sources (ATP, GTP) and nutrients (amino acids, nucleosides) in the first stages of development. Most of the chemical energy is spent in the process of translation, highly demanding energetically. The energy charge, the real energy parameter in ATP-based biochemical systems, holds that recycling byproducts of ATP hydrolysis is as important as keeping ATP at high constant concentration. Erasing the expressed information (mRNAs and proteins) after use is also crucial in biological systems, especially for minimal cells which cannot expend their volume as quickly as real cells. As a consequence, implementation of controlled mRNA and protein degradation seems also indispensable, a task still challenging to achieve *in vitro*.

The information is essentially the DNA genome providing the minimal cell with its functions and the regulation of their expression. With a DNA program large enough, one can solve most of the limitations aforementioned (mechanical robustness with a robust cell wall, sophisticated metabolism to exploit basic energy source in the

environment, replication of the DNA, synthesis of the necessary machineries, phospholipids synthesis, messenger and protein degradation, etc.). However, the execution of such large DNA program *in vitro* has not been demonstrated yet. It is estimated that about 200–400 genes are required to boot up a minimal cell from scratch that would be alive in ideal laboratory conditions [21,50]. Whereas the construction of such program into a single genome is nowadays technically possible [51], the design of its regulation, post-transcriptional modification and its compression is much more challenging. The behavior, *in vivo*, of synthetic 'non-Darwinian' DNA programs composed of well-characterized parts is still a serious challenge, due for a large part to our inability to characterize spurious crosstalks in synthetic networks. The problem also resides in coupling the digital information contained in DNA to the dynamics of self-reproduction processes, which are mainly analog. A purely Edisonian approach based on high-throughput techniques, liquid handling robotics and microfluidics for example, together with machine learning algorithms can accelerate the prototyping of such synthetic minimal genomes. Nonetheless, it is not clear that a sufficient level of synthetic regulation with favorable variability can be reached so that Darwinian evolution can take over the design of the information content and its computation. The creation of a sufficiently large population of minimal cells and physical cycles of reactions seems also a prerequisite for evolution to play a role [52].

Some of the major milestones toward the bottom-up synthesis of a minimal cell are summarized in Table 1: self-organization, metabolism and information. The integration of such achievements is necessary to construct an evolvable minimal cell.

The cell-free TX-TL approach to bottom-up minimal cells

Cell-free transcription-translation (TX-TL) systems have recently become valuable platforms for synthetic biology [53••]. These kits are now used to construct biochemical systems *in vitro* over a wide range of scales. They are also a primary choice to construct minimal cells. The TX-TL machineries are extracted from living cells to execute genetic information *in vitro*. These systems integrate ATP regeneration systems. About 1 mg/ml of soluble proteins can be synthesized in test tube reactions with commercial kits, which corresponds to about 30 μM of active eGFP. With an average cytoplasmic protein concentration of 500 nM in *Escherichia coli* [54], about 40–60 genes could be in principle expressed to either execute sophisticated gene regulations or to recapitulate complex biomolecular self-assemblies. The cell-free synthesis of bacteriophage T7 (40 kbp, about 60 genes) concurrently with its genome replication in a single test tube TX-TL reaction demonstrates that complex natural DNA program can be expressed into functional entities *in vitro* [55••]. This result also shows the enormous gap that has to be filled between natural DNA programs and elementary man-made gene circuits executed *in vitro*. The recent development of an efficient *E. coli* cell-free TX-TL toolbox that use the endogenous sigma factor transcription set, rather than bacteriophage RNA polymerases, opens new possibilities.

Numerous efforts are spent to fuel cell-free TX-TL systems with new energy sources and to recycle byproducts of reactions. Saccharides (glucose, maltose, maltodextrin) are now used to activate glycolysis. This approach shows that complex metabolic reactions can

Table 1

Some of the major steps achieved toward the bottom-up construction of a minimal cell using cell-free TX-TL for each of the three modules: container (self-organization), metabolism and information

Container (self-organization)	Metabolism	Information
Cell-free TX-TL synthesis of ribosome from DNA [58••]	Polyphosphate based metabolism for cell-free TX-TL (Caschera and Noireaux, unpublished)	First all *E. coli* cell-free TX-TL platform [53••]
DNA directed synthesis of bacterial cytoskeletal components in liposomes [59•]	High yield of *in vitro* protein synthesis exploiting di-and-polysaccharides [56••]	Total Synthesis and genome replication of a phage in a cell-free TX-TL system [55••]
Cell-free TX-TL synthesis of the bacteriophages T7 and phiX174 from their genome [55••]	Cell-free platform for metabolic engineering [60]	Replication of DNA directing cell-free protein synthesis [61]
Cell-free TX-TL expression and membrane reconstitution of ATP synthase [62]	Lipid synthesis in liposomes from encoding DNA [63]	First simple cell-free TX-TL gene circuits in test tubes [64]
Cell-free TX-TL synthesis of a functional aquaporin in liposome [65]	Invention of the T7 hybrid cell-free TX-TL system [66]	Cell-free expression of natural operon [67]
Cell-free TX-TL in cell-sized liposomes [43,68]	Inverted membrane vesicles (IMVs) in cell-free TX-TL and oxidative phosphorylation [69]	
	Long-lived semi-continuous cell-free TX-TL [70]	

Long-lived cell-free expression inside liposomes [43]: cell TX-TL of alpha-hemolysin, self-assembly of the membrane channel, nutrients exchanges between inner and outer compartments.

be recapitulated *in vitro* in the background of TX-TL. The total protein production in batch mode of the most recent system exceeds 2 mg/ml [56••]. The successful application of machine learning algorithms for the optimization of cell-free protein synthesis is a promising avenue to improve biological networks [18].

Cell-free TX-TL has been carried out in liposomes, but has rarely gone beyond the expression of reporter genes. The low efficiency of the available kits limits the size of the genetic information that can be expressed into liposomes. In addition, large fluctuations of protein synthesis are observed among single liposome population whatever the technique used, which requires extensive characterization [57•]. Important steps have been achieved, but the realization of complex genetically programmed self-assembled functions such as division or DNA replication in cell-sized vesicles is still lacking. With the development of new versatile and powerful cell-free TX-TL platforms [53••], such accomplishments seem attainable. The major progress made in cell-free expression of integral membrane proteins is a windfall for bottom-up minimal cell synthesis. Yet, the development of the phospholipid bilayer as a real active interface from the internal expression of genes has to be done. The encapsulation of cell-free TX-TL into liposome made of various phospholipids types is also a bottleneck in the field. Beyond phosphatidylcholine, complex membranes are still hard when complex biochemical reactions are used.

Perspectives

The bottom-up synthesis of a minimal cell in the laboratory is still far away but significant advances are being made for each of the pieces making this complex puzzle. The integration and connection of the necessary molecular machineries in cell-sized volumes remains the big challenge. This research area motivates the development of quantitative approaches and platforms for cell-free biology, as well as inspires the creation of artificial chemical systems. The construction of cell analogs using biological components is a formidable playground for multidisciplinary education, from soft matter to metabolic engineering and from genomics to the theory of cellular automata. While tinkering with molecular machineries in test tube reactions is still a necessary step, it is also time to take modern approaches to accelerate the comprehension and the design of complex biochemical systems *in vitro*. Machine learning and high-throughput techniques, for example, are suitable methods to break down the vast parameter space found in such systems.

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