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Journal of Materials Chemistry

Cite this: *J. Mater. Chem.*, 2011, **21**, 18887

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FEATURE ARTICLE

Compartmentalized reactions as a case of soft-matter biotechnology: synthesis of proteins and nucleic acids inside lipid vesicles†

Pasquale Stano,[a] Paolo Carrara,[a] Yutetsu Kuruma,[b] Tereza Pereira de Souza[c] and Pier Luigi Luisi*[a]

Received 23rd May 2011, Accepted 22nd July 2011
DOI: 10.1039/c1jm12298c

In this mini-review we would like to summarize the recent advances in the field of protein and nucleic acid synthesis inside lipid vesicles (liposomes). This research, which originated within the origin of life community, is now recognized as an example of synthetic biology. Current approaches are based on the convergence of liposome technology and cell-free *in vitro* technology. In particular, in addition to the classical liposome preparation methods, the new water-in-oil droplet transfer method appears very interesting for progressing in the assembly of these cell-like systems, possibly in combination with microfluidic devices. As an alternative to cell extract, the use of a transcription/translation kit composed of purified components is also presented as a new tool for carrying out protein synthesis inside liposomes. Data presented in the literature are collected and shortly discussed, and the potential relevance of this new soft-matter biotechnology in various research fields is also commented on.

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1. Introduction

In recent years there has been an increasing interest in micro-compartmentalized reactions, *i.e.*, chemical and biochemical transformation occurring inside microscopic compartments, generally lipid-based ones, such as lipid vesicles (liposomes)[1] and water-in-oil (w/o) emulsion droplets.[2] Although under different perspectives, these studies share common technological features and present similar conceptual traits. This is the realm of femtolitres reaction volumes (also including attolitres and picolitres), where—depending on the concentration of the reactants—stochastic behaviour can be a characteristic signature, ultimately due to the discreteness of the number of molecules and to the paths that determine the formation of the compartment. Due to their similarity with cells, it is not surprising that lipid micro-compartments have been explored (i) as cell models for understanding the basic properties of compartmentalized systems and investigating the emergence of living cells from mixtures of non-spatially organized reactants, or (ii) for constructing synthetic cells.[1a–d,3] The common innovative feature essentially concerns the shift from the classical analytical (dissecting) approach to the knowledge, to the rediscovered synthetic (or "constructive"[4]) one.[1d,5] Probably, this is one of the most intriguing epistemic facets of synthetic biology,[6] sometimes not fully appreciated by the synthetic biology community. In fact, as stated in the *incipit* of a recently published review, "we are much better at taking cells apart than putting them together".[7] From this consideration it becomes clear that a shift toward a synthetic approach becomes useful for gaining insights into physico-biochemical facts that characterize living things.

In this review we will first describe the historical steps that led to the establishment of the current approaches, shortly discussing the relevance of this new soft-matter biotechnology for investigations in the field of origin of life, synthetic biology, and biotechnology. In particular, we will present the concept of "semi-synthetic" minimal cells, pioneered in the 1990s by Luisi's group in Zurich,[1a,d,8] and now expanded into synthetic biology.[9] The discussion will be focused only on lipid vesicles. The field of reactions in w/o emulsion droplets will be not treated here; the interested reader is referred to recently published reviews.[2b,c] Similarly, other encapsulating systems like gels,[2f] layer-by-layer capsules,[2g] and polymer-based vesicles (*i.e.* polymersomes[2h]) are not treated here.

We will schematically show the general experimental strategies for carrying out micro-compartmentalized reactions inside lipid vesicles. Finally, a full list of published work is given, and the most representative cases are commented on in more detail.

Not intended to be exhaustive with respect to compartmentalized reactions, this review covers only some specific technological aspects. In particular, here we will not present the classical arguments of the origin of life research,[10] or the concept of autopoiesis[11] as a theoretical framework for defining life at the cellular level and for guiding the design and construction of minimal cells.[11b,12] These and other issues, such as the minimal genome[1a,13] have been discussed recently.[1]

[a]*Biology Department, University of Roma Tre, Rome, Italy. E-mail: luisi@mat.ethz.ch*
[b]*Department of Medical Genome Sciences, Graduate School of Frontier Sciences, The Tokyo University, Japan*
[c]*Pharmacy Institute, Friedrich Schiller University, Jena, Germany*
† This article is dedicated to the memory of Alec D. Bangham (1921–2010).

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2. Lipid vesicles as biomimetic cell-like compartments

Since the time of their discovery by Alec Bangham in the 1960s ("phospholipids spontaneously form closed membrane structures in the presence of water"),[14] it was realized that liposomes (lipid vesicles) could be used as a model of cellular membranes. In addition, due to their distinctive morphology, physico-chemical properties, and especially their ability to encapsulate a large variety of biochemical solutes, liposomes and other kinds of vesicles are used as cell models (in particular, fatty acid vesicles as primitive cell models).[1a,f,h,15]

Vesicles are formed in aqueous solutions by the self-assembly of lipids or other surfactants in the form of bilayers (or monolayers in the case of bola amphiphiles), which close on themselves to form a hollow sphere, containing a portion of the aqueous phase. The architecture of a vesicle implies the emergence of a separation between the internal and the external worlds, thanks to the existence of a physically stable *boundary*, which is typically semi-permeable. Amphiphiles forming the boundary are not physically frozen, but display a wide range of dynamics, from vibration to axial rotation, from flip-flop to 2D diffusion, and a dynamical exchange with the soluble amphiphile fraction, always co-existing in the solution ("monomer" to aggregate equilibrium, described by k_{on}/k_{off} parameters and by the critical aggregation concentration).[16] From the morphological standpoint, vesicles are typically classified according to their shape and size (Fig. 1). The simplest morphology is the unilamellar vesicle.

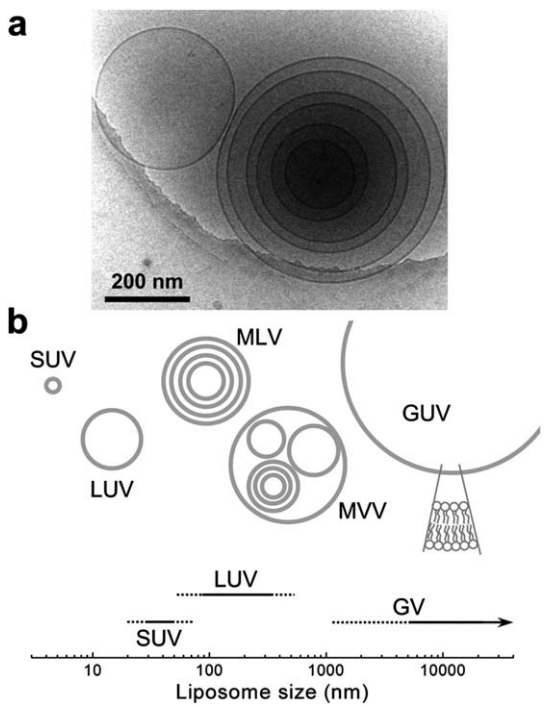

Fig. 1 (a) Cryo-TEM image of unilamellar and multilamellar POPC vesicles; (b) schematic drawing of vesicles with different morphologies and approximate size (below): small unilamellar vesicles (SUVs), large unilamellar vesicles (LUVs), multilamellar vesicles (MLVs), multivesicular vesicles (MVVs), and giant unilamellar vesicles (GUVs) (drawing not to scale). Panel (b) is reproduced from ref. 1*c*, with permission of John Wiley & Sons.

This is a sphere closed by one lamella (the bilayer). Oligo- and multi-lamellar vesicles are composed of a few or several (concentrical) lamellae, whereas multivesicular vesicles are vesicles that contain other vesicles in non-concentrical way. Depending on the size, unilamellar vesicles can be small (diameter $d < 50$ nm), large ($d \approx 100$ to 200 nm), or *giant* ($d > 1$ μm). Giant vesicles can be visualized by light microscopy, whereas submicrometric ones require electron microscopy.

The permeability of vesicle membranes is a strong function of their chemical composition (especially chain length) and heavily depends on the chemical nature (and size) of the permeant species as well. Classical phosphatidylcholine membranes are permeable to water (permeability coefficient across egg phosphatidylcholine vesicle membranes $\sim 10^{-4}$ cm s^{-1}), but almost not permeable to salts and charged compounds. For example, the permeability of a sodium ion is 10^{10} times less than water.[17] The physical state of the bilayer (solid-like or liquid-like) affects permeability, which can have a peak value (for some lipids and for some solutes) at the phase transition temperature T_m.[18] These considerations on permeability are relevant for two key aspects of the use of lipid vesicles as compartments for enzyme-catalysed complex reactions. Firstly, it allows the stable encapsulation of biomacromolecules in their inner aqueous phase, so that a true cell-like spatial-dynamic organization can be supported. Secondly, its semi-permeable character implies that some solutes can permeate it, perhaps slowly, but allow the external feeding of liposome compartments, that can therefore become open "*bioreactors*", in the sense that they can exchange matter with the environment. In the next sections, we will see how the natural permeability of lipid bilayers can be enhanced by the use of membrane channels or by sublytic concentrations of detergents.

3. Why protein synthesis inside lipid vesicles?

The roots of studies on protein synthesis inside lipid vesicles lie in origin-of-life oriented research. In particular, the interest to run complex enzyme reactions inside liposomes stems from the attempts to construct simple cell-like models.

A commonly accepted scenario in origin of life studies is that primitive cells consisted of lipid compartments that have encapsulated a sort of minimal metabolism based on ancient RNA, or ancient peptides, or both. In particular, the RNA world hypothesis affirms that the first "replicant" molecules were ribonucleic acids, which could act in principle as templates for replicating their sequence, and as catalytic species, as well (the "ribozymes"). Moreover, it is also assumed that along the path toward the first living cell, intermediate structures endowed with a primitive metabolism existed.

How is it possible to establish an experimental research program on these topics? The central idea is that by *constructing* cell models that resemble primitive cells, it is indeed possible to understand the self-organization principles that underlie the transition from non-living molecules to living cells, and therefore to prove that in certain specific conditions, it is indeed possible to observe the emergence of living-like behaviour only according to physico-chemical laws.

Within this broad research field, a major role is played by biopolymerization reactions. The synthesis of RNA, DNA and proteins from their constituents NTPs, dNTPs, and amino acids

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paves the way to the onset of complex metabolic dynamics based on macromolecular catalysis and template-directed polymerization (however, template-free polymerization reactions are of even higher interest for origin of life studies).

It is therefore not surprising that the first investigations on biopolymerizations inside lipid vesicles aimed to produce ribonucleic acids, in particular to condense ADP into poly(A) (catalysed by polynucleotide phosphorilase)[19,20] and to replicate a template RNA (catalysed by Qβ replicase).[21] Later on, the focus was also shifted to polypeptide synthesis (poly(Phe)),[22] and finally to protein synthesis.[23]

All these concepts and approaches are linked together by the notion of the "minimal cell", namely, the cell composed of the minimal number of different molecules, that still display minimal living properties, as self-maintenance, self-reproduction and the capacity to evolve. So, in order to build a minimal cell in the laboratory, one has to firstly assess whether it must be used for simulating primitive conditions (and therefore using primitive molecules), or for studying the essence and the origin of cell organization and dynamics (also using modern molecules). The common trait is that the object under study (a 'cell') is built in the laboratory starting from separated components. The construction of minimal cells, from this viewpoint, is often referred to as bottom-up, to distinguish it from other approaches, where the step of "putting molecules together" is missing. For example, a minimal cell could also be obtained by eliminating unnecessary elements from an existing living cell, or by inserting a minimal genome in a host cell.[24] However, the terminology in this young field is not yet crystallized.

Minimal cells constructed with modern molecules, such as DNA, enzymes, *etc.*, are also interesting for biotechnology. Here the interest is shifted from primitive molecules to other—equally important—aspects, as the self-organization of molecules and networks, but limited to a minimal complexity. These minimal cells, often called "semi-synthetic"[1a,d,5b] to emphasize the use of modern enzymes and nucleic acids (Fig. 2), have attracted the interest of several researchers, and are promising models for understanding cellular life, and tools that might be exploited for future biotechnology based on cellular structures.

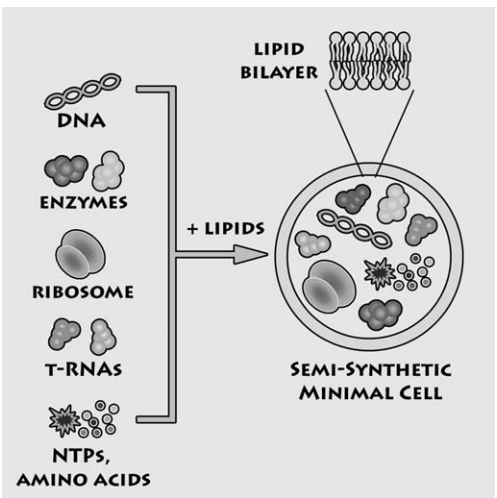

Fig. 2 Semi-synthetic minimal cells are constructed by assembling the minimal number of biomolecules such as DNA, enzymes, ribosomes, tRNAs, amino acids, NTPs, *etc.* within a lipid compartment (liposome). The ultimate goal is the achievement of a minimal living cell from non-living components. Reproduced from ref. 5*b* with permission of Elsevier.

This review is focused on semi-synthetic minimal cells, whereas the peculiar aspects of primitive cell approaches will not be commented on in detail (see ref. 1); some key reports are however listed in this review.

For the full development of semi-synthetic cell technology, the synthesis of a functional protein inside liposome is a vital step. In fact, proteins are central to all natural cell activities. The *in situ* generation of enzymes and structural proteins is therefore a prerequisite for constructing functional cells, for example those which will be able to process a substrate, or to replicate their genetic material, to move or to respond to chemical messengers by means of surface-mediated receptors, to communicate, and so on. In the concluding remarks we will outline some possible applications of semi-synthetic cells as new self-organized and compartmentalized soft materials for basic science and technology.

4. General aspects of the synthesis of proteins and other biomacromolecules inside lipid vesicles

It is interesting to remark that the current approach for synthesizing proteins and other biomacromolecules as RNAs and DNAs inside lipid vesicles is actually a convergence of two pre-existing technologies, namely liposome technology[25] and cell-free technology.[26] According to the current state of the art (see below) quite probably the trend will quickly include also microfluidic devices as a tool for generating, manipulating and analysing giant lipid vesicles.

Let us now describe the general strategy for constructing enzyme- and nucleic acid-containing vesicles. The solutes of interest (enzymes, nucleic acids, low molecular weight compounds) must be encapsulated inside vesicles. Typically, vesicle formation and solute encapsulation occur contextually. This means that the vesicle preparation methods, and the chemical nature of lipids and of the solutes, both play a role in determining the extent of encapsulation (entrapment efficiency) and give rise to a wide range of possibilities. Experiments can be designed in order to co-entrap all required components for the occurrence of an internalised reaction, or only a part of them, leaving out some species that later can be added externally, for example by exploiting the membrane semi-permeable character. In some specific cases, as for giant vesicles, solutes can be micro-injected inside the vesicle after its formation. This provides a route to filling vesicles with the desired mixture, but—being a manual technique—it allows the construction of only a limited number of vesicles.

Largely regardless of the preparation method, when lipid vesicles are formed from a solute-containing solution, it is expected that the vesicle populations will display a certain degree of heterogeneity with respect to solute capture efficiency. This is obvious when vesicles with different morphologies and size are compared, but it is true also for vesicles of similar shape and size. There are at least two reasons for this. The first one is the heterogeneity of vesicle formation mechanisms, which could be strongly reduced by methods that allow a very reproducible mechanism of vesicle formation. The second one is the stochastic

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nature of microscale phenomena, which cannot be eliminated. Under certain conditions, these effects can be enhanced or dumped. For example, the encapsulation of a protein present at 3 μM bulk concentration by 100 nm (diameter) vesicles corresponds to an average expected encapsulated number of *ca.* 1 protein per vesicle (for spherical unilamellar vesicles, $N_{avg} \approx 2.52 \times 10^{-6}\ C_{\mu M}\ R^3_{nm}$). It is therefore expected that stochastic fluctuations will bring about the formation of empty vesicles, as well as of vesicles containing 2 or more protein molecules. By increasing the solute concentration or the vesicle size, this intrinsic stochastic effect tend to decrease (in relative terms), but extrinsic phenomena, due to the heterogeneity of vesicle formation mechanism, are still present, so that a difference in solute filling has been observed even for giant vesicles prepared by the natural swelling method.[27] As we will show below, microfluidic-based GVs preparation methods have the advantage of reducing vesicle heterogeneity. Moving from one-solute to multi-solute encapsulation, another important consideration concerns the molecular *composition* of each vesicle's content. In fact, not all vesicles that are expected to contain *M* different solutes will actually co-entrap all of them, but also it is not obvious that the relative abundance of the solutes will be constant in all vesicles. In other words, missing just one of the *M* different solutes implies that the vesicle will not be able to sustain a certain reaction of interest. Having all solutes but in different ratios can strongly affect the course of internalised reaction, for example by leading to a premature stop or facilitating side reactions. Again, these considerations become critical especially for small vesicles and low solute concentrations, but experimental evidence on running complex reactions such as protein synthesis in large and giant vesicles reveals a large heterogeneity in intra-vesicular reactivity. Being fascinated by this topic, we have recently started an investigation on the analysis and exploitation of vesicle diversity.[28]

With these consideration in mind, the general experimental strategy for building vesicle bioreactors consists in: (1) the vesicle formation/solute encapsulation step (for microinjection strategy (GVs only) the vesicle formation step precedes solute encapsulation); (2) the removal or inhibition of externally present (non-entrapped) reactants; and (3)—in some cases—the addition of missing compounds to activate/feed the internalised reaction. The fourth and final stage is the analysis of the reaction product. Two illustrative examples are represented schematically in Fig. 3.

Some important aspects of compartmentalized reactions are commented on in the next section, together with a short discussion on cell-free systems for protein synthesis. Finally, a full list of studies on biopolymerization reactions inside liposomes is provided as an analytical table, and some work will be highlighted with further comments.

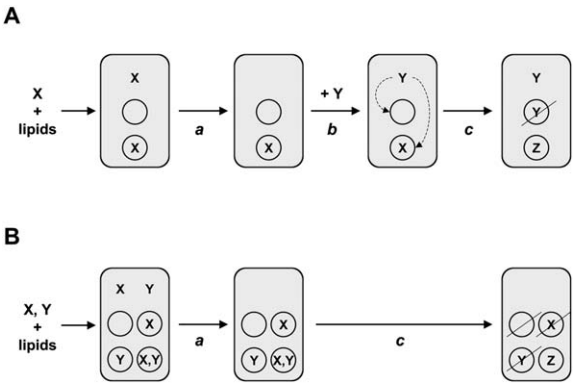

Fig. 3 Carrying out compartmentalized reactions (two examples). (A) By forming liposomes in the presence of the compound X, empty and X-containing liposomes are formed in the most general case (also depending by the experimental conditions), and part of X remains unencapsulated. After removal of free X (step *a*) the second (permeable) component Y is added (step *b*). Only liposomes that contained X successfully produced the final product Z (step *c*). (B) In the case of two or more reactants X, Y that are simultaneous entrapped inside liposomes, more possibilities should be considered (in this example, there is no external addition of a missing reactant). In particular, some liposomes might not contain all elements required. After removal of free unentrapped solutes (step *a*), the final product will be produced only in those liposomes that have co-entrapped X and Y (step *c*). Clearly, experimental conditions will affect the amount of empty and filled liposomes (*e.g.* solute concentration, liposome formation mechanisms, and post-formation processing, such as freezing-and-thawing). Reproduced from ref. 1*c*, with permission of John Wiley & Sons.

5. Current technologies

5.1. Classic liposome preparation methods

There are several methods for preparing lipid vesicles.[29] All include a key hydration step that drives the association of lipid molecules, previously deposited as dried material (thin film or freeze-dried pre-formed vesicles) or solubilized in organic solvents (ethanol, diethyl ether) into liposomes. The morphology and size of produced vesicles is an extremely sensitive function of the preparation method, chemical nature of lipids, and other conditions (buffer type, pH, ionic strength, osmolarity, temperature, vesicle manipulation, *etc.*). Moreover, GVs preparation generally requires special techniques.[30]

Hydration of lipid thin films has been widely used for preparing vesicles that carry out biopolymerization reactions. The lipid thin film is hydrated by a solution containing the solutes to be entrapped, and large (often multilamellar or multivesicular) vesicles are generally obtained. These are typically processed in order to reduce their size and lamellarity by a mechanical *extrusion*.[31] Higher entrapment yields are obtained by hydrating a "liposome-cake", derived by the freeze-drying of pre-formed empty vesicles.[15c,32] Both methods are advantageous because the solutes of interest are exposed only to lipids, without the presence of solvents or detergents. GVs can be prepared by special film hydration methods: natural swelling[33] and electro-swelling.[34] Only the first one has been used for building a system capable of synthesizing proteins.[35]

The use of the ethanol injection method[36] for making protein-synthesizing vesicles has also been reported.[37,38] The advantage of the method is that it can give sub-micrometric unilamellar vesicles without extrusion, but it exposes enzymes and nucleic acids to ethanol (<5% v/v).

All these methods, however, although quite successful (especially the hydration of thin lipid film and of freeze-dried liposome cake) suffer from a couple of limitations. The first one concerns the impossibility of controlling the solute encapsulation. One has

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to optimise conditions in order to let synthetic cells assemble only by self-organization. Actually, this can be an advantage when the interest is focused on these aspects, as in the case of the recently reported anomalous solute super-entrapment.[38,39] The practical and theoretical problems of solute entrapment and liposome formation mechanisms have been discussed above. When complex multimolecular systems have to be entrapped within lipid vesicles the content variability can limit the use of such constructs in biotechnological processes. The second is the presence, outside vesicles, of a large amount of the same reaction mixture that needed to be entrapped, so that the external reaction must be eliminated. As a matter of fact, only a minor fraction of reaction mixture is typically entrapped (roughly <10–20%).

These two aspects, from the practical viewpoint, somehow constrain the full development of semi-synthetic cell technology, even if they did not hamper scientific achievements as those that will be discussed below. As shown in section 5.2, a novel method for vesicle production was established. This is very promising for the future development of liposome-based bioreactors.

5.2. The droplet transfer method

A few years ago, Weitz and co-workers[40a,b] reported the preparation of giant vesicles by transferring a lipid-coated water-in-oil (w/o) droplet from an oil phase to an aqueous phase, by crossing a lipid oil/water interface, improving a method previously reported by Xiao[40c] (Fig. 4, details are given in the figure caption[41]).

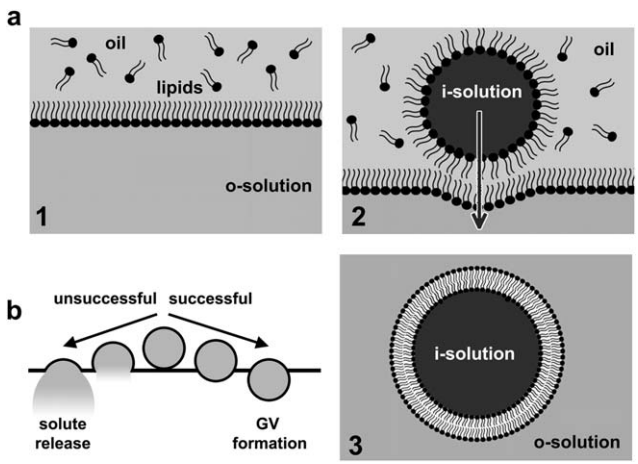

Fig. 4 (a) The droplet transfer method.[40] (1) A solution of lipids in oil (hydrocarbons) is stratified over an aqueous solution (outer-solution: "o-solution"). In a second step (2), a lipid-stabilized water-in-oil emulsion, prepared by emulsifying a small aliquot of an aqueous solution (inner-solution: "i-solution") in lipid containing oil (the lipid can be different than that one used for preparing the oil-water interface in (1)), is poured above the oil/water interface prepared in (1). Water droplets tend to fall down toward the interface due to their higher density when compared to oil. By crossing the lipid-containing oil/water interface (1) a droplet is covered by a second lipid leaflet, being transformed into a vesicle. The vesicles are generally collected by centrifugation. (b) Depending on the conditions (local conditions, *i.e.*, nature of the oil/water interface, or global conditions, *i.e.* nature of compounds used) the droplet might not successfully produce a vesicle, being instead broken at the interface and releasing its content (i-solution) in the o-solution.[42] Panel (A) is reproduced from ref. 41 with minor modifications, with permission of Elsevier.

Ideally, this method overcomes the two limitations described for classical liposome preparation methods. First of all, all solutes initially present in the w/o droplet become contained in the corresponding vesicle. This means that the experimenter has a strong control of vesicle content, because it is easy to control the w/o droplet content. Clearly, the considerations of compositional diversity in the aqueous phase of w/o droplet content still remains (because these droplets ultimately derive from stochastic fragmentation/coagulation processes of a large macroscopic volume that is emulsified into the oil phase), but it is mitigated by the large size of droplets, that reduce (but not eliminate) inter-droplet diversity.[28c]

Secondly, the external vesicle phase should not contain free solutes. Actually, practical experience indicates that a number of droplets might break during the interface crossing, so that solutes are also released in the aqueous phase.[42] Despite these "minor" problems, the w/o droplet transfer method is a very promising one, especially for constructing liposome-based reactors, and indeed it has been already used.[41,43–45]

We also started an experimental investigation on the droplet transfer method. In particular we wanted to set up a standard protocol for encapsulating cell-free extracts into GVs by means of this method. Fig. 5 is a fluorescence micrograph of a GFP-producing GV prepared by the droplet transfer method. Although successful also in our hands, we believe that the method requires further optimisation in order to really become the technological standard for semi-synthetic cell generation. In particular, we have observed a very limited number of GFP-producing GVs, probably because the cell extracts that needs to be encapsulated, very rich in proteins and nucleic acids, affect the delicate surface force balance operating when w/o droplets cross the oil/water interface (*i.e.*, the release of droplet content in the aqueous phase competes with the lipid coating/transfer process, see Fig. 4B). Our investigation shows that the chemical nature of lipids at the oil/water interface is the main factor for determining the amount of GVs produced by the droplet transfer method (Carrara *et al.*, unpublished results).

In addition, the droplet transfer method has already been used by several authors not only for the aim of constructing cell models, but also for investigations on osmotic-driven dynamic changes in GVs[42a] and even lipid raft microdomains.[42b]

5.3. Microfluidic-assisted liposome preparation

The most relevant novelty in the field of vesicle production comes from the microfluidics world. This young technology has rapidly evolved, and found a very important application thanks to the convergence with the use of w/o compartments *à la* Tawfik–Griffiths.[2a] In recent years, in fact, a very rapid growth of interest toward w/o droplets generated and studied in microfluidic channels has been observed, mainly for *in vitro* selection and evolution, high-throughput screening, and more in general to investigate molecular diversity.[2b,c,46] Clearly, the key factor for these applications is the small volume of w/o droplets (in the picolitre range), the easy preparation, and their very good compartmentation efficiency, which translate into miniaturization, and into the consequent possibility of exploring a broader range molecular diversity. Research on w/o droplets, however, does not aim at constructing realistic cell

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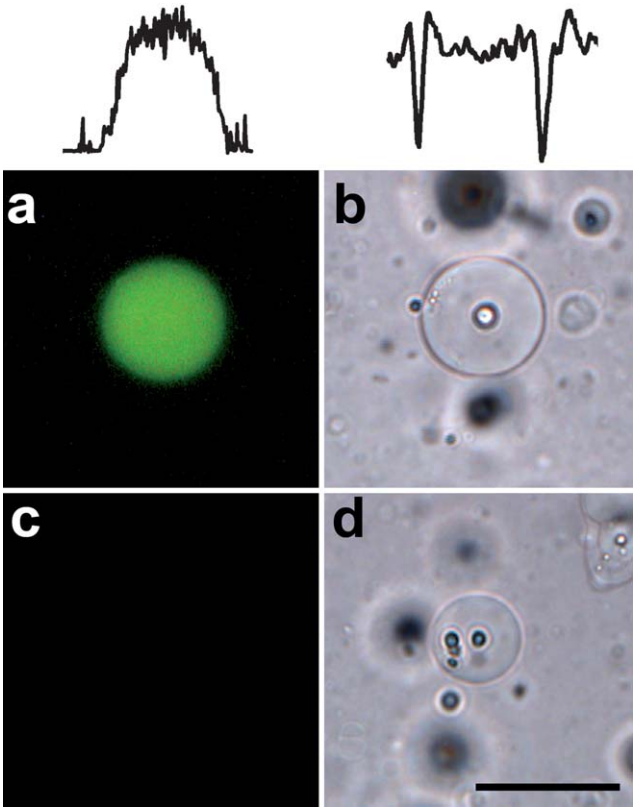

Fig. 5 EGFP synthesis inside GVs produced by the droplet transfer method. EGFP encoding DNA (40 ng μL^{-1}) was mixed with *E. coli* cell extracts (Promega) as described elsewhere.[38] 5 μL of such mixture was emulsified in 500 μL of 0.5 mM POPC in mineral oil (M5904 Sigma) by pipetting. The obtained w/o emulsion was gently poured above 500 μL of 0.5 mM POPC in mineral oil, previously stratified over 500 μL of 25 mM Tris–acetate (pH 7.5), 100 mM potassium acetate, 10 mM magnesium acetate, 1 mM DTT buffer. After centrifugation (2000 rpm, 10 minutes) the vesicles were incubated at 37 °C for 2 hours and then visualized by fluorescence microscopy. The number of EGFP producing vesicles was quite low. Panels (a and b): cell-free extracts plus DNA; panels (c and d): cell-free extracts without DNA (negative control); panels (a and c): fluorescence imaging; panels (b and d): bright-field imaging. On the top, an equatorial profile of micrographs' pixel luminosity (panels a and b). Size bar represents 50 μm.

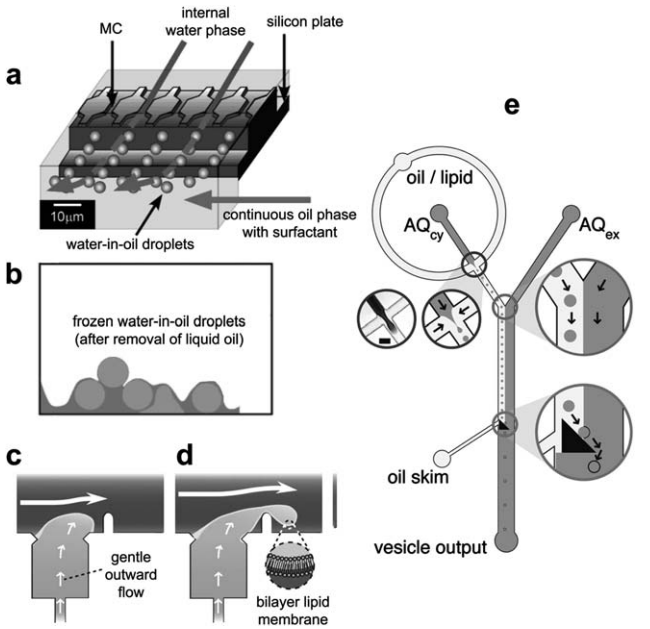

Fig. 6 Three novel methods for GVs production, based on microfluidics. First method: (a) Surfactant stabilized w/o droplets are firstly generated by microfluidic channeling, then transformed into GVs by freezing the droplets (−10 °C) and surfactant replacement, followed by an evaporation process (b) and a final hydration and GVs recovery.[47] Second method: (c) A gentle outward flow further bends out the bilayer, previously assembled from w/o/w liquid micromanipulation in a specifically designed microfluidic channel. Next, shear forces (d) generate GVs.[48] Third method: (e) Microchannel generated w/o droplets and outer aqueous solution are tangentially mixed in microdevices. A properly designed triangular guide mediates the phase transfer of the droplets from the oil flow through the interface and into the outer solution flow.[49] Panels (a and b) reproduced from ref. 47 with permission from American Chemical Society; panels (c and d) reproduced from[48] with permission from John Wiley & Sons; panel (e) reproduced from ref. 49 with permission from the American Chemical Society.

models, even if some experiments have been done in this direction.[2d,e,28c]

Is it possible to prepare (giant) lipid vesicles by means of microfluidic devices? To the best of our knowledge, there have been three reports that reply positively to this question,[47–49] and they are commented on in Appendix 1, and illustrated in Fig. 6. It is possible that this technology will be optimised soon for constructing semi-synthetic cells, so that a controlled encapsulation of enzymes and other macromolecules could be achieved efficiently.

5.4. The PURE system

The second main ingredient of protein synthesis inside lipid vesicles is the complex multi-molecular machinery that synthesizes proteins from DNA or RNA templates. A historical sketch of the development of current cell-free technology can be found in a recently published book (see ref. 26*b*, chapter 1). The typical set of macromolecules for the cell-free protein synthesis derives from cell extracts, *e.g.* extracts from *Escherichia coli*, wheat germ, rabbit reticulocytes, insect cells, and human cells.[50] These kits are functioning very well in diverse research fields but are limited by the fact that their exact composition is not known since it based on the cell extract. As black boxes, they carry out their protein synthesis function, but only partially fit the requirements of synthetic (constructive) biology. In this regard, a breakthrough advancement has been accomplished by the group of Takuya Ueda (University of Tokyo), who assembled a cell-free protein synthesis kit from individually purified components.[51] This reconstituted kit, known with the brand name of the PURE system (protein synthesis using recombinant elements), is composed of four modular yet coupled subsystems, namely: (i) transcription, (ii) translation, (iii) amino acid charging onto tRNA (aminoacylation), (iv) energy regeneration, see Fig. 7. Its composition is given in Table 1.[51b] As it can be seen, the PURE system is composed of 36 individually purified His-tagged proteins, purified ribosomes, and tRNAs mix. It can be considered as a standard chassis for synthetic biology (http://partsregistry.org/Main_Page). Since 2006, it became widely

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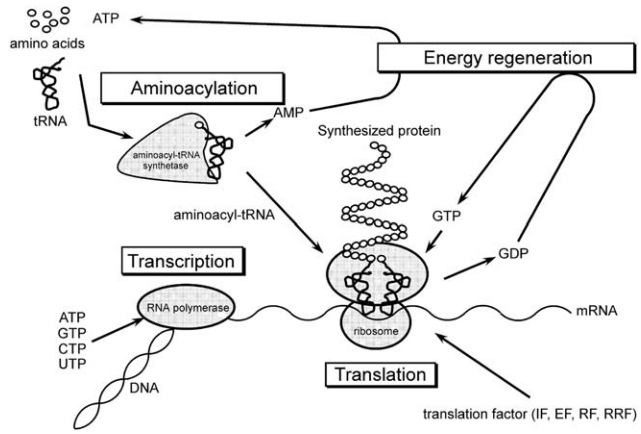

Fig. 7 The PURE system (protein synthesis using recombinant elements) is composed of 36 individually purified His-tagged proteins, purified ribosomes, tRNAs mix, amino acids, nucleotides. Four modules can be distinguished: transcription, translation, aminoacylation and energy regeneration. The ultimate energy source is creatine phosphate. Reprinted from ref. 51*b*, with permission from Elsevier.

used for accomplishing protein biosynthesis inside liposomes,[52,53] although the yield of synthesized proteins are generally lower than those obtained with cell extracts (for a review, see ref. 54). Note, however, that for simpler biopolymerization reactions inside liposomes, such as DNA transcription, RNA replication, PCR, or poly(U)-poly(Phe) synthesis, *in vitro* mixtures of well-defined composition were already used (see Section 6.2).

5.5. Eliminating the external reaction

The second point essentially consists of mechanical removal of unentrapped solutes by vesicle centrifugation/filtration/chromatographic gel filtration, or in the addition of a compound that inhibits the external reactions or destroys the external solutes (*e.g.*, protease, nuclease, non-permeable inhibitors, *etc.*). Sometimes even simple dilution is effective to quench external protein synthesis.[55] The best strategy has to be decided according to the vesicle type and the scope of the experiment.

5.6. Feeding methods

When one or more compounds need to be added to vesicles, which already contain enzymes or other reactants, several possibilities have been envisaged and experimentally tested (Fig. 8). These are: (1) passive diffusion, generally possible for small and not heavily charged substrates (a typical example is the feeding of an enzyme-containing vesicle with its substrate, a review on these systems has been recently published[56]); (2) fusion of two or more vesicles thanks to electrostatic, depletion or molecular recognition forces (this strategy has been recently proposed as a way to stepwise increase the vesicle content and lead to functional vesicles starting from non-functional ones[57]); (3) the direct microinjection of reactants into immobilized GVs;[58] (4) the passive diffusion across a membrane hold at or near its melting temperature, because membrane defects causes an enhancement of permeability coefficient of some species (typical is the use of DMPC vesicles at 23 °C[1f,20,59]); (5) the passive diffusion across a membrane doped with detergents at sublytic concentrations (for example, see the cholate-induced selective permeabilization of POPC vesicles[60]); (6) the formation of membrane pores by some proteins (the pore allows the passage of molecules smaller than a cut-off value), like α-hemolysin[43,61] or porins.[62] All these mechanisms work because there is a differential permeability when substrates and enzymes are considered. In particular, all these methods ensure the entrance of an externally added substrate *without* releasing macromolecules from within.

Table 1 Composition of the PURE system (reproduced from ref. 51*b* with permission from Elsevier)[a]

Component	Concentration
Translation factors	
IF1	2.7 μM
IF2	0.4 μM
IF3	1.5 μM
EF-G	0.26 μM
EF-Tu	0.92 μM
EF-Ts	0.66 μM
RF1	0.25 μM
RF2	0.24 μM
RF3	0.17 μM
RRF	0.5 μM
Amino acyl-tRNA synthetases (RSs)	
AlaRS	1900 U mL^{-1}
ArgRS	2500 U mL^{-1}
AsnRS	20 mg mL^{-1}
AspRS	2500 U mL^{-1}
CysRS	630 U mL^{-1}
GlnRS	1300 U mL^{-1}
GluRS	1900 U mL^{-1}
GlyRS	5000 U mL^{-1}
HisRS	630 U mL^{-1}
IleRS	2500 U mL^{-1}
LeuRS	3800 U mL
LysRS	3800 U mL^{-1}
MetRS	6300 U mL^{-1}
PheRS	1300 U mL^{-1}
ProRS	1300 U mL^{-1}
SerRS	1900 U mL^{-1}
ThrRS	1300 U mL^{-1}
TrpRS	630 U mL^{-1}
TyrRS	630 U mL^{-1}
ValRS	3100 U mL^{-1}
Other enzymes	
MTF	4500 U mL^{-1}
Ribosomes	1.2 μM
Creatine kinase	4 μg mL^{-1}
Myokinase	3 μg mL^{-1}
Nucleoside diphosphate kinase	1.1 μg mL^{-1}
Pyrophosphatase	2 U mL^{-1}
T7 RNA polymerase	10 μg mL^{-1}
Energy sources	
ATP	2 mM
GTP	2 mM
CTP	1 mM
UTP	1 mM
Creatine phosphate	20 mM
Other components	
20 amino acids	0.3 mM
10-Formyl-5,6,7,8-tetrahydrofolic acid	10 mg mL^{-1}
tRNAmix (Roche)	56 Abs260

[a] The components are solubilized in 50 mM HEPES–KOH pH 7.6; 100 mM potassium glutamate, 13 mM magnesium acetate, 2 mM spermidine, 1 mM DTT. One unit of activity was defined as the amount of enzyme that catalyzes the formation of 1 pmol of amino acyl-tRNA in 1min.

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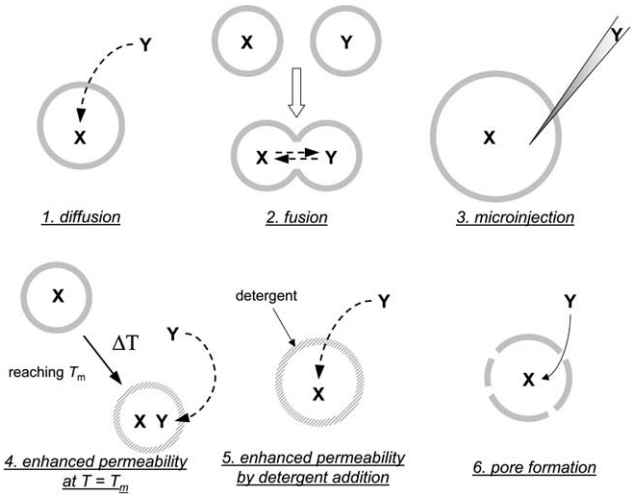

Fig. 8 Experimental methods to delivery a second reactant Y inside an X-containing vesicle. (1) Free (passive) diffusion of Y; (2) fusion between two or more vesicles;[57] (3) microinjection of Y inside a giant vesicle;[58] (4) Keeping the vesicles at the phase transition temperature T_m (or by thermal cycles around T_m);[1f,20,59] (5) free diffusion of Y into detergent-doped membranes (by adding detergents at sublytic concentration);[60] (6) entrance through pores, formed by α-hemolysin, porins.[43,62] Reproduced from ref. 1*c* with permission from John Wiley & Sons.

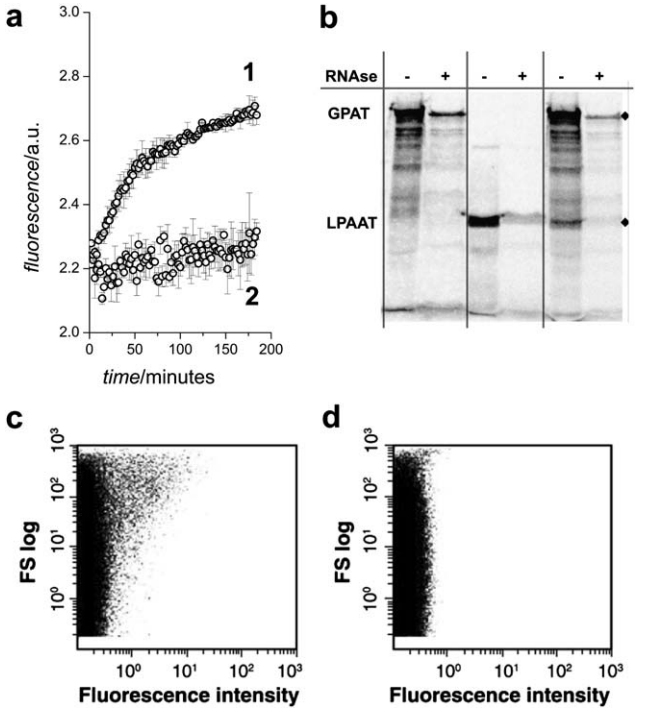

Fig. 9 Some examples of intravesicle protein detection. (a) Fluorescence *versus* time profiles of EGFP expression inside small vesicles, as revealed by batch fluorescence;[38] curve 1: RNAse A added after liposome formation; curve 2: RNAse A added before liposome formation. (b) Autoradiography of polyacrylamide gel after electrophoretic separation of the synthesized proteins (GPAT and LPAAT) inside liposomes. The two enzymes are expressed separately in liposomes with or without RNAse A (lanes 1–4 from the left), or co-expressed (lanes 5–6 from the left). Note that when RNAase A is added externally, after vesicle formation, only proteins synthesized inside liposomes are detected, and the corresponding bands are weaker.[65] (c and d) Flow-cytometry detection of liposome subpopulations expressing GFP. Each dot represents a liposome, plotted on a 2D dotplot *versus* a scattering signal (*y*-axis) and green fluorescent signal (*x*-axis). The subpopulation of liposomes that expresses GFP is evident in panel (c), whereas it is absent in panel (d), where DNA was not coentrapped.[64] Panel (a) reproduced from ref. 38 with permission from Wiley; panel (b) reproduced from ref. 65 with permission from Elsevier; panels (c and d) reproduced from ref. 64 with permission from Elsevier.

The elegant example provided by α-hemolysin pore (cut-off: 3 kDa) on protein-synthesizing GVs shows how a complex catalytic network composed of dozens of enzymes and nucleic acids can be effectively compartmentalized and externally fed by low molecular weight compounds, without being released in the environment.[43]

Moreover, it should be considered that, in addition to reactant entrance, small products can also leave the vesicles. Removal of the products can be relevant when their accumulation can be harmful for the internal reaction network, or when their release is needed for specific applications or studies. Interestingly, the importance of solute release was one genuine outcome of numerical modelling.[63]

5.7. Analytical methods

The production of proteins (or nucleic acids) inside lipid vesicles can be quantified with or without destroying vesicles. For example, the production of green fluorescent protein (GFP), or the production of enzymes that can act on fluorogenic substrates can be easily followed by fluorescence assays, and if giant vesicles are used, the fluorescence from each individual giant vesicle can be recorded. It is not a surprise, therefore, than the first studies on protein expression inside liposomes involved GFP as model protein. In other cases, co-encapsulated radioisotope-labeled amino acids are incorporated into the internally produced protein, which can be quantified after its extraction from liposomes. The production of enzymes can be confirmed by specific assays. Examples of protein quantification[38,64,65] are shown in Fig. 9. It is also interesting to note the difference between averaging and not-averaging measurements. For example, flow cytometry and fluorescence microscopy are not averaging techniques, because they can be used to quantify fluorescence from each single particle, whereas bulk fluorescence measurements or protein extraction level out the differences between compartments. In non-averaging methods, the "diversity" between vesicles is explicitly taken into account, so that the sample can be indeed seen as a population of individuals, ranked along a certain parameter (solute content, reaction yield, size, *etc.*). This 'diversity' implies that selective/competitive processes can arise in a population of semi-synthetic cells, making this an attractive model for studying the dynamics of primitive cell populations, as already noted in some reports.[28a,b,66]

5.8. Computational methods

Although rapidly progressing, the set of experiments on biopolymerization reactions inside lipid vesicles has not yet generated a parallel series of studies in terms of numerical simulations.

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Diverse reports have been published by Mavelli (in collaboration with Ruiz-Mirazo), who has developed a dedicated software platform for simulating micro-compartmentalized reactions by stochastic modelling.[63,67] The internalised synthesis of biopolymers has been investigated by coupling diffusive transport and internally catalysed reactions. One of the most recent analyses[67c] involved the *in silico* simulation of an RNA synthesis inside self-reproducing liposomes (*i.e.*, a model of ribozyme-based primitive cells, as proposed by Szostak *et al.*[10c]). With respect to numerical modeling of cell-free protein synthesis kits, to the best of our knowledge there are no systematic analyses that combine experimental and *in silico* studies. The group of Yomo reported the reciprocal effects of varying the concentration of the PURE system components on the yield of synthesized protein in bulk phase,[68] whereas Foy and co-workers have recently simulated stochastically the protein synthesis path.[69] Stochastic simulations play a major role for studying these systems by considering the small reaction volumes (and therefore the limited number of solutes) that distinctively feature reactions inside liposomes. The combination of experimental and theoretical methods might allow a clearer interpretation of observation and a development of a more detailed model for reactions in microcompartments; we therefore hope that further advancements will be done in this direction.

6. State of the art

6.1. Protein synthesis inside liposomes

In Table 2 we have collected a full list (to the best of our knowledge) of published work on protein synthesis inside conventional (sub-micrometric) and giant lipid vesicles.[22,23,35,37,38,43–45,52,53,64,71–74] Notice that in order to synthesize a protein from DNA, RNA must be first synthesized, and this is implicit in records of Table 2. The explicit syntheses of nucleic acids (DNA and RNA, intended as target molecules) in lipid vesicles are instead commented on below.

When a protein is synthesized inside vesicles, the main difference with bulk expression is given by the small encapsulated volume. The sum of internal volumes of all liposomes is only a small fraction of the physical volume of the sample. The overall efficiency is further decreased by the fact that not all vesicles synthesize the protein, or because there are differences in individual vesicle performances, due to the heterogeneity of their content. A detailed study of the overall protein yield, by varying the method of liposome preparation and other parameters, can be found in the work of Mastrobattista.[70] Their study, based on β-galactosidase expression, has shown the effects of lipid concentration, cholesterol content, charged lipids, and PEGylated lipid. After optimisation, an intra-vesicle yield of 30–40 μg mL^{-1} of liposomes (60 mM lipid) was obtained.

It is not possible to generalize these conclusions for all systems, so that it is still useful to carry out optimisation in each case of interest.

In the following, we will comment on the records presented analytically in Table 2, giving emphasis to the historical development of this research (experimental conditions are specified in Table 2). The reason is that such studies were not intended to improve the amount of synthesized proteins, but are very often proof of concepts for showing that compartimentalized systems of desired complexity and performance could be indeed prepared in the lab.

The first entry in Table 2 refers to the pioneering work of Oberholzer, Nierhaus and Luisi who successfully reported the synthesis of poly(Phe) inside conventional submicrometric vesicles, by using poly(U) as template.[22] The first evidence of the synthesis of functional folded protein (GFP) was instead given by the group of Yomo in 2001, by using the method of rehydration of freeze-dried liposomes.[23] The same group was able to apply flow cytometry as a new tool for quantitative detection of reactions occurring inside lipid vesicles.[23,52,64,72–74] Soon after, the synthesis of GFP in giant vesicles prepared by the natural swelling method was reported.[35]

Two impressive cases of complex bioreactor design were reported in 2004. The first one, again from the Yomo group,[64] was a case of a compartmentalized two-step genetic cascade reaction, *i.e.*, the first gene was expressed to give a well folded RNA polymerase, which in turn transcribed the second gene to give the final well folded GFP as the final product. The second one comes from the Libchaber laboratory.[43] Giant vesicles, prepared by the method of w/o droplet transfer, were made permeable to low molecular weight compounds only thanks to the presence, in the lipid membrane, of a α-hemolysin pore (consisting of a α-hemolysin heptamer). The very important fact is that the pore self-assembles *in situ* thanks to the internal synthesis of α-hemolysin. It was shown that such vesicles could be fed with fresh nutrients (releasing by-products) for about 100 hours. The next technical advancement was reported in 2006/2007 when, independently, two studies shifted from cell extracts to the PURE system as a transcription/translation kit.[52,53] Another major achievement was due again to the group of Yomo, consisting of the establishment of a reciprocal RNA replication (from sense strand to antisense strand and *vice versa*) under Qβ-replicase catalysis.[72] The innovative aspect was that the Qβ-replicase was itself produced by translating the RNA code, by means of ribosomal apparatus. In other words, the genetic material (RNA) encoded for a function that ultimately leads to its own replication.

Aimed at the experimental verification of the smallest vesicle size that could support protein synthesis, in 2009 we demonstrated that GFP could be synthesized in 200 nm (diameter) vesicles.[38] This result, which was somehow unexpected on the basis of the very low probability of co-entrapping *ca.* 80 different macromolecules into the same small compartment, actually started a research program on self-organization properties of lipids and solutes during vesicle formation (see Appendix 2).

Baigl, Inoue and co-workers[44] have investigated the synthesis of GFP in vesicles prepared by the droplet transfer method, firstly reported by Noireaux and Libchaber[43] and also evidenced by us in Fig. 4. The novelty here is given by the fact that, for the first time, the progress of GFP production was followed in each individual vesicle, providing an estimate of the compositional diversity of giant vesicles derived from w/o emulsion droplets. For example the fluorescence produced inside each GV could vary by factors 0.3–2 around the average. Our preliminary study on w/o droplets confirms that an intrinsic inter-compartment heterogeneity is always present, even for simple 1-solute systems. For multi-solute systems, these variations combine in complex

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Table 2 List of published work on protein synthesis inside vesicles[f]

#	Lipids	Preparation method	Template	T&T kit	Product	Detection	Note	Ref.
1	POPC	Film	RNA (Poly(U))	Ribosomes	Poly(Phe)	Radioactive labelling	First ribosomal polypeptide synthesis	22
2	Lipid mix[a]	Film	DNA	Cell extracts	GFP	Flow cytometry, fluorescence microscopy	First 3D folded protein synthesis	23
3	POPC	Injection	DNA	Cell extracts	GFP	Batch fluorescence		37
4	DOPC/ DOPG 10/1	Natural swelling	DNA	Cell extracts	GFP	Fluorescence microscopy		35
5	Lipid mix[a]	FD	DNA (2 genes)	Cell extracts	T7 RNA polymerase and GFP	Flow cytometry	2-Steps cascade reaction	64
6	Egg PC	Droplet transfer	DNA (also 2 genes)	Cell extracts	GFP and α-hemolysin	Fluorescence microscopy	Self-assembled pore allows entrance of nutrients	43
7	Lipid mix[b]	FD	DNA	PURE system	GFP	Flow cytometry	Pure system	52
8	POPC	FD	DNA	PURE system	GFP	Fluorescence microscopy		53
9	Egg PC	Droplet transfer	DNA	PURE system	GFP	Fluorescence microscopy		71
10	Lipid mix[c]	Film	DNA (2 genes)	PURE system	GPAT and LPAAT	Radiolabelling	First example membrane protein	65
11	Lipid mix[d]	FD	RNA	PURE system	Qβ-Replicase and β-galactosidase	Flow cytometry and fluorescence microscopy	RNA replication	72
12	Lipid mix[d]	FD	DNA	PURE system	β-Glucuronidase	Flow cytometry		73
13	POPC	Film and injection	DNA	Cell extracts and PURE system	GFP	Batch fluorescence	Small vesicles	38
14	Egg PC	Droplet transfer	DNA, RNA	PURE system	GFP	Fluorescence microscopy	Individual tracking	44
15	DOPC	Droplet transfer	RNA	Cell extracts	GFP	Microscopy		45
16	Lipid mix[e]	FD	DNA	PURE system	GFP	Flow cytometry and fluorescence microscopy		74

[a] EggPC : cholesterol : DSPE–PEG5000 (1.5 : 1 : 0.08) (molar ratio). [b] POPC/PLPC/SOPC/SLPC/cholesterol/DSPE–PEG5000 129 : 67 : 48 : 24 : 180 : 14. [c] POPC : POPE : POPG : cardiolipin (50.8 : 35.6 : 11.5 : 2.1). [d] POPC/cholesterol/DSPE–PEG5000 (58 : 39 : 3). [e] (Different ratios of POPC/POPG/POPE)/cholesterol/DSPE–PEG5000 (58 : 39 : 3). [f] Film: hydration of thin lipid film, injection: ethanol injection method, FD: rehydration of previously freeze-dried liposomes.

and often unpredictable ways, justifying the product final broad distribution of the product.[28c]

All entries of Table 2 refer to the synthesis of water-soluble proteins inside lipid vesicles. In addition to GFP, clearly chosen as reporter protein for its easy detection, T7 RNA polymerase, α-hemolysin, and β-galactosidase have been successfully synthesized. What about membrane proteins? Although the use of cell-free extract was already known as a tool for expressing membrane proteins *in the presence* of lipid/detergent particles as micelles and in some cases vesicles (see chapter 3 of ref. 26*a*, and chapters 8–9 of ref. 26*b*), the first example of synthesis of functionally active membrane proteins *inside* vesicles was reported only recently.[65] The system was composed of a couple of membrane-associated enzymes that accomplish the first two steps of the lipid salvage pathway, namely the sequential acylation of glycerol-3-phosphate to diacylphosphatidic acid. The two enzymes are acyltransferases, and the final goal of the study was the enzyme-catalyzed synthesis of diacylphosphatidic acid inside lipid vesicles, as a step toward the reproduction of semi-synthetic minimal cells. Interestingly, this work demonstrated that for a successful approach, the lipid composition is a key factor. In fact, lipids used for preparing liposomes should simultaneously: (i) form vesicles with good entrapment yield, (ii) be chemically compatible with the protein synthesis machinery (several recent studies have pointed out that possible interferences exist[22,70,74,75]), and (iii) support the protein insertion/folding and consequent enzyme activity (the effect of lipid composition on the activity of membrane proteins has been recently remarked upon).[76]

There is indeed an increasing interest toward membrane protein synthesis,[77] and very recently this was extended to lipid vesicles (on their outer surface, or more rarely inside them). Clearly, an external synthesis is accompanied by larger amounts of protein, but in some cases, due to the vectorial geometry of membrane proteins, an internal synthesis might be necessary, not only for constructing semi-synthetic cells, but also for biotechnological applications.

As in the case of cytosolic proteins, membrane proteins can also be synthesized by cell-free systems, which is generally complemented by detergents/lipids for providing a hydrophobic microenvironment where the protein preferentially binds in correct conformation. So, current strategies are based on trial-and-error tests, by simply coupling the cell-free system and proper amphiphilic compounds (but in the specific case of membrane insertion, lipid vesicles are typically used). In the simplest cases the success depends on a spontaneous membrane insertion of the synthesized protein. This phenomenon is due to the hydrophobic interaction between the protein and lipid membrane. Although the spontaneous insertion can be effective for some membrane protein insertions, it is not considered that any type of membrane protein can be correctly integrated.

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Whether a spontaneously inserted protein can be functional or not depends on the nature of the membrane protein and on the membrane lipid composition. For example, POPC vesicles help in solubilizing glycerol-3-phosphate acyltransferase, but do not contribute in the activation of the enzyme.[65]

It is especially difficult to control the membrane orientation of complex proteins which contain multi-membrane-spanning domains. In fact, in a living cell, such spontaneous insertion does not occur. Most membrane proteins require additional regulation after, or during, their synthesis. For instance, in bacteria, the assembly between lipid membrane and secretory proteins is mediated by a cytosol factor, SecA,[78] which is needed to translocate the protein through cellular membrane. For the integral membrane protein, a nascent polypeptide elongated on ribosome is escorted onto membrane surface by other cytosol factors, the signal recognition particle (SRP) and the SRP receptor (SR),[79] and then directly integrated into lipid bilayer environment. It must be noted that in both types of membrane proteins, they must pass through the specific membrane pore, which is composed of heterotrimer membrane proteins, SecYEG. Also for these systems, lipidic environment is important. Nishiyama *et al.* have recently reported that a novel unique glycolipid has been found to be essential for the process of SecYEG dependent protein membrane integration.[80]

If all of these factors were well studied and adapted, this would allow the full control of the membrane insertion (or secretion) system, so that it could be integrated in the technology of synthetic cells.

6.2. Nucleic acid synthesis inside liposomes

The topic of nucleic acid synthesis inside liposomes is also very well studied and has several implications in origins of life studies and biotechnology. In fact, the first attempts to compartmentalize RNA were inspired by modeling RNA/lipid worlds in a joint fashion[10c] The goal was the observation of RNA production (ideally ribozyme-catalyzed RNA replication) in self-reproducing vesicles.[12a,19,21] This would have brought to a minimal cell containing RNA molecules that would have acted simultaneously as templates and catalysts (polymerase, lipid synthase, *etc.*). Unfortunately these difficult objectives have been not yet achieved, even if recent and elegant advancements in ribozyme chemistry have been reported.[81] There have been several successful attempts for nucleic acid synthesis in liposomes, each exploiting a different strategy and aimed at exploring different aspects to these systems.

6.2.1. The polynucleotide phosphorilase (PNPase) system. ADP can be polymerized to poly(A) in a template-free reaction catalysed by PNPase. This reaction is also known as the "Oparin's reaction" because it was also studied in coacervates (see ref. 1–3 cited in ref. 19). In all studies, ADP was externally added to PNPase-containing liposomes. In the first work (1994), PNPase was entrapped inside extruded fatty acid vesicles, and simultaneously to the ADP addition, oleic anhydride was also given to the vesicles, triggering vesicle grow and division[19] (the issue of vesicle self-reproduction drives several studies that involve biopolymerization reactions inside liposomes and more in general research on minimal cells, the interested reader could take as a good starting point a recently published review[82]). Such a system was a model for nucleic acid synthesizing- and self-reproducing vesicles. ADP could penetrate into oleate vesicles thanks to the relatively high permeability of fatty acid membranes when compared to classical phospholipid ones. In the same year a similar model system was realized by using extruded DMPC liposomes.[20] ADP could permeate the bilayer membrane, when it was kept at 23 °C (*i.e.*, at the T_m of DMPC). As mentioned above, at the phase transition temperature membrane defects are often associated to the lipid reorganization. A third case was realized by entrapping PNPase inside extruded POPC liposomes, whose membrane was made partially permeable after the addition of sodium cholate (a surfactant) at sublytic concentration (POPC/cholate 5/4 mol per mol).[60]

6.2.2. The Qβ-replicase system. Another way to synthesize RNA is to exploit the catalysis of Qβ-replicase, an RNA-dependent RNA polymerase that was also used by Spiegelman in his studies on *in vitro* RNA replication and evolution (see ref. 9*e* and 10 cited in ref. 21). Qβ-replicase was entrapped inside extruded oleate vesicles, together with an RNA template and NTPs. Also in this case, feeding by oleic anhydride served to couple internal RNA synthesis with vesicle self-reproduction.[21] More recently, as described in the paragraph on protein synthesis, the group of Yomo elegantly proposed a new—more complex—design where the Qβ-replicase is encoded by the template RNA, and synthesized *in situ* thanks to ribosomes.[72]

6.2.3. Polymerase chain reaction and DNA synthesis. DNA polymerase, entrapped in POPC extruded vesicles together with a template DNA, primers, and dNTPs, was instead the key enzyme for carrying out the polymerase chain reaction inside vesicles. Thermal cycles allow the amplification of DNA strands.[83] A new report on PCR inside lipid vesicles has been described this year. The PCR components were entrapped into large and giant vesicles prepared by the rehydration of freeze-dried liposomes (POPC–DSPE–PEG_{5000}–cholesterol (65 : 5 : 30)) and the progress of PCR reaction was followed by flow-cytometry, by staining double-stranded DNA with SYBR-Green I (as in real-time PCR). It was shown that the amplification of 1229 bp DNA was successfully carried out inside vesicles, and that the efficiency of reaction was better in large vesicles.[84]

Additional examples of DNA synthesis inside liposomes are provided. The *E. coli* Klenow fragment was entrapped in liposomes composed of the lipid mixture (POPC/cholesterol/DSPE–PEG_{5000} (58 : 39 : 3 mol : mol)), together with template DNA and dNTPs. DNA was then produced at 25 °C within vesicles prepared by the dehydration–rehydration method.[85] The intravesicle isothermal (65 °C) DNA replication was realized by coentrapping the components of the thermophilic helicase-dependent amplification system (a mix of thermostable proteins including UvrD helicase, single-strand binding protein, and DNA polymerase) into large (5 μm) POPC vesicles prepared by the thin film hydration method.[86]

6.2.4. DNA transcription by RNA polymerase. The DNA dependent synthesis of RNA has been carried out inside liposomes. The enzyme RNA polymerase was coentrapped with DNA inside giant vesicles, prepared by the natural swelling

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method, composed of DOPC/DOPG 10 : 1.[87] The same reaction was carried out in POPC giant vesicles prepared by the electro-swelling method, by injecting the reaction mixture (T7 RNA polymerase, DNA template and NTPs) with a microneedle.[58b]

The enhanced NTPs permeability across the DMPC bilayers, held at T_m (23 °C) or subjected to temperature cycles (from 23 to 37), was a good way to give them entry into a previously prepared RNA polymerase-containing DMPC conventional liposomes.[1f,59]

6.2.5. Enzyme-free oligomerizations. In addition to the above mentioned examples, enzyme-free oligomerization reactions for producing nucleic acids (and oligopeptides as well) have been reported inside vesicles. These systems are designed to model primitive cells, showing how activated building blocks could have reacted together to give the early bio-oligomers that eventually evolved into functional macromolecules as we know them. Some relevant examples are the spontaneous oligomerization of NCA-amino acids,[88] amino acids thio-esters,[89] AMP/UMP under dehydrating conditions,[90] or imidazoyl-activated nucleotides.[91]

Further detailed comments on nucleic acid polymerization reactions inside liposomes can be found in Monnard.[1g]

It should be finally added that lipid vesicles are also used for reconstituting cytoskeletal elements. This is a very interesting research avenue that nicely complements the synthesis of proteins inside vesicles. Current research focused on bacterial cell division machinery (FstZ),[92] membrane tube networks,[93] actin–membrane interaction,[94] filopodia formation,[95] and membrane scission,[96] as it has been recently reviewed by Liu and Fletcher.[7] Similarly, it has been shown that actin filaments can be anchored to the interior GVs walls through the spectrin/ankyrin proteins, producing tightly packed actin bundles.[97] More recently, actin polymerization was also triggered inside liposomes prepared by the droplet transfer method;[41] moreover, a mixture of F-actin and heavy meromyosin (acto-HMM) was encapsulated within vesicles prepared by the droplet transfer method, and after ATP entrance *via* a membrane pore (α-hemolysin), vesicles were shown to change shape, due to changes attributable to the sliding between F-actin and HMM in the compartmentalized protein network.[98]

7. Concluding remarks

Here we have shown the recent efforts in constructing cell-like particles in the laboratory, thanks to the advancements in liposome and cell-free technologies. Great emphasis is given to the synthesis of proteins inside vesicles, because it paves the way to the establishment of a more complex intravesicle dynamics, based on the expression of enzymes, membrane channels, protein receptors, *etc.* The fast-progressing microfluidic technology, when successfully blended with current experimental approaches, might allow an effective and controlled high-throughput production of cell-like particles.

What are the potential applications of such compartmentalized systems? There are several possibilities, some of them similar to the use of enzyme-containing vesicles.

From the viewpoint of basic science, the bottom-up laboratory construction of a lipid compartment that is capable of displaying some simple living functions, such as protein synthesis, or a simple built-in metabolism. It will allow the study of these processes in confined microspaces without the background interference of other cellular processes (note that here the term "bottom-up" is intended as denoting the assembling of simpler components into a system). These minimal experimental models have also the advantage of a possible system-level numerical analysis, so that mechanistic hypotheses can be checked. Clearly, the extrapolation of *in vitro* results to *in vivo* conclusions should be taken cautiously, but nevertheless we believe that the act of constructing a model system is still a challenge for checking our understanding of its organization.

The second aspect, still related to basic science, belongs to conceptual issues in theoretical biology and to the definition and construction of living entities. As soon as the semi-synthetic cell technology will advance, it will become possible to cross the "boundary" between non-living and living constructs.[1b,11b,99] Realizing that a living cell has been indeed constructed in the lab will help us in defining life from the operative viewpoint, and demonstrate that the property "life" emerges from the organization of non-living molecules, without the need of vitalistic or supranatural forces. This in turn will provide a proof of concept for the origin of life on earth, even if modern molecules are used instead of primitive ones.

From the biotechnological viewpoint, several new methods could rely on the availability of semi-synthetic minimal cells or simpler lipid vesicle constructs. Here the major features of these systems are the (i) semi-permeable character of the membrane, (ii) the possibility of reconstituting membrane enzyme or membrane receptors, (iii) the confinement of complex multienzyme solutions (and therefore their protection from external agents). For example, new assays could be developed by incorporating lipid vesicles into micro-patterned chips, as cell models for screening huge libraries of compounds, or for permeability tests or cell-vesicle interactions. Additional uses in analytical chemistry and for laboratory purposes are conceivable.

Being already used as drug delivery agents, lipid vesicles with internalised sensing/metabolic models could be used as a tool for nanomedicine. A recently published paper devises their use as a "nanofactory".[100] These particles, injected in the body as currently happens for drug carriers, should recognize a target tissue by means of surface receptors, sense their environment thanks to sensing modules (based on surface derivatization), and produce an effective drug where it is needed thanks to internal metabolic modules. Similar applications for enzyme therapy and gene delivery could be based on synthetic cell technologies. Shifting from one-particle to many-particles, especially if more complex behaviour could be implemented in the next generation of synthetic cells, a layered or multi-layered population of liposome-based cells in physical contact could be a model for biofilms or biological tissues.[42,101] Finally, a fascinating area of study would be the development of artificial communication between natural and synthetic cells as a prerequisite for more advanced applications.[102a,b] A direct physical junction between a liposome and a cell has also been reconstructed and exploited to transport chemicals from a "synthetic" to a natural cell.[102c] The concept of multicompartment assemblies is also interesting in these perspectives.[103]

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Appendix 1. Microfluidic-assisted liposome preparation

Here we comment on four recently published papers on the production of GVs by microfluidic-devices. The first one[47a] prepares surfactant-stabilized w/o droplets by a microfluidic device, and after droplet freezing, and surfactant/lipid exchange, the droplets are transformed in GVs by resuspension in a buffer (Fig. 6A). The procedure forms homogeneously sized GVs in reproducible manner. In the second procedure,[48] a planar lipid bilayer was first formed at a microfluidic T-junction, then the GV is firstly "blown up" by a flow of aqueous solution onto the lipid bilayer, and later detached by mechanical shear force (Fig. 6B). The third method[49] combines the w/o droplet transfer method with microfluidic manipulations of droplets, and consists of a w/o generation module that has been engineered in order to let each droplet be transferred to an aqueous phase by means of a properly designed triangular post (Fig. 6C). In the fourth method[47b] a microfluidic device is used to generate w/o droplets that are collected and transformed to GVs by interface transfer, in a two-step fashion. At the moment, these methods have not been used for building semi-synthetic cells, or to encapsulate enzymes, but quite probably in next years the joint convergence of w/o droplet transfer method and microfluidics will allow the reproducible preparation of a large number of GVs with a good control of encapsulated solutes. In other words, this would correspond to a wet microbiotechnology for assembling synthetic cells from their constituents (aqueous components plus lipids). Currently it is not clear whether this will become soon a reality, or whether the anticipated problems on the robustness of the droplet transfer method will hinder its realization. But suppose for a moment that this technology would be available. What is gained and what is lost when compared to classical preparation methods? The big advantages are the reproducible production of giant vesicles with similar size and content, the possibility to decide what is the composition of each aqueous phase trapped inside liposomes, and the virtual absence of free solutes in external solution. In other words, this technology would correspond to an artificial cell "producing machine".

A first limitation is that the method is valid for GVs only, but the advantage of observing individual GVs by light microscopy (in contrary to submicrometric vesicles) somehow counterbalances this limitation. The second one is that this method, bypassing the hydration step that is instead present in all other classical methods, cannot be used for revealing self-organization processes that underlie the formation of lipid compartments from separated molecules (lipids, enzymes, *etc.*). The almost total control on vesicle formation mechanism is certainly an advantage in biotechnological terms, but a disadvantage when the focus of the study is shifted toward the investigation of self-organizing patterns. For example, a recent study of the latter type has highlighted that the spontaneous formation of cell-like systems brings about an enhancement solute entrapment and protein synthesis, shortly commented on in Appendix 2.[38,39]

Studies on the mechanical properties of GVs in microchannels have been reported.[104] This might open a broader perspective for microfluidic technologies and lipid vesicles.

Appendix 2. Spontaneous concentration of solutes inside liposomes

For protein synthesis inside vesicles, it is necessary that the about 80 different macromolecules, typically present at the concentration of about 0.1–1 μM, are co-entrapped in the same liposome. When the PURE system[51] is used, it can be calculated that the probability of coentrapping at least one copy of each macromolecule inside a vesicle with diameter 200 nm is around 10^{-26}. In contrast, experimental evidence[38] shows that a small amount of protein is indeed synthesized, *e.g.*, about 1 protein molecule every 100 vesicles. In our opinion, this ratio can be better interpreted, for instance, as 10 protein molecules produced inside 1 active vesicle out of 1000 inactive vesicles, revealing that only a small fraction of vesicles actually contains all macromolecules required for synthesizing a protein. In other words, this hypothesis suggests that most vesicles, as expected from the Poisson statistics, poorly entrap the transcription/translation kit, whereas a few of them originated by an anomalous pathway that allows a super-concentration of solutes (by a factor 20 or more) inside vesicles. In order to test this hypothesis, a thorough investigation was started aimed at demonstrating which was the real occupancy distribution of macromolecules in each vesicle. Direct vesicle visualization, made possible by cryoTEM, has shown that the entrapment frequency of ferritin[39a] and ribosomes[39b] inside liposomes does not follow the expected Poisson distribution, but it is shaped as in power-laws, revealing the presence of many "empty" vesicles (no or very little entrapped solute), and a long decreasing tail with extremely crowded vesicles. Similar direct evidence was also obtained when cell extracts or the PURE system were encapsulated.[39b] In other words, lipid vesicles, thanks to the interplay between their formation mechanism and the interaction with the solutes, can concentrate solutes in their aqueous cavity, even when prepared in diluted solutions. It appears that surface phenomena control this unexpected behaviour that is currently under further experimental investigation and theoretical modeling.

Abbreviations

ADP	adenosine diphosphate
DMPC	1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphates
DOH	1-decanol
DOPC	1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine
DOPG	1,2-dioleoyl-*sn*-glycero-3-phosphatidylglycerol
DSPE-PEG5000	1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-PEG5000
Egg PC	phosphatidylcholine extracted from egg yolk
GPAT	glycerol-3-phosphate acyltransferase
GFP	green fluorescent protein
GVs	giant vesicles
LPAAT	lysophosphatidic acid acyltransferase
LUVs	large unilamellar vesicles
MLVs	multilamellar vesicles
MVVs	multivesicular vesicles

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NTP	nucleotide triphosphates
PCR	polymerase chain reaction
Phe	phenylalanine
PLPC	1-palmitoyl-2-lauryl-*sn*-glycero-3-phosphatidylcholine
PNPase	polynucleotide phosphorilase
Poly(A)	poly(adenilic acid)
Poly(Phe)	poly(phenylalanine)
poly(U)	poly(uridylic acid)
POPC	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine
POPE	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylethanolamine
POPG	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylglycerol
PURE system	protein synthesis using recombinant elements (PURE) system
RNA	ribonucleic acid
SLPC	1-stearoyl-2-lauril-*sn*-glycero-3-phosphatidylcholine
SOPC	1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine
T&T	transcription and translation
T7	promoter T7
T_m	lipid phase transition temperature
cryo-TEM	cryogenic transmission electron microscopy

Addendum

The PCR synthesis of DNA inside GVs, which also undergo growth-division after lipid precursor addition, has been published during the preparation of this article.[105]

Acknowledgements

This work derived from our recent involvement in studies on the construction of semi-synthetic minimal cells, funded by the Sixth Framework EU Program (SYNTHCELLS: Approaches to the Bioengineering of Synthetic Minimal Cells 043359), HFSP (RGP0033/2007–C), ASI (I/015/07/0), PRIN2008 (2008FY7RJ4); and further expanded thanks to networking initiatives as SynBioNT (UK), and the COST Systems Chemistry action (CM0703). T. P. Souza has been supported by the Alexander von Humboldt Foundation as a post-doctoral fellow. We thank Dr Frank Steiniger and Prof. Alfred Fahr (University of Jena, Germany) for technical and scientific collaboration at cryo-TEM imaging.

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Published on 04 October 2011. Downloaded by Michigan State University on 22/01/2016 04:19:48.

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