Development of an artificial cell, from self-organization to computation and self-reproduction

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This article describes the state and the development of an artificial cell project. We discuss the experimental constraints to synthesize the most elementary cell-sized compartment that can self-reproduce using synthetic genetic information. The original idea was to program a phospholipid vesicle with DNA. Based on this idea, it was shown that in vitro gene expression could be carried out inside cell-sized synthetic vesicles. It was also shown that a couple of genes could be expressed for a few days inside the vesicles once the exchanges of nutrients with the outside environment were adequately introduced. The development of a cell-free transcription/translation toolbox allows the expression of a large number of genes with multiple transcription factors. As a result, the development of a synthetic DNA program is becoming one of the main hurdles. We discuss the various possibilities to enrich and to replicate this program. Defining a program for self-reproduction remains a difficult question as nongenetic processes, such as molecular self-organization, play an essential and complementary role. The synthesis of a stable compartment with an active interface, one of the critical bottlenecks in the synthesis of artificial cell, depends on the properties of phospholipid membranes. The problem of a self-replicating artificial cell is a long-lasting goal that might imply evolution experiments.

Defining Life

Since Schrödinger's classic book *What Is Life?* (1), the definition of life has always been a puzzle with a variety of partial answers, none really satisfying. One answer is that life is a coded system with mutation and error correction (2). The information is encoded into DNA (the genotype) and the genetic code allows translating this information into proteins (the phenotype). The genetic code is universal, and the genome can support mutations, recombination, duplication, and partial transfer from organisms to organisms. Error correction limits the risk of melting the information into random sequences. This is fine, but life is also metabolism with a permanent absorption and transformation of nutrients from the environment (3). A constant flux of energy is needed to sustain the operation of this living dynamical system out of equilibrium. But life is also self-reproduction (4). Cells originate from preexisting cells by cell division. The DNA genome is replicated, the cell self-reproduces as well as the complete organism. Is self-reproduction a constraint of evolution present at each step and imposing severe design constraints or a possible definition of life? Or is life an emerging phenomenon resulting from complex chemical reactions, developing networks and cycles until, at a certain critical size of this network, life starts as an emerging property (5)? Within this approach, life is a dynamical system where signal to noise is just marginal; a living system positions itself at the edge of chaos. Finally the only generally accepted theme is that life is the result of evolution, natural selection, and adaptation (6), a historical phenomenon. Thus there is not a clear and concise definition of life.

A Living Cell Is Synthesized from a Living Cell

It is noticeable that, in the biological world, quantization is introduced because all organisms are built from cells and all cells originate from preexisting cells. This basic feature was pointed out by Virchow in 1858 in his cellular pathology book (7), with the now famous "Omnis cellula e cellula." Not only is life composed of cells, but also the most remarkable observation is that a cell originates from a cell and cannot grow in situ. In 1665, Hooke made the first observation of cellular organization in cork material (8) (Fig. 1). He also coined the word "cell." Schleiden later developed a more systematic study (9). The cell model was finally fully presented by Schwann in 1839 (10). This cellular quantization was not a priori necessary. Golgi proposed that the branched axons form a continuous network along which the nervous input propagates so that neurons, given their huge extension, will not be part of the cell theory (11). Cajal then showed that neurons are indeed cells (12).

De Novo Construction of a Cell

However complex living cell organization and functions are, it is today conceivable to synthesize a cell from its basic elements. This approach aims at a global understanding of cellular life, in particular, the cooperative link between its three essential components: the DNA information, the compartment, and the metabolism (13). The synthetic cell, built from scratch, would be a unique compartment with a structure and an organization similar to a bacterium. ATP and GTP would be used as the energy source in the first stages of the development. For the information part, the synthetic DNA programs would be expressed with the transcription and translation machineries extracted from an organism. The physical boundary of the artificial cell would be a phospholipid bilayer. In aqueous solutions, phospholipids self-assemble spontaneously into cell-sized vesicles, a process driven by the hydrophobic interaction between the fatty linear chains. Lipid bilayers are also the natural template for membrane proteins. Membrane proteins, essential to any living system, carry out exchanges of materials and information between the inside and outside world of the cell. Water is evidently an essential part of life, for vesicle formation as well as for molecular interactions and protein folding.

The main goal of an artificial cell built up from the bottom is its ability to self-replicate, evidently a formidable task. Before entering into more details, we discuss briefly the theoretical and the experimental content related to this project.

Von Neumann's scheme of cell-reproducing automata: a computational approach to cellular life

Von Neumann's theory of self-replication for natural and artificial automata (4) is a useful guideline to understand the logical parts that compose self-reproducing systems, including living cells (Fig. 2*A*). Let us notice that von Neumann theory predated the discovery of the structure of DNA. The information part of the automaton is defined as a tape I, which is both a code and a program. This memory, only used for computation, is a Turing-

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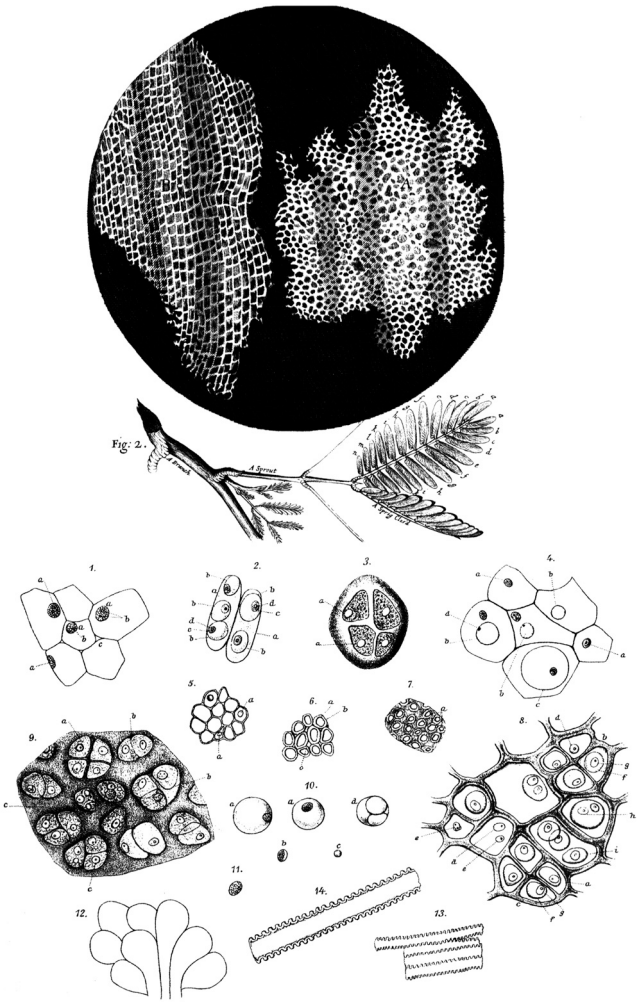

Fig. 1. Cells observed by Robert Hooke and Theodor Schwann. (*Top*) Cells observed through a slice of cork with a microscope in 1665 by Robert Hooke (8). Hooke saw a pattern of holes that he named cells (each cell about 20 μm). In the figure on the left, the cells are split the "long ways" and show "diaphragms" between cells as described by Hooke. A branch of the cork oak tree is shown below. Reprinted from ref. 8. (*Bottom*: images 1 to 14) Plant and animal cells observed by Theodor Schwann in 1839 (10) (figures 1, 2, 3, and 14 are from plants, figures 4, 5, 6, and 7 are from a fish, and figures 10, 11, and 12 are from an animal eye). Reprinted from ref. 10.

like machine containing the data and the instructions. The second part D of the automaton is composed of parts A, B, and C. The universal constructor A constructs the offspring part's D according to the instructions encoded in the tape I, whereas the copier B replicates the instruction tape I. The controller C controls the operations of A and B. The new automaton follows the same scheme to self-reproduce. The parts used in this scheme have direct counterparts in real living cells (Fig. 2*B*): Tape I is the DNA genome, part A is the transcription/translation machinery (proteins and RNA), part B is the DNA replication machinery (proteins), and part C is the regulation (proteins and RNA). This logic of self-reproduction applies well to automata but what about the artificial cell? In particular, what meaning should we give to instructions I in the case of a genome where there are no well-defined instructions?

Beyond von Neumann: Self-Organization and Metabolism

Although useful to understand the global architecture of a self-reproducing cell, von Neumann concepts do not capture the entire essence of cellular life. In bacteria, the DNA program represents only 1% of the volume of the organism. Life is not only a program;

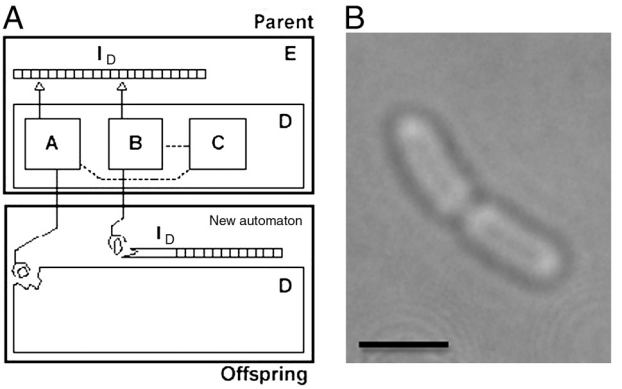

Fig. 2. A self-reproducing automaton and a bacterium. (*A*) Von Neumann's logic. A universal constructor (part A) constructs the hardware (part D) of the offspring automaton according to the instruction I. The copier B makes a copy of the software part of the offspring automaton, the instruction tape I. Part A and part B are controlled by the regulatory part C. (*B*) Phase contrast microscopy image of the division of an *E. coli* cell (scale bar: 1 μm).

it relies also on many other fundamental nongenetic properties. Molecular self-organization (Fig. 3*A*) and molecular crowding (Fig. 3*B*), for example, are critical processes in living cells. The phospholipid membrane displays a wealth of properties necessary for cellular organization, such as the formation of domain patterns (14). The phospholipid bilayer is, in turn, a template for the self-organization of macromolecular protein structures (Fig. 3*C*). In *Escherichia coli*, cross-linked networks and polymers responsible for cell rigidity and cell shape are assembled from the lipid bilayer (15). At the cellular level, one can wonder how a DNA program is developed to take advantage of self-organization processes. It is one of the motivations of the bottom-up approach to artificial life: It also addresses fundamental questions on nongenetic processes. Assembling a synthetic cell unfolds the importance of physical aspects that are, in vivo, regulated by already evolved gene networks. We shall see later, for instance, how the osmotic pressure favors exchanges of small nutrients by increasing the permeability of the lipid bilayers.

Questions and constraints related to metabolism are also excluded from the von Neumann theory of self-reproducing automata. A living organism is an open system made of hundreds of chemical reactions whose properties go beyond the DNA program. A continuous uptake of energy and a continuous elimination of reaction by-products are critical for any living systems as well as for a synthetic cell. The adenylate energy charge $(([ATP]+\frac{1}{2}[ADP])/([ATP]+[ADP]+[AMP]))$ is an index that captures the energy state of a system (16). This index also contains the inhibitory action of ADP and AMP on the activity of enzymes. Enzymes using ATP have a strong nonlinear response to the energy charge index. As a consequence, the energy charge has to be maintained at high level. In *E. coli*, for instance, the energy charge index is maintained between 0.8 and 0.9 during cell growth (17). Accordingly, the construction of an artificial cell requires the development of an artificial environment. The external medium has to be an isotonic nondissipative feeding solution that maintains physiological conditions by exchanges of low molecular mass nutrients, nucleotides, and amino acids, in particular, through the phospholipid membrane. The volume of the external medium has to be orders of magnitude larger than the vesicle to drain off the by-products of reactions by diffusion from the internal compartment.

The bottom-up approach to synthesize an artificial cell addresses a wide variety of questions on soft matter physics, computation, and chemistry. Many experimental problems have to be solved at the same time to construct a stable compartment that can be further developed. Selective exchanges, osmotic pressure problems, and efficient transcription/translation are among the issues that must be solved to obtain an initial workable microscopic

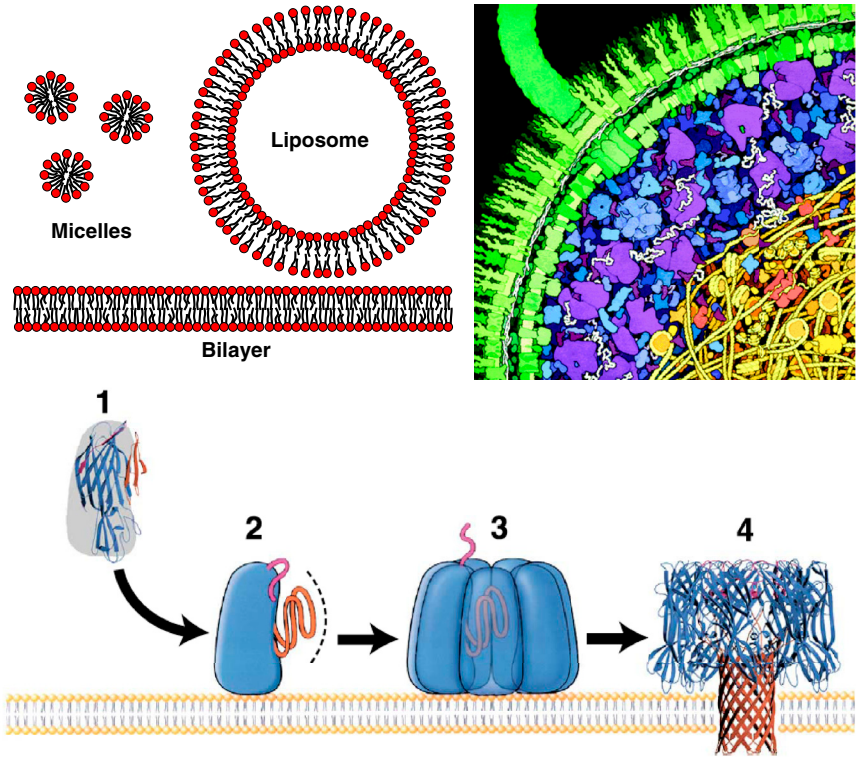

Fig. 3. Example of nongenetic processes. (*A*) Cross-sections of the three structures that self-organize upon the dispersion of phospholipids in aqueous solutions. Phospholipids molecules are composed of a hydrophilic head (red circles) and of two hydrophobic chains (dark region). The interior of spherical micelles is composed of hydrophobic chains only. Bilayer structures arranged in spherical shapes with aqueous solutions inside and outside are called liposomes or vesicles. Large planar bilayers can also be formed. (*B*) A picture illustrating molecular crowding in the cytoplasm of *E. coli*. In green: the phospholipid membrane, the cell wall, and a flagellum. In purple: the ribosomes synthesizing proteins (white). In blue: the high protein concentration in the cytoplasm. In yellow: DNA with interacting DNA binding proteins [Reproduced with permission from Dr. David S. Goodsell (Copyright 2000, David S. Goodsell).] (*C*) α-hemolysin pore formation, an example of protein self-organization at the membrane. The soluble monomer (step 1) interacts with the bilayer (step 2) to form a prepore composed of seven monomers (step 3) before pore formation (step 4) through the membrane [Reproduced with permission from ref. 103 (Copyright 2003, Elsevier).]

vesicle. The implementation and the coordination of complex functions, such as volume expansion and fission, can in principle be solved with DNA programs. So far, no artificial cell capable of self-reproduction has been synthesized using the molecules of life, and this objective is still in a distant future. Nevertheless, the research in this field highlights the essential missing links to achieve self-reproduction. This research redefines our knowledge of the basic functions of a cell, and our understanding of the physical and chemical processes associated with unicellular life.

Let us notice that a pure computational approach to synthetic life has been achieved recently with the pioneering work on *Mycoplasma genitallium* (18). An artificial cell was synthesized by knocking out the genome of a living organism to a minimum set of genes. This reductive top-down approach demonstrates that bacterium can be reprogrammed with synthetic genomes, which opens a wide range of biotechnological applications. Top-down and bottom-up approaches to synthesize artificial cells have different perspectives and they use different techniques, but they both require the development of original methods and ideas (19).

Bottom-up Development of an Artificial Cell: Broad Considerations

For the experimenter, the real question is, how can a synthetic cell be developed as a DNA programmable phospholipid vesicle? The effort does not focus only on the preparation of a DNA program. The experimenter studies how the program that is being developed connects to the other parts, the membrane and the other nongenetic processes in particular. As the DNA program gets larger, the experimenter tries to understand how complex DNA subprograms, designed for vesicle division for instance, connect to the whole code-script. As opposed to living cells, the entire program of the artificial cell has to be devised, which is a difficult problem, as we shall see. The metabolism of the phospholipid vesicle increases as it is built up. The experimenter needs to understand how the energy uptake and the elimination of by-products can be carried out and optimized, which addresses questions on transport and exchanges.

In the second part of the article, we follow von Neumann's concepts of self-reproducing machine to discuss the construction of an artificial cell. The universal constructor, essential to translate the DNA program, takes a central place in the experiments. The computational framework imposed by von Neumann's scheme, however, does not exclude the importance of noninformational processes from the discussion.

The Molecular Approach to Artificial Cell: Experiments, Possible Extensions, and Limitations

Universal Constructor: From Information to Proteins. DNA is a chemically and mechanically robust biopolymer that serves as a code and a memory. DNA has almost no enzymatic or catalytic activity. In living cells, as well as in a DNA-programmed artificial cell, proteins perform the tasks, each one being encoded by a DNA gene. These nanomachines have a wide range of functions. Proteins can be catalysts, molecular motors, or membrane channels. Proteins self-assemble in large structures to carry out complex functions, such as flagella for motility (20). Proteins also interact physically with DNA to regulate gene expression. The transfer of the DNA sequence information to proteins is carried out in two steps: the transcription and the translation. During transcription, an RNA polymerase protein copies double stranded DNA into a single stranded messenger RNA. Transcription is rather simple compared to translation, which requires on the order of 100 proteins and tRNA molecules. The main component of translation is the ribosome, a large macromolecule that translates messenger RNAs into proteins (Fig. 3*B*), using tRNA for the translation of one codon at a time. In bacterial cells, the coupled transcription/translation process takes on the order of 1 min for a gene of 1,000 base pairs, from the beginning of transcription to the synthesis of a functional protein. DNA genes encode all of the transcription and translation components. Transcription and translation machineries, common to all living organisms, are the key part of a universal constructor, which can construct all of its own components. In an artificial cell, the transcription/translation machineries must be physically present to carry out the expression of the first DNA programs. In the long run, transcription and translation machineries must be efficient and viable enough to boot up the expression of a minimal synthetic DNA genome.

Universal Constructor in the Test Tube. Transcription/translation extracts are the main systems to carry out gene expression in vitro. Originally prepared to study biological processes in vitro (21), modern cell-free systems have been developed for large-scale protein synthesis in response to an increasing number of applications in biotechnology (22). A cytoplasmic extract, prepared from a living organism, provides the translation machinery. The endogenous genetic material (DNA and messenger RNAs) is removed from the extract during the preparation. The transcription of genes is carried out by adding a bacteriophage RNA polymerase to the extract. Typical cell-free expression systems are prepared from *E. coli*, wheat germ, and rabbit reticulocytes. The best cell-free systems can deliver 1 mg/mL of reporter proteins in batch mode. The decrease of the energy charge is one of the main factors that limits gene expression to a few hours in batch mode reactions. The protein synthesis rate is exponentially dependent on the energy charge (23). Only a slight decrease in the ATP concentration induces a dramatic change in the protein synthesis rate. Changes of pH during the reaction (24) and the fast degradation of some amino acids (25) are the other important factors responsible for limiting expression to a few hours in batch mode reactions. Ultimately, the cell-free protein synthesis stops due to the consumption of the finite resources present in the reaction.

The PURE system, a breakthrough in constructive biology, is another technology to carry out gene expression in vitro (26). In the PURE system, the translation machinery of *E. coli* is entirely reconstituted from purified components and ribosomes, whereas the transcription is driven by a bacteriophage RNA polymerase. The protein production with the PURE system is slightly inferior compared to cell extracts. As opposed to cell extracts, the entire composition of the PURE system is known. In contrast, cell-free extracts contain the complete cytoplasmic part of a cell with many other molecules that are not involved in transcription/translation. These additional components carry out other essential functions or can be used to implement important functions. For example, chaperonins present in the extract are necessary for protein folding. Purified chaperonins have to be added to the PURE system to increase the amount of functional proteins produced (27). The endogenous proteases AAA + present in *E. coli* extracts can be used for specific protein degradation (28). These features make *E. coli* extracts advantageous compared to the PURE system and to eukaryotic systems such as wheat germ or reticulocytes.

The invention of continuous transcription/translation reactions was a major step in cell-free expression. Spirin and coworkers were the first to show that cell-free expression can work over long periods of times (29). Continuous exchanges between the cell-free reaction (1 mL) and a reservoir of nutrients (a 10-mL buffered solution of nucleotides and amino acids) are carried out through a dialysis membrane of molecular mass cutoff of 10 kDa. The protein synthesis rate observed over a few hours in batch mode reactions can be maintained a few days in continuous mode. This experiment demonstrated that the viability of the translation machinery in vitro is in the range of the doubling time of a cell-free reaction (30). For a cell-free reaction initially composed of 10 mg/mL of proteins, the synthesis of 10 mg/mL of proteins would take a few days. However, the cell-free synthesis of an amount of proteins that is equal or greater than the initial reaction protein content has not been proven yet. An artificial cell can in principle rely on the transcription/translation machinery provided by the extract for at least one generation. Important ongoing efforts are also made to improve the energy regeneration in cell-free reactions (31, 32).

A bottleneck of cell-free expression systems is the protein synthesis rate, which is 200–300 times smaller than the protein synthesis rate in vivo (33). For *E. coli* extracts, the cytoplasmic protein content of the cells (200–300 mg/mL) is diluted 10 times during crude extract preparation (30 mg/mL). In a cell-free reaction, the best protein production occurs at a crude extract protein concentration of 10 mg/mL typically. Because of this dilution, the translation initiation and elongation rates are one order of magnitude smaller than the in vivo rates (33).

The transcription used in cell-free systems poses serious problems for the development of large DNA programs. Modern cell-free systems use bacteriophage RNA polymerases with their specific promoters because they are the most efficient polymerases. Informational processes have been reconstituted with such hybrid cell-free systems (34–38). It is hardly conceivable, however, that a synthetic cell would work with a DNA program made of bacteriophage promoters only. The number of bacteriophage promoters and the regulation of these promoters with operator sites are too limited to build a sufficiently large DNA program. Unlike other information systems, such as electrical circuits, gene networks are not constructed with elementary blocks that can be repeated. Although many gene motifs have similar functions to electrical circuits, such as switches, amplifiers, and filters, they cannot be used in series or parallel arrangements (39). Rather, genetic modules work with specific transcription factors and their respective DNA promoters and operators. The recent preparation of a universal T7 transcription system extends the possibilities to express genes from various organisms, but it does not solve for the lack of modularity (40). Compared to bacteriophage transcription, the structure of bacterium promoters allows for a much larger modularity of transcription, even with a single transcription factor. The recent development of an *E. coli* cell-free system driven by the endogenous RNA polymerase as efficient as conventional bacteriophage cell-free systems opens previously undescribed possibilities to develop DNA programs (41).

Another serious bottleneck of cell-free systems is the lack of procedures to control the degradation of both messenger RNA and protein. Gene expression obeys the source–sink dynamics, in vivo as well as in vitro. As it is well known in information theory, erasing the information is as fundamental as its generation and more costly in energy (42). The degradation of both messenger RNAs and proteins needs to be controlled to get an economical and an efficient bookkeeping of the information processing. In particular, messenger RNAs lifetime must be small to avoid a huge amplification and accumulation of unnecessary information. It is critical for an artificial cell that has a fixed volume in the early stages of development. The recent development of methods to control the global inactivation rate of messenger RNAs and the degradation rate of proteins in an *E. coli* cell-free system partly resolve the source–sink unbalance (28).

Universal Constructor in Synthetic Vesicles. In vitro transcription/translation inside cell-sized synthetic phospholipid vesicles, at a level comparable to test-tube batch mode reactions, was demonstrated recently. Ishikawa et al. expressed GFP through a transcriptional activation cascade inside small liposomes (43). Expression inside the submicronic vesicles was measured by flow cytometry. Noireaux and Libchaber carried out cell-free expression in large unilamellar vesicles observed by fluorescence microscopy (44) (Fig. 4). In this work, the vesicles were formed in a solution containing all the basic nutrients necessary to feed the reaction, whereas DNA and cell-free extract were encapsulated inside the liposomes. To create a selective exchange of small nutrients through the vesicle membrane, the pore-forming protein α-hemolysin was expressed inside the vesicles (Fig. 3*C*). α-hemolysin, a water-soluble protein, self-assembles into membrane channels of diameter 1.2 nm (45), which corresponds to a molecular mass cutoff of 2–3 kDa. This cutoff was adequate to feed the vesicle with nutrients and to eliminate the by-products of reactions by diffusion. The authors showed that expression could be sustained for up to 4 d. This work was also a real example of a DNA toolbox-solving problem where the three parts, information–compartment–metabolism, were worked out together. Among thousands of gene products, whose functions have

been reported in literature, one gene was used to implement a previously undescribed feature that improves the properties of the system. In this particular case, the channel was formed through the lipid bilayer by the self-organization of seven α-hemolysin proteins, a general type of processes found in living systems. Furthermore, this work highlighted the problems related to the osmotic pressure and some potential advantages. A fine balance of the osmotic pressure was required to prevent the vesicles from bursting after formation. The authors showed that cell-free expression of EGFP inside vesicles could last a longer time compared to batch mode reactions carried out in a test tube (Fig. 4). In that case, exchanges through the membrane were facilitated by the spontaneous formation of nanopores due to the osmotic pressure (46). Although many problems remain to be solved, efficient cell-free expression inside synthetic phospholipid vesicles was a major step in the constructive approach to artificial cells.

Program Tape and Controller C: DNA Genome and Regulation. As mentioned earlier, the artificial cell project discussed in this article consists of synthesizing DNA programmable phospholipid vesicles. Let us notice that RNA could be used to program synthetic cells. The RNA world hypothesis suggests that RNA could have been both the information and the catalyst molecules of primitive cells (47, 48). The catalytic and autocatalytic activities of RNA have been experimentally demonstrated and the translation process is largely based on RNA. RNA protocell models have been proposed (49). Although conceivable, an RNA-based artificial cell capable of self-reproduction seems far more complicated to build than a DNA cell. An entire RNA program would need to be invented and synthesized.

Many recent experimental and theoretical efforts have been made to determine the minimal size of a synthetic DNA genome for self-reproduction (50–57). It is now accepted that 200–400 genes are required to get an artificial cell capable of self-reproduction over many generations in realistic laboratory conditions. This estimation was confirmed by the top-down reductive work carried out on the genome of *Mycoplasma genitallium*, which has the smallest genome known yet (18). This work required the development of previously undescribed cloning methods to reconstitute genome-size DNA plasmids (58–60). DNA programs of smaller size can be also prepared by recombination or direct assembly of synthesized oligonucleotides (61–63). To synthesize a DNA genome, one can imagine that genes or entire operons would be copied from simple organisms such as bacteria or bacteriophages. Most of the genes in microorganisms are smaller than in higher organisms, and they encode for single-function proteins (64, 65).

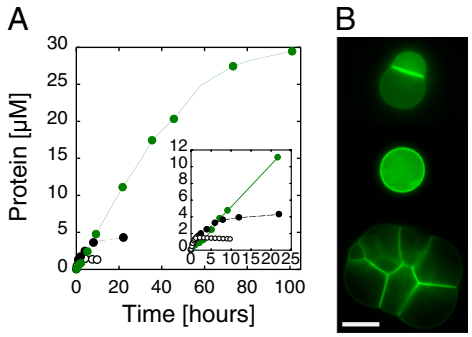

Fig. 4. Protein expression in a test tube, in a vesicle, and in a vesicle with pores. (*A*) Kinetics of expression of eGFP in a test tube (open circles), in a phospholipid vesicle (closed dark circles), and expression of α-hemolysin-eGFP in a phospholipid vesicle (closed green circles). (*Inset*) Blowup of the first 20 h. (*B*) Fluorescence microscopy images of α-hemolysin-eGFP expressed inside vesicles after a few hours of expression [(*Top* to *Bottom*) a vesicle doublet, a single vesicle, and an aggregate of vesicles]. Scale bar: 10 μm. [Reproduced with permission from ref. 43 (Copyright 2004, National Academy of Sciences).]

The construction of synthetic genome-size DNA programs is feasible, although technically challenging. The problem of synthesizing a large DNA plasmid resides in the network complexity that arises far below 200 genes. Based on current knowledge, a deterministic approach to synthesize a plasmid of a few hundred genes does not seem reasonable. Networks composed of a few genes only become rapidly unpredictable in their behavior. Dozens of articles have been written on the bacteriophage Lambda bistable switch, a paradigm in gene expression processes (66). The genome of coliphage Lambda, 48.5 kb long, encodes for 30 proteins approximately. After entering a bacterium *E. coli*, the decision between the two alternative pathways, lytic or lysogenic, is made by a genetic bistable unit consisting of three operators overlapping two promoters oriented in opposite direction. Despite being one of the most studied elementary gene networks, its mechanism has not been entirely uncovered yet (67).

A combinatorial assembly of DNA subprograms seems more appropriate to get a synthetic DNA genome large enough to program an artificial cell for self-reproduction. Sets of operons encoding for the essential genetic modules can be found in minimum genome lists. Practically, operons could be copied from living organisms and either recombined or ligated to make synthetic genomes. This approach precludes a comprehensive path to DNA programming on large scales, because the analytical design of DNA programs is currently conceivable only for a small number of genes with simple regulation. Programming synthetic phospholipid vesicles is therefore a choice of taking a detailed approach limited to a small number of DNA subprograms or a global approach that bypasses a comprehension of the DNA subprograms networking. In particular, the development of a regulatory part, represented as part C in the von Neumann schematic (Fig. 2*A*), to coordinate the different DNA subprograms in time may require evolution experiments.

With more and more DNA subprograms characterized, finding the correct piece of information becomes an issue. Among the available resources, the BioBricks Foundation is certainly the most interesting initiative to find synthetic DNA programs (68–70). This registry of standard biological parts catalogs DNA information programs of different sizes, namely parts, devices, and systems whose products have been characterized. The registry, which works as an open wetware where any individual can add parts, is becoming a giant toolbox of DNA subprograms. In return, all the elements present in the registry are available for free.

In continuous transcription/translation cell-free reactions, the maximum protein production is on the order of 5 mg/mL (with bacteriophage transcription). The average copy number of proteins in the *E. coli* cytoplasm is 500 nM (71). With an average protein size of 50 kDa, the synthesis of 200–400 gene products corresponds to a protein concentration of 5–10 mg/mL. Hence, the protein production of cell-free expression systems is in the range of self-reproducing synthetic cell.

Encapsulation, Active Membrane, and Division. With a program tape and a universal constructor, von Neumann's scheme captures correctly the fundamental working structure of real living cells. The idea of compartment, however, is rather simplified. Von Neumann's model does not include the generation of the offspring's external boundary that encapsulates the program tape and the different parts of the constructor. The generation of the outer boundary of the whole automata could be included in part A's function without deteriorating the scheme. Still, the scheme does not catch the technical difficulties to fabricate a stable cell-sized compartment. The physical boundary of bacterial cells consists of a phospholipid bilayer and a cell wall, a polymeric network made of amino acids and sugar molecules (Fig. 3*B*). The phospholipid bilayer is an active interface where membrane proteins control the information between the cytoplasm and the environment. The cell wall, anchored to the lipid membrane,

provides the structural strength to the bacterium. During cell division, synthesis of the membrane and cell wall have to be synchronized with the binary fission. In view of such complex mechanisms, involving tens of proteins all encoded in the DNA program, the fabrication of a stable compartment with an active interface is one of the most challenging steps in the synthesis of a DNA-programmed artificial cell. The genetic program and the transcription/translation machinery, composed of hundreds of components, have to be encapsulated inside a phospholipid vesicle. The bilayer needs to be developed as an active interface, which requires the insertion of integral membrane proteins. Let us notice that the artificial cell could, in principle, be made of more elaborate envelopes. For example, Fox showed that heated amino acids can polymerize into small vesicles in the presence of water (72). Vesicles could also be made out of synthetic block–copolymers (73).

Natural lipids form small unilamellar spherical vesicles spontaneously upon suspension in aqueous solutions due to the favorable packing parameter of phospholipids (74, 75). Soft matter and protocell research have provided many ways to fabricate cell-like compartments with pure amphiphilic molecules (76–78). Some of these methods are used to encapsulate pure proteins inside phospholipid vesicles to study cellular processes. For example, quantitative experiments on the deformation of vesicles upon the polymerization of cytoskeleton proteins have been performed (Fig. 5 *A*–*C*) (79–83). These experiments, however, do not incorporate the information flow. Recently, two different encapsulation techniques were used to achieve efficient in vitro gene expression in synthetic phospholipid vesicles (43, 44). The demonstration that a simple lipid bilayer can enclose mixtures as complex as cell-free reactions was an important step in the field of synthetic cell. Whereas a transcriptional activation cascade was carried out inside small unilamellar vesicles obtained by the swelling method (43), an emulsion technique was used to create large continuous vesicle bioreactors (44) (Fig. 4). A precise balance of the osmotic pressure was required to prevent the vesicles from bursting within seconds after formation. The osmotic pressure is one of the relevant parameters for the synthesis of an artificial cell. The polymerization of a robust cell wall anchored to the lipid bilayer necessitates the insertion of integral membrane proteins (IMPs) for which no significant results have been obtained yet.

Many ongoing studies are performed to characterize protein synthesis inside liposomes. The internal structure of large multilamellar vesicles, obtained by the freeze-dry method, was studied by quantitative measurements of gene expression (84). The encapsulation of the PURE system inside small unilamellar vesicles of 100 nm in diameter revealed a strong heterogeneity of the entrapment below the micrometer size (85, 86). Amphipathic peptides, which insert spontaneously into lipid bilayers, and autonomous pore-forming proteins have proven to work when they are expressed inside synthetic vesicles (87). The polymerization of cytoskeletal proteins at the membrane is currently investigated (Fig. 5*D*). The formation of membrane patterns or domains with different phospholipid compositions has been also investigated (87). Such physical properties of biomembranes could theoretically facilitate division mechanisms (88). Finally, quantitative methods are developed to study molecular self-organization processes using cell-free expression (89).

The efficient insertion of IMPs into the bilayer is the real current limitation to the development of an active interface. In *E. coli*, the integration of membrane proteins inside the lipid bilayer occurs via the SecYEG pathway, itself composed of membrane proteins (90). Recently, techniques to express a large amount of functional IMPs in vitro have been developed (91). IMPs, expressed in batch mode reactions, were integrated into the bilayer of small unilamellar vesicles added to the solution (92). This was observed without the addition of membrane protein insertion mechanisms suggesting that *E. coli* cell-free extracts contain a remaining strong activity for inner membrane protein

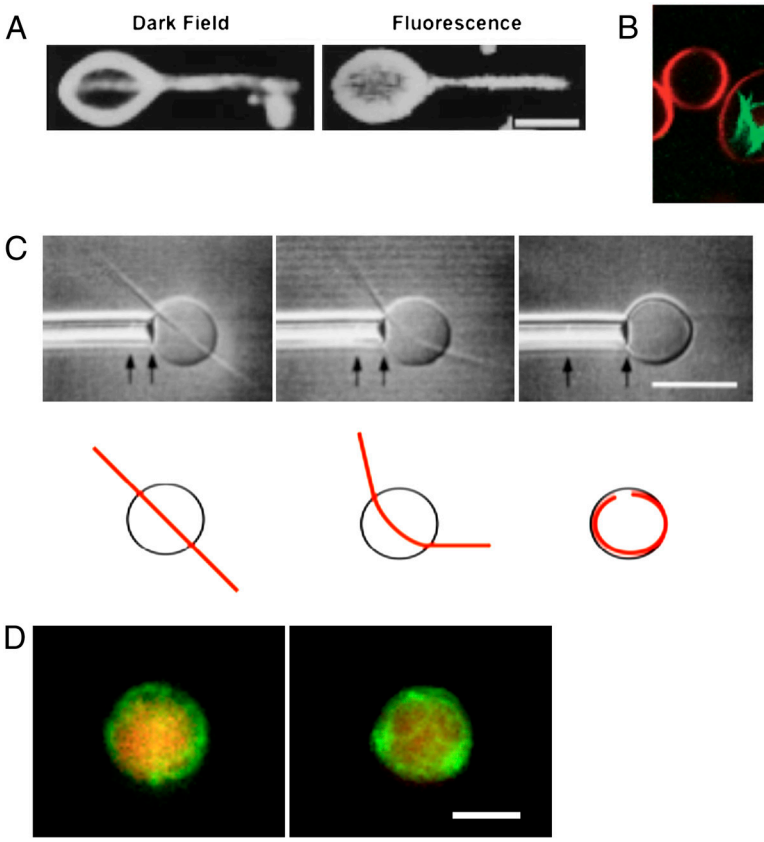

Fig. 5. Self-organization of polymeric structures inside phospholipid vesicles. (*A*) Filamentous actin (F-actin) with fascin in a liposome. Scale bar 5 μm. [Reproduced with permission from ref. 79 (Copyright 1999, Elsevier).] (*B*) F-actin and anchoring proteins (ankyrin and spectrin) in a liposome. Red: lipid, Green: F-actin. Scale bar 5 μm. [Reproduced with permission from ref. 81 (Copyright Wiley-VCH Verlag GmbH & Co.).] (*C*) Microtubule in a liposome. Buckling of microtubule using micropipette aspiration (the arrow show the position of the vesicle in the pipette). Microtubule is shown as red line, whereas membrane is a black line in the schematic illustration. [Reproduced with permission from ref. 83 (Copyright 1996, American Physical Society).] (*D*) Cell-free expression of the bacterial actin MreB and its associating membrane protein MreC inside a liposome. Green: YFP-MreB, Red: Rhodamine-BSA (used as a fluorescent cytoplasmic marker). YFP-MreB form filaments as it interacts with MreC. Scale bar: 10 μm.

insertion. With the PURE system, IMPs can be also expressed and integrated into phospholipid bilayers, although with a much lower efficiency (93). Despite many efforts, the development of an active membrane, using only the internal gene expression, has yet to be demonstrated.

Physical and chemical constraints limit the size of living organisms to a minimum of 250 nm ± 50 nm (94). The upper limit to the size of synthetic cells seems to be around a few micrometers, certainly below 10 μm. The production of each protein at a sufficient concentration from a single gene copy is cost-effective and faster in small volumes. An increase in size by a factor of two requires an increase of protein production by one order of magnitude. Furthermore, some DNA programs, which could be used to program the vesicles, encode for processes that do not work above a certain size. The FtsZ ring, responsible for bacterial fission, cannot self-assemble inside cylinder-shaped phospholipid vesicles that have a diameter greater than 10 μm (95). A small vesicle, however, is detrimental for the development of an active interface because the surface to volume ratio is inversely proportional to the radius.

Vesicle division is one of the upcoming challenges in the artificial cell project. Vesicle division addresses both computational and nongenetic questions. On one hand, the process of vesicle fission at constant volume cannot occur spontaneously (13). On the other hand, multiple subprograms have to be coupled and coordinated to carry out division. Fission, governed by 10 genes in *E. coli* (96), is coupled to volume expansion, which necessitates the synthesis of new phospholipids, and cell wall synthesis. Although polymerization of cytoskeletal proteins is currently being investigated (Fig. 5*D*), volume expansion by phospholipid synthesis, recently programmed with a couple of genes (93), could not be observed.

The Copier: DNA Replication and Segregation. In both living cells and von Neumann's self-reproducing automata, DNA replication is a central part because it contains the memory from which the offspring is generated. In vitro DNA replication and amplification is extensively used in laboratories through the PCR. PCR has been carried out successfully in phospholipid vesicles (97). Natural processes such as thermal convection can also drive PCR because steady circular flow occurs between hot and cold regions, and DNA is exposed to thermal cycling (98, 99). DNA replication by PCR, however, cannot be used for a synthetic cell because thermal cycling at high temperature would denature the proteins making the transcription and translation machineries. No simple enzyme-free solution exists to carry out DNA replication in vitro. The DNA replication of bacteriophage T7, which requires only four proteins and some nutrients (100), is an interesting alternative for a synthetic cell.

In addition to the process of replication, partitioning the duplicated genetic materials is also a key step for self-reproduction. The question is whether a simple mechanism can be found to synchronize cell division with DNA replication. Otherwise, the various functions (volume expansion, fission, and DNA replication) will have to be solved by programming the vesicle. This is one of the main questions raised in this article and the challenges faced by the experimenter: What are the cooperative links between computational and nongenetic processes necessary to synthesize an artificial cell?

Conclusions

The artificial cell project is a framework, a laboratory to test many hypotheses. The concept of phospholipid vesicle automated with DNA programs is becoming a reality, although many experimental challenges have yet to be resolved. Advances in cell-free transcription/translation reactions allow the expression of many genes. The development of a DNA program, however, remains a very complex hurdle. Evolved ready-to-use DNA programs can be tested and are part of the solution. Yet, one might need to evolve previously undescribed programs in the laboratory, a difficulty that living cells do not have.

Any cell is a crowded environment where self-organization sets in and leads to unexpected developments (Fig. 3*B*). Molecular crowding, confinement, and adsorption are inherent properties of biological systems (101). Entropic effects can lead to aggregation, and local gradients to various patterns. All those effects, independent of the DNA program, are questioned through the artificial cell project. Let us notice that the evolution approach to synthetic life provides possible nongenetic scenarios for membrane growth and vesicle division (49, 102). How such processes can be used in a DNA-programmed artificial cell is not clear.

Finally in this scenario complex proteins and DNA subprograms, given to us by evolution, are used. Another approach, closer to what is believed to be the early soup, could use simpler elements. Various RNA polymers could be encapsulated in vesicles. In such small boundary conditions, burgeoning chemical reactions would be tested (47).

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