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PERSPECTIVE

Toward an artificial cell based on gene expression in vesicles

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Abstract
We present a new experimental approach to build an artificial cell using the translation machinery of a cell-free expression system as the hardware and a DNA synthetic genome as the software. This approach, inspired by the self-replicating automata of von Neumann, uses cytoplasmic extracts, encapsulated in phospholipid vesicles, to assemble custom-made genetic circuits to develop the functions of a minimal cell. Although this approach can find applications, especially in biotechnology, the primary goal is to understand how a DNA algorithm can be designed to build an operating system that has some of the properties of life. We provide insights on this cell-free approach as well as new results to transform step by step a long-lived vesicle bioreactor into an artificial cell. We show how the green fluorescent protein can be anchored to the membrane and we give indications of a possible insertion mechanism of integral membrane proteins. With vesicles composed of different phospholipids, the fusion protein alpha-hemolysin-eGFP can be expressed to reveal patterns on the membrane. The specific degradation complex ClpXP from *E. coli* is introduced to create a sink for the synthesized proteins. Perspectives and subsequent limitations of this approach are discussed.

1. Introduction

Biological systems are much more complicated than the usual systems of physics, and a living cell is an incredibly complex machine. Living cells reunite most of the fundamental properties of physics and chemistry to combine molecules into a propagating system that can adapt to its environment. Because cellular life is the only known system that uses a program to replicate, move, invade space and evolve, it can be seen as an ultimate property, a model to build machines and foster the imagination. On the basis of current knowledge of cellular function and the progress in molecular biology and soft condensed matter, we have devised a constructive approach to build an artificial cell inspired by von Neumann's work on automata—self-reproducing machines [1, 2].

Historically the first breakthrough in programmable machines came with the introduction in 1936 of the abstract Turing machine, by Alan Turing [3]. This program concept gave birth to computer science and led to the construction of new automated mechanical devices known as robots. Later, von Neumann used the concept of the universal Turing machine to develop the theory of a self-reproducing machine based on a universal constructor [1, 2]. It is remarkable that, without knowing the basic mechanisms of biological information, von Neumann was able to draw universal concepts that mimic some functions and self-reproduction of cellular life. His self-reproducing constructor is capable of reading an input tape, interpreting the data, constructing a configuration it describes by using an arm, and generating a copy of that tape. This model can be translated for a cell

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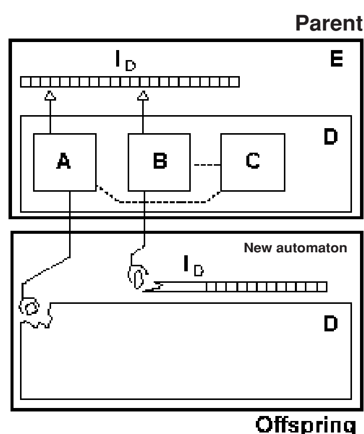

Figure 1. The basic composition of von Neumann automata. A universal constructor A can construct another automaton according to instruction I. The copier B can make a copy of the instruction tape I. The controller C, which combines A and B, allows A to construct a new automaton according to I and B to copy instructions from I and attach them to the newly created automaton and separates the new automaton from the system A + B + C. An automaton D consists of A, B and C. An instruction tape I_D describes how to construct automaton D. An automaton E consists of D + I_D.

(figure 1): the instruction tape I is the DNA, part A corresponds to the RNA polymerase and the translation machinery, part B the DNA polymerase and the replication machinery, part C the transcription and translation regulation.

Here, we present a new experimental approach to building an artificial cell. A synthetic genome, used as DNA software, is designed step by step to program a phospholipid vesicle that encloses a cell-free expression system used as the hardware. Although the long-term objective is the self-reproduction of the vesicle-bioreactor, the different steps of this novel approach produce molecular systems that display some of the properties of life. In particular, with a DNA program, how to transform a synthetic lipid bilayer into an active interface, how to create specific degradation of the synthesized messengers and proteins and how to destabilize the membrane to initiate division of the vesicle are among the challenging steps of this approach.

2. Building an artificial cell: cell-free approach and significance

Technically, it is now possible to assemble in the laboratory a synthetic genome composed of a few tens of genes. When encapsulated in a lipid vesicle, this custom-made DNA program would be used as a software to develop the functions of the vesicle. Experimentally, the bottleneck is to find a system to carry out efficiently, *in vitro*, the two essential steps of gene expression: transcription of DNA and translation of messenger RNA. Transcription is not so much a problem. One can start with the powerful bacteriophage RNA polymerases with their specific promoters. Even though the possibility of building genetic circuits is reduced, the transcription machinery involves only one protein and the reaction conditions are flexible; it can thus easily be coupled to translation. Translation involves many more components, about one hundred, and the reaction conditions depend on very precise adjustments of some parameters, such as magnesium and salt concentrations. Apart from the non-affordable reconstitution of translation by Shimizu and co-workers [4], a cell-free expression extract is the best available system to express proteins *in vitro*. These cytoplasmic extracts are prepared from cells rich in ribosomes. *E. coli* bacteria and wheat germ have mainly been used. They are optimized for translation and their endogenous genetic content (DNA and messenger RNA) is removed so that exogenous genes can be expressed. In the last two decades, most of the problems that limit extract activities have been solved and cell-free expression has been considerably improved. Endogenous nuclease activity has been reduced, intensive degradation of certain amino acids has been identified, the protein productivity has been increased by extract condensation and important efforts have been made to improve the energy supply. However, even with an ATP regenerating system, expression in batch mode is limited and can be maintained for only a few hours, 8–10 h being a maximum [5]. This problem has been solved by the introduction of the continuous cell-free coupled transcription-translation [6]. In the most recent version of this system, the continuous-exchange cell-free system (CECF), the reaction byproducts are continuously removed and the consumable substrates are supplied through a polymeric dialysis membrane (figure 2(*a*)). The porous membrane, with a molecular mass cutoff around 10 kDa, retains the proteins within the reaction chamber (1 ml) while allowing the nutrients of the feeding solution (10 ml) to diffuse continuously into the reaction chamber. With such a system and with our actual capacity in molecular cloning, a cell-free approach to build an artificial cell seems conceivable.

However, given the present knowledge of the structure and function of living cells, especially prokaryotic, one can ask: why is it interesting to build an artificial cell? One of the answers is that the complexity of living cells is such that only a partial description can be given. Quantitative measurements *in vivo* are still difficult to come by. Freedom to change the parameters is so limited that one cannot investigate the complete parameter space for a given mechanism. An artificial cell with a minimal number of components is, in contrast, well suited to permit easier access to the study of various cellular mechanisms. The studies of enzymatic and metabolic mechanisms outside of their context allow us to extract the basic phenomena. A minimal cell would enable us to go beyond quantitative measurements of isolated mechanisms, and understand how functions are coupled together and how complexity is established step by step.

The realization of a minimal cell highlights all the physical constraints that must be overcome in living organisms. One of the key properties of life, self-reproduction, varies drastically between prokaryotic and eukaryotic cells. This raises another important question: can we infer mechanisms different from existing ones with an artificial cell? Building an artificial cell offers alternatives to two other aspects of life. First, one can distinguish the importance of non-genetic processes. For example, the phospholipid membrane has a special status;

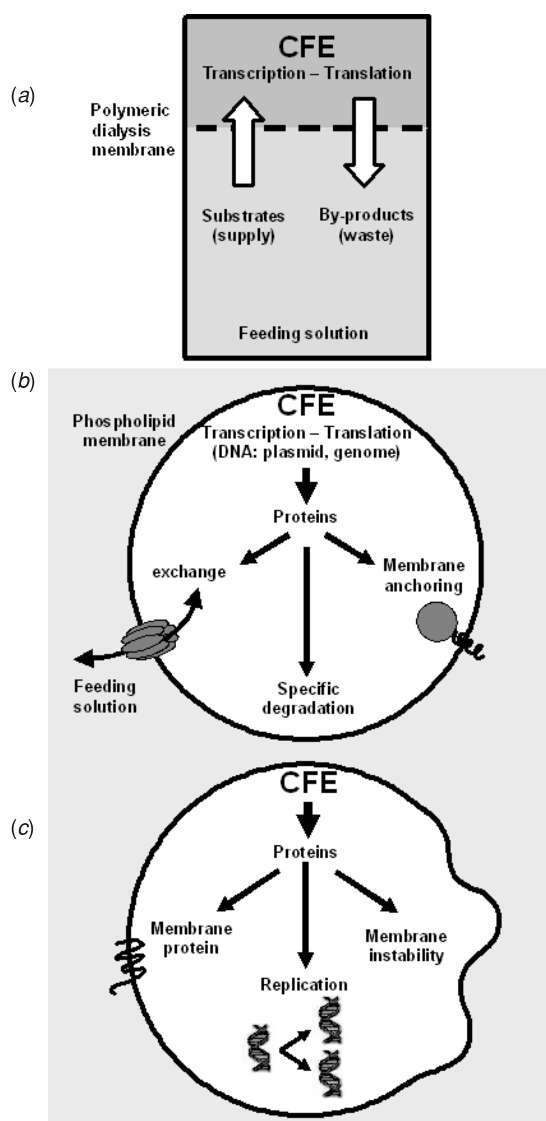

Figure 2. (*a*) The continuous exchange cell-free system (CECF). The reaction on top is separated from the supply reservoir below by a dialysis membrane. Expression is sustained by diffusion of nutrients (nucleotides, amino acids) from the feeding compartment to the reaction. The hydrolyzed nucleotides and the byproducts of the reaction are diluted in the feeding compartment. (*b*) Present development on an artificial cell based on cell-free gene expression. The toxin alpha-hemolysin from *Staphylococcus aureus* allows a continuous expression of genes inside the vesicle. The synthesized soluble proteins can be anchored to the membrane or degraded specifically. (*c*) Future elements to further develop the system towards a self-reproducing machine. Integral membrane proteins have to be inserted in the bilayer to develop an active interface. Membrane instabilities have to be generated to induce fission of the membrane. This has to be coupled to the insertion of newly synthesized phospholipids. Finally DNA has to be replicated.

it has a broad phase diagram that leads to various self-organizations and its own reproduction is different from any other. We have already shown how simple it is to form a vesicle [7]. A membrane is a source of symmetry breaking and instabilities and it will be interesting to understand how its properties combine with gene expression. Engineering

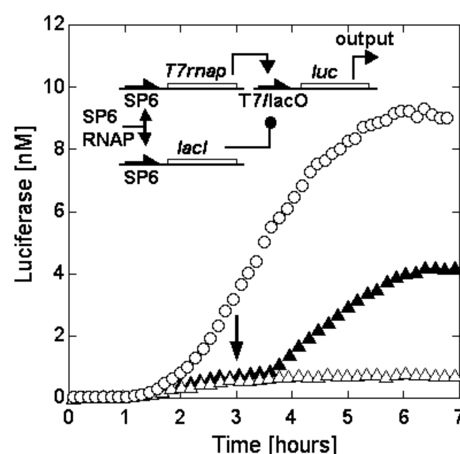

Figure 3. A batch mode three-gene circuit expressed in a wheat germ extract [17]. Three genes were cloned in three different plasmids: SP6-*lacI*, SP6-*T7rnap* and T7/lacO-*luc* (pIVEX2.3d-lacO-*luc*). Co-expression of the three genes with (open circles) and without (open triangles) IPTG at $t = 0$ (0.005 nM SP6-*T7rnap*, 0.1 nM SP6-*lacI* and 0.5 nM T7/lacO-*luc* plasmids). IPTG (0.5 mM) was added after 3 h to induce Luc production (arrow, closed triangles).

an artificial cell also opens prospects for biotechnical and biomedical applications [8]. Among them, drug delivery and biosensors are the most promising. Finally, the vesicle bioreactor we describe hereafter is also an unprecedented tool that can be used as a mini laboratory to test new molecules such as small peptides, and mutated or engineered proteins [9]. One can also try to use synthetic compounds in concert with biological molecules to circumvent some of the problems. This, we hope, will help us move toward a synthetic man-made machine that can propagate itself.

3. Progress toward an artificial cell based on cell-free gene expression

To build an artificial cell based on cell-free extract, one has first to measure the capacity and flexibility of such systems to express elementary genetic networks [5]. To develop an artificial cell based on DNA, a large number of genes have to be expressed at the same time to form protein networks. The reaction (10–20 μl in volume) is assembled in a 1.5 ml tube and incubated at room temperature ($\sim$25 °C). A wheat germ extract was used for these experiments because the RNA polymerases of *E. coli* and bacteriophages T7 and SP6 are functional. The expression lasts up to 6–8 h and reaches a few micromolars of protein, enough for biochemical reactions. This extract offers other properties useful to build networks: mRNA lifetime can be modified, calibration of expression is easy, and finally the prokaryotic proteins used in the networks are not present in the extract itself. A two-gene cascading network showed how important is the messenger lifetime. A three-gene network was built to create a switch (figure 3), based on the lac operon of *E. coli*. This circuit showed the capacity of a cell-free expression system to carry out activation and repression *in vitro* and the possibility of switching on the expression of a gene at will.

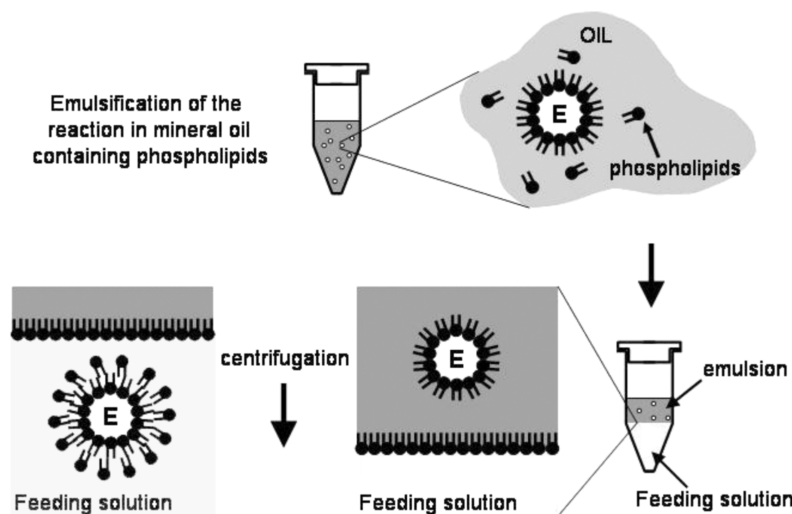

Figure 4. Preparation of the vesicles. The reaction is emulsified in oil containing phospholipids. The small droplets are stabilized with a monolayer of lipids at the interface oil-reaction (E for extract). The emulsion is placed on top of the feeding solution. A monolayer of phospholipids is formed at the biphasic interface that gives a perfect configuration to form vesicles. After centrifugation, the vesicles containing the reaction are recovered in the feeding solution.

The expression of more complex genetic networks requires a longer-lived expression system, at least one day. In batch mode, expression stops because of the decrease of the adenylate energy charge [10]. The translation rate decreases exponentially with the decrease of the ATP level [11]. Continuous expression systems for large-scale protein production have been invented to solve this problem (figure 2(*a*)) [6]. Our long-term objective is to obtain a synthetic system that can self-reproduce on the basis of an internal genetic program; therefore, expression has to occur in a phospholipid vesicle. No other known biological or synthetic barrier gives as many possibilities and as much flexibility to functionalize and establish controllable exchanges between inside and outside. Cell-free expression in emulsion droplets [12, 13] brings expression to the scale of the cell but does not allow continuous expression because exchanges with the solvent phase are difficult. A two-step method (figure 4) has been devised to go from a homogenous expression reaction in batch mode to a heterogeneous cell-free reaction in synthetic phospholipid vesicles with a size from 1 to 50 μm in diameter. A variant of this method has been proposed elsewhere to form asymmetric vesicles [14].

In our first experiment, a wheat germ extract with a plasmid reporter and a bacteriophage RNA polymerase was encapsulated in the vesicles. Outside of the vesicles, the same wheat germ extract was used without plasmid and polymerase. In this configuration, the osmotic pressure is almost matched and hundreds of vesicles are formed. Inside the vesicles, protein synthesis level and duration was identical to batch mode. A second set of experiments was done with a buffered feeding solution outside the vesicles and an *E. coli* extract inside. To obtain real exchange between the two compartments and create a long-lived vesicle bioreactor, the toxin alpha-hemolysin was used to form membrane pores. In the context of our experimental model we found that this toxin is the appropriate solution to establish exchange with

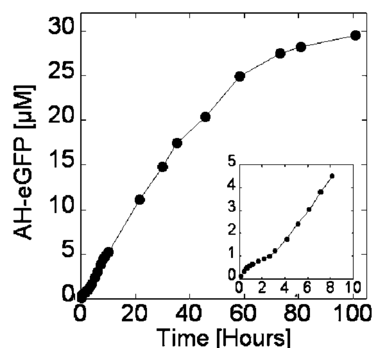

Figure 5. Kinetics of expression of alpha-hemolysin-eGFP inside a vesicle [16]. Closed circles: 0.5 nM pIVEX2.3d-alpha-hemolysin-eGFP expressed in RTS500 cell-free expression system. Inset: blowup of the first 10 h of expression of alpha-hemolysin-eGFP.

the environment and improve considerably the lifetime of the bioreactor. With a pore size of 1.4 nm, corresponding to a molecular mass cutoff around 3 kDa [15], a selective permeabilization was realized. Hence, all the energy/nutrients compounds are easily exchanged, whereas DNA, RNA and proteins stay inside the vesicle. To visualize the toxin and quantify the pore concentration as a function of time, a fusion alpha-hemolysin-eGFP was engineered with the reporter at the C-terminus of the pore. Accumulation of the toxin at the membrane and in the extract was observed. Moreover, the expression was observed to last four days with a maximum protein production between 30 and 40 μM (figure 5). Thus, a one order of magnitude increase in protein production and duration of expression is obtained when compared to batch mode. The protein permeabilizes the membrane and induces its own expression prolongation by making a positive feedback where more pores means more protein production.

To further develop this system and get closer to a self-reproducing object, numerous problems have to be solved both

at the technical and methodological level. One of the first problems, we encountered, was fusing a reporter gene to the gene of interest. Note that the observations are essentially done with fluorescent proteins. The reporter gene has to be fused to the gene studied. Thus, suitable linkers that do not affect the function of either protein are required. Furthermore, osmotic pressure seems to impose a continuous expression of the pore to maintain an efficient exchange. This could be solved by addition of polymer or inert proteins in the feeding solution to counter the osmotic effect. Finally, one of the crucial problems in this system is the lack of an active integral membrane protein secretion mechanism in the extract. In the following, we present the progress made to develop an artificial cell based on cell-free gene expression (figure 2(*b*)), with new results concerning specific protein degradation, membrane anchoring, membrane protein insertion and membrane domains.

3.1. Specific protein degradation

The reconstitution of networks requires a long-lived and powerful expression of proteins. Yet, it is also essential to have a sink for the synthesized proteins [16]. The degradation, though, has to be specific so as to keep the extract undamaged, discarding only the produced proteins. This can be achieved with the ATP dependent ClpXP complex from *E. coli*, which degrades only proteins that contain specific short signals in the N- or C-terminal [17]. This mechanism is relatively simple and it can be reconstituted in an appropriate buffer with the two proteins ClpP and ClpX and a tagged protein as a target. To test this system, we purified the two proteins forming the complex and two tagged eGFP. In one case, the reporter protein bears the ssrA signal for degradation, and in the other case the signal contains two mutations so as to prevent degradation (ssrA/DD). Our results confirm that specific degradation of the ssrA bearing protein occurs in bulk and in vesicles (figure 6). This mechanism works also in *E. coli* extract (data not shown).

3.2. Active membrane

We have already shown [7] that one can anchor soluble proteins to the membrane. This was done using the short amphipathic peptide 18L that spontaneously inserts into the phospholipid membrane. More recently, we developed this approach and found two other peptides to anchor cytoplasmic proteins to the membrane. The peptides Bombolitin III and Melittin are very efficient vectors to anchor eGFP to the membrane (figure 7) [18, 19]. The binding in these cases is relatively uniform with the exception of small aggregate formation. However, despite their lytic activities, neither Bombolitin III nor Melittin induces prolongation of expression or destabilization of the vesicles. Another class of molecules, the coat proteins of several bacteriophages, has been reported to interact with lipidic membranes. These proteins are models of membrane protein translocation independent of specific insertion mechanism [20]. We expressed the two coat proteins G8P and G9P of the phage M13 as potential vectors to anchor proteins to the membrane and to test the capacity of the artificial cell to reconstitute such processes. When expressed in the vesicles, the two viral proteins interacted clearly with the membrane. In the case of G8P, binding to the membrane was observed when eGFP was fused either in N- or C-terminal of the coat protein. As the N-terminal part of G8P can cross the bilayer, it could be used to bind proteins to the exterior leaflet of the vesicle [21]. Note that it is important to be able to insert integral membrane proteins into the bilayer to develop an active interface between inside and outside. Yet, cell-free expression systems do not have an active mechanism to insert integral membrane proteins. *In vivo*, these mechanisms include several proteins, a few

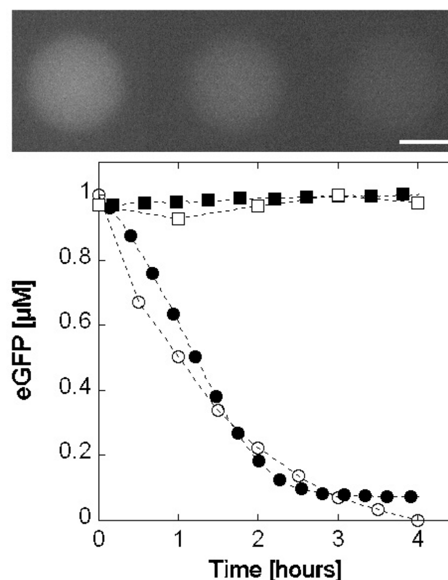

Figure 6. Specific degradation of eGFP with the complex ClpXP of *E. coli* reconstituted in a buffer. Top row: fluorescence images of the degradation of His-eGFP-ssrA by the complex ClpXP: at time $t = 0$ (left), $t = 1$ h (center) and $t = 2$ h (right) (scale bar, 10 μm). Curves: kinetics of degradation in bulk (closed symbols) or in vesicles (opened symbols). 1 μM of His-eGFP-ssrA (circles) or His-eGFP-ssrA/DD (squares), 1.5 μM of His-ClpX$_6$ and 1.5 μM of ClpP-His$_{14}$ were used.

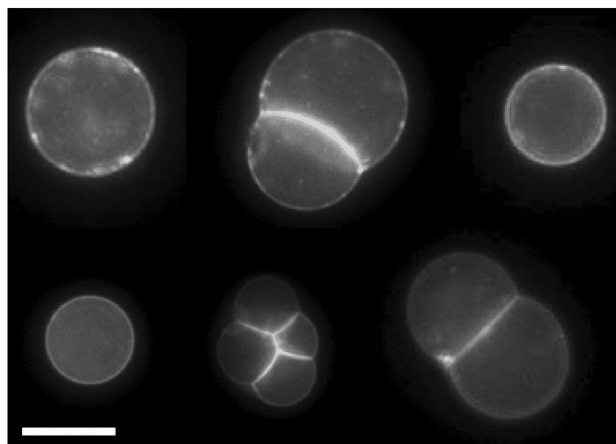

Figure 7. Binding of eGFP to the membrane with different peptides. (L-α-lecithin, RTS500 cell-free expression system, one day of expression). Top row: BombolitinIII-eGFP (left and center), Melittin-eGFP (right). Bottom row: G9P-eGFP (left and center), eGFP-G8P (right). (Scale bar, 10 μm.)

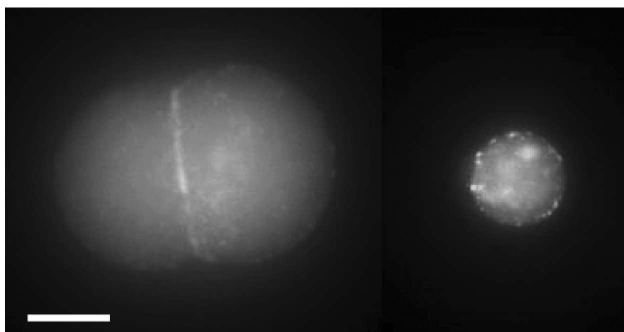

Figure 8. Interaction of Mistic-eGFP with the membrane of vesicles (L-α-lecithin, RTS500 cell-free expression system, one day of expression). On the left, one can see the binding of the fusion protein at the interface between the double vesicle. On the right, the fusion protein forms aggregates at the membrane. (Scale bar, 10 μm.)

of them being integral membrane proteins, which makes the process difficult to achieve in artificial cells. To overcome this obstacle, we tried to make use of the protein Mistic from *Bacillus subtilis* discovered recently [22]. This protein was reported to have the capacity to insert integral membrane proteins in functional form without any other mechanism. In our test, a fused eGFP at the C-terminus of Mistic was expressed in vesicles. Despite the fact that eGFP is hydrophilic, we observed an interaction of the fusion protein with the membrane (figure 8). Note though, that the binding is not uniform and a lot of aggregates are formed.

3.3. Membrane phospholipid composition

The phospholipid composition of the membrane is another important aspect of the artificial cell. Phospholipids display a very large and rich phase diagram of self-organized structures and conformations [23]. Mixtures of different phospholipids (length of the hydrophobic chain and nature of the polar head) and cholesterol induce fluid domains in bilayers. In particular, mixtures of phosphatidylcholine, sphingomyelin and cholesterol are known to form domains in a range of temperature compatible with gene expression [24]. This non-genetic aspect can be used to break the space symmetry in the vesicles. Hence, we have attempted to generate mixes of different phospholipid types. We expressed the toxin alpha-hemolysin fused to eGFP as a reporter. The main experimental problem that we encountered was the dissolution of phospholipids and cholesterol in mineral oil, which is the solvent of choice used to form the emulsion. Until now we have used lecithin (phosphatidylcholine from egg yolk, a mixture of palmitic, stearic, oleic, linoleic). This mixture dissolves well in oil and emulsification is relatively easy under gentle vortex. In the case of sphingomyelin and lecithin mixes, dissolution poses problems even at low concentration and emulsification becomes difficult. This results in a reduction of vesicle formation. Moreover, cholesterol, which dissolves very well in the oil, inhibits emulsification even at low concentration. It also inhibits the binding of alpha-hemolysin to the membrane (data not shown). While the fusion protein alpha-hemolysin-eGFP is uniformly distributed at the membrane with lecithin, distribution of the pore at the membrane is significantly modified in the presence of sphingomyelin (figure 9). Domains of high and low concentrations of the pore were obtained. In this case, depletion of the fusion protein corresponds to the presence of sphingomyelin. It was quite surprising that the protein would react to the lipid composition. In another set of experiments, a short chain phospholipid was used (DHPC, diheptanoyl-PC). On this occasion, large and clear domains were formed in the membrane (figure 9). The pore could not insert into the membrane in the presence of the short lipids. If the problem of phospholipids' solubility can be solved, a much larger phase space can be studied. Until now, we found that mineral oil is the most suitable solvent, but a mixture of alkanes can also be tested to facilitate both the solubilization and emulsification [14].

4. Future developments

To pursue this cell-free approach (figure 2(*c*)), the extracts have to be prepared differently. With the available *E. coli* extract, transcription can be performed only with bacteriophage RNA polymerases. This significantly limits the construction of genetic networks. Moreover, available extracts are prepared such that they are to be fed through a polymeric dialysis membrane. Thus, some difficulties are encountered encapsulating them in vesicles because of osmotic pressure

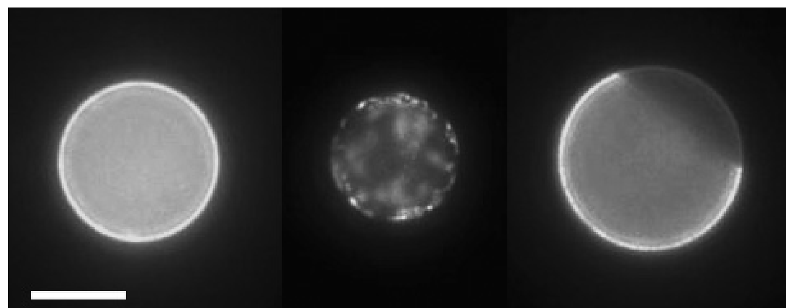

Figure 9. Fluorescence images of alpha-hemolysin-eGFP expressed in vesicles (RTS500 cell-free expression system) after one day of expression The fusion protein is expressed in vesicles made of L-α-lecithin (left), a mixture of L-α-lecithin (90%) and sphingomyelin (10%) (center), a mixture of L-α-lecithin (95%) and DHPC (5%) (right). (Scale bar, 15 μm.)

build-up. Like most of the bacteria, the membrane of the artificial cell will have to be reinforced with an external network that fulfils the role of peptidoglycane. Also, extracts have been optimized for large-scale protein production. This implies that messengers have a long lifetime, which is detrimental for the realization of networks [5]. The synthesized messengers have to be degraded specifically without damaging the extract. This aspect is critical; delays and thresholds have to be created if we want to introduce time-dependant behavior. Another important point is that the extract is a complex, partly unknown, solution in which we use only the translation machinery. Thus, interference with other existing proteins has to be either avoided or clearly characterized.

On basis of the simplest organisms and on the actual knowledge of prokaryotic genomes, it is estimated that a minimal cell that can reproduce would function with between 200 and 350 genes [25, 26]. Even though this is a small number, technically it is a challenge. Throughout the present work, it becomes evident that a minimal synthetic genome has to be tailored with large fragments each containing numerous genes, encoding for entire metabolic pathways for example. For DNA replication and the development of a mini genome, the use of a bacteriophage genome, or part of it, seems the most suitable solution. For instance, the phage T7 has its own replication machinery and it needs very few proteins from the host (*E. coli*). The synthetic genome must also contain a set of genes to order the introduction of all the different parts composing the software.

To divide the vesicle, one needs to couple two mechanisms: a source of phospholipids to insert into the membrane and a membrane destabilization process. Insertion of lipid into a bilayer is feasible [27]. Instabilities at the membrane can be produced either with biopolymers or by budding mechanisms, using membranes of different composition [28]. This is still an open problem, with no significant results as yet. Finally, insertion of integral membrane proteins into the bilayer is a key element to develop an active interface, an essential part of the project. In our recent work, we propose to use the protein Mistic as a minimal vector to insert integral membrane proteins. If this process works, it will be used to insert a complete and efficient mechanism of integral membrane protein secretion.

What we have described is a deterministic approach to construct an artificial cell, and clearly we face problems that appear difficult to solve. On the basis of our present knowledge, it seems that one cannot use a purely engineering approach to synthesize life in the laboratory. A living cell is the result of a long evolutionary process to reach its living working point. Our future goal is to imagine some laboratory evolution schemes, using a large number of vesicles, with slightly different genetic content, and a defined selection pressure.

Acknowledgments

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References

[1] von Neumann J 1951 The general and logical theory of automata *Cerebral Mechanisms in Behavior: The Hixon Symposium* ed L A Jeffress (NewYork: Wiley)

[2] von Neumann J 1966 *Theory of Self-Reproducing Automata* (Champaign, IL: University of Illinois Press)

[3] Turing A M 1936 *Proc. London Maths. Soc.* **42** 230–65

[4] Shimizu Y, Inoue A, Tomari Y, Suzuki T, Yokogawa T, Nishikawa K and Ueda T 2001 Cell-free translation reconstituted with purified components *Nat. Biotechnol.* **19** 751–5

[5] Noireaux V, Bar-Ziv R and Libchaber A 2003 Principle of cell-free genetic circuits assembly *Proc. Natl Acad. Sci. USA* **100** 12672–7

[6] Spirin A S, Baranov V I, Ryabova L A, Ovodov S Y and Alakhov Y B 1988 A continuous cell-free translation system capable of producing polypeptides in high yield *Science* **242** 1162–4

[7] Noireaux V and Libchaber A 2004 A vesicle bioreactor as a step toward an artificial cell assembly *Proc. Natl Acad. Sci. USA* **101** 17669–74

[8] Pohorille A and Deamer D 2002 Artificial cells: propects for biotechnology *Trends Biotech.* **20** 123–8

[9] Chiu D T *et al* 1999 Chemical transformations in individual ultrasmall biomimetic containers *Science* **283** 1892–5

[10] Atkinson D E 1968 The energy charge of the adenylate pool as a regulatory parameter: interaction with feedback modifiers *Biochemistry* **7** 4030–4

[11] Matveev S V, Vinokurov L M, Shaloiko L A, Davies C, Matveeva E A and Alakhov Y B 1996 Effect of the ATP level on the overall protein biosynthesis rate in a wheat germ cell-free system *Biochim. Biophys. Acta* **1293** 207–12

[12] Tawfik D S and Griffiths A D 1998 Man-made cell-like compartments for molecular evolution *Nat. Biotechnol.* **16** 652–6

[13] Pietrini A V and Luisi P L 2004 Cell-free protein synthesis through solubilisate exchange in water/oil emulsion compartments *Chem. Biochem.* **6** 1055–62

[14] Pautot S B, Frisken J and Weitz D A 2003 Engineering asymmetric vesicles *Proc. Natl Acad. Sci. USA.* **100** 10718–21

[15] Song L, Hobaugh M R, Shustak C, Cheley S, Bayley H and Gouaux J E 1996 Structure of staphylococcal alpha-hemolysin, a heptameric transmembrane pore *Science* **274** 1859–66

[16] Elowitz M B and Leibler S 2000 A synthetic oscillatory network of transcriptional regulators *Nature* **403** 335–8

[17] Singh S K, Grimaud R, Hoskins J R, Wickner S and Maurizi M R 2000 Unfolding and internalization of proteins by the ATP-dependent proteases ClpXP and ClpAP *Proc. Natl Acad. Sci. USA.* **97** 8898–903

[18] Argiolas A and Pisano J J 1985 Bombolitins: a new class of mast cell degranulating peptide from the venom of the bumblebee *Megabombus pennsylvanicus J. Biol. Chem.* **260** 1437–44

[19] Rex S and Schwarz G 1998 Quantitative studies on the Melittin-induced leakage mechanism of lipid vesicles *Biochemistry* **37** 2336–45

[20] Rasched I and Oberer E 1986 Ff coliphages: structural and functional relationships *Microbiol. Rev.* **86** 401–27

[21] Wickner W 1976 Asymmetric orientation of phage M13 coat protein in *Escherichia coli* cytoplasmic membrane and in synthetic lipid vesicles *Proc. Natl Acad. Sci. USA.* **73** 1159–63

[22] Roosild T P, Greenwald J, Vega M, Castronovo S, Riek R and Senyon C 2005 NMR structure of Mistic, a

membrane-integrating protein for membrane protein expression *Science* **307** 1317–21
[23] Lipowsky R 1991 The conformation of membranes *Nature* **349** 475–81
[24] Baumgart T, Hess S T and Webb W W 2003 Imaging coexisting fluid domains in biomembrane models coupling curvature and line tension *Nature* **425** 821–4
[25] Zimmer C 2003 Tinker, Tailor: can Venter stitch together a genome from scratch? *Science* **299** 1006–7
[26] Gil R, Silva F J, Pereto J and Moya A 2004 Determination of the core of a minimal bacterial gene set *Microbiol Mol. Biol. Rev.* **68** 518–37
[27] Chen I A, Roberts R W and Szostak J W 2004 The emergence of competition between model protocells *Science* **305** 1474–6
[28] Staneva G, Angelova M I and Koumanov K 2004 Phospholipase A2 promotes raft budding and fission from giant liposomes *Chem. Phys. Lipids* **129** 53–62