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Novel directions in molecular systems design: The case of light-transducing synthetic cells

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ABSTRACT

Important progresses have been achieved in the past years in the field of bottom-up synthetic biology, especially aiming at constructing cell-like systems based on lipid vesicles (liposomes) entrapping both biomolecules or synthetic compounds. These "synthetic cells" mimic the behaviour of biological cells but are constituted by a minimal number of components. One key aspect related to this research is the energetic needs of synthetic cells. Up to now, high-energy compounds have been given in order to drive biochemical reactions inside the vesicle lumen. In order to be autonomous, synthetic cells must produce their own biochemical energy from available energy sources. At this aim we started a long-term research program focused on the construction of photoautotrophic synthetic cells, starting with the reconstitution, in active and highly oriented form, of the photosynthetic reaction centre in giant lipid vesicles (Altamura et al., PNAS 2017, 114, 3837–3842). Here we comment this first milestone by showing the synthetic biology context wherein it is developed, the future steps, and the experimental approach that might allow such an achievement.

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Bottom-up SB and the construction of cell-like systems

When, in the early 1990s, pioneer research on the construction of autopoietic minimal cell-like systems was carried out at the ETH in Zurich, mainly thanks to the efforts of P. L. Luisi, P. Walde, and T. Oberholzer,[1-4] the term 'synthetic biology' (SB) was known only to the science historians who recalled the book "*La Biologie Synthétique*" by Stéphane Leduc (1853–1939).[5] Leduc's approach to the synthesis of life (based on inorganics and osmotic growths, resulting also in the so-called "chemical gardens") may appear naïve today, but it was based on a profound, fundamental, and still open question, *i.e.*, can we build a living cell from inanimate matter?

This important and still unsolved question, has been addressed again and again in the history of science, as done by the above-mentioned Swiss team more than 25 years ago. In particular, their pioneering approach was based on the increased knowledge of biological systems, and, importantly, developed under the elegant theoretical framework of the autopoiesis.[6-8] Today, this research has become a rich and fecund research program of SB, the modern biology branch born in the 2000s by applying engineering principles to biology.[9,10] In particular, the question is addressed within the bottom-up SB approach (Fig. 1a).

The core idea is that it should be possible to build synthetic cells starting from biological molecular parts (lipids, proteins, nucleic acids, …), and even from non-biological ones, like carrier polymers[11] or functionalised carbon nanotubes,[12] metal nanoparticles exhibiting catalytic activity,[13] fluorescent compounds for diagnostic or analytic purposes.[14] By combining both these different kind of constituents, it would be possible to form hybrid structures designed for performing specific tasks or suitable for mimicking complex cellular behaviours in agreement with the constructive-knowledge paradigm (this paradigm can be summarized by the famous Feynman's sentence: "What I cannot create, I do not understand"). In order to construct synthetic cells, self- and/or guided-multimolecular assembly should be mastered. The

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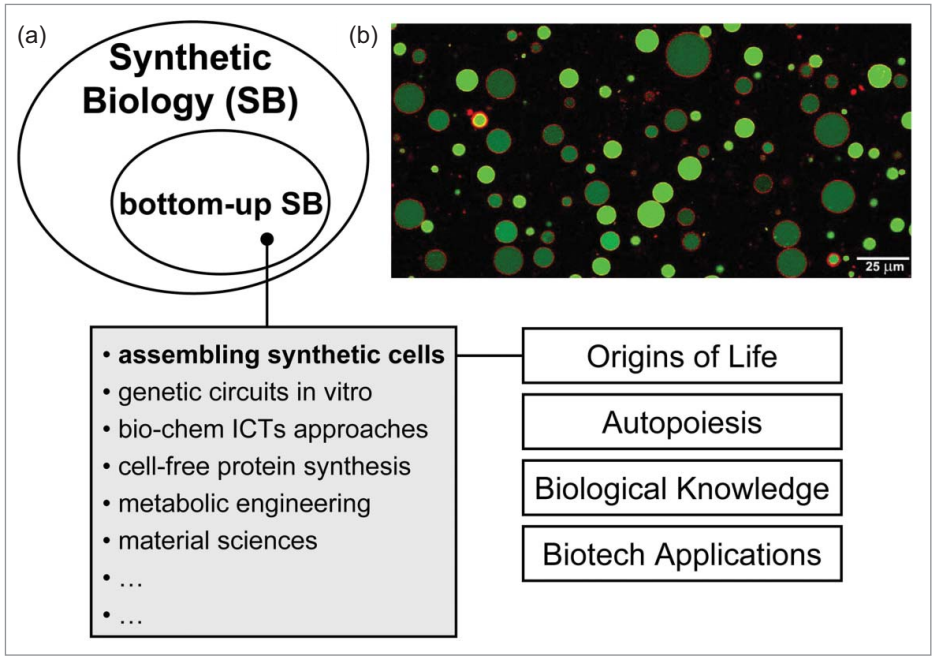

Figure 1. Bottom-up approach to synthetic biology. (a) bottom-up approaches in SB, also called cell-free SB approaches, or "chemical" synthetic biology foresee the investigation on a number of topics, among which the assembling synthetic cells from separated components is a major one. In particular, this kind of synthetic cells should be seen as opposed to the alternative practice based on the introduction of a synthetic chromosome in a living cell deprived of its own native genetic material.[49] (b) Giant lipid vesicles are often used for constructing cell-like systems. The picture shows calcein-filled vesicles whose membranes have been stained by Trypan Blue. Reproduced from;[50] an open access publication distributed according to the CC-BY license.

long-term goal of this research is the construction of a *living* and *minimal* synthetic cell. It might look as a sort of Faustian dream, but actually is strongly connected with cutting-edge biotechnology.

Clearly, the construction of synthetic cells is very challenging, but it has become evident that a lot of knowledge can be extracted even by the exploration of intermediate steps along this route. The construction of synthetic cells contributes in several ways to improve our scientific vision, and stimulate technical progresses. For example: (1) it helps understanding the self-assembly and self-organization mechanisms that led to the onset of early life; (2) it represents a practical way to implement the theory of autopoiesis in the chemical/biochemical domain; (3) it provides a set of tools for generating knowledge according to the "understanding-by-building" Feynman's paradigm; (4) it contributes to develop novel biotechnological tools based on cell-like artificial systems, for bioassays, diagnostics, nanomedicine, and more; (5) it is a simplified matrix for rigorous quantitative models and mechanistic enquiring of molecular systems (whose study in living cells is hampered by the presence of 'noisy' background processes).

Today, the efforts toward the construction of synthetic cells in the laboratory are based on the convergence and integration of four elements: liposome technology,[15] cell-free systems,[16,17] microfluidics,[18] and modelling.[19-22] Most of previous research, reviewed in refs. 23–25 were focused on investigating the formation and the properties of microcompartments (mainly lipid vesicles, or liposomes) and on studying reactions inside the liposome aqueous lumen. Giant vesicles (GVs, Fig. 1b) have been often used because of their size ($> 1\ \mu$m), which allows direct visualization by light microscopy. For its theoretical and practical importance, protein synthesis inside GVs has been widely explored and can now be considered generally well understood. Coupled transcription-translation systems can be encapsulated inside the vesicle lumen, so to synthesize water-soluble proteins from their DNA sequences. The resulting functionalised vesicles perform specific functions embodied in the synthesized proteins and in their activity.

The need of implementing an energy-production module in synthetic cells

In this rapidly evolving and challenging scenario, we recently reported a first step toward the construction of a molecular device capable of producing chemical energy (i.e., ATP) inside synthetic cells.[26] Our work has been innovative both for being a novel and perhaps decisive topic in synthetic cell research, and for the employed methods/achieved results. In particular, we have

reconstituted the *Rhodobacter sphaeroides* photosynthetic reaction centre (RC) in the membrane of GVs by a novel methodology allowing the physiological orientation (ca. 91%) of RC in the lipid membrane. In this procedure RC retains its photo-activity, being able to produce, under red-light illumination, a proton gradient across the GVs membrane (ca. 0.061 pH units per minute). Moreover, it maintains its functionality for at least one day.

The work represents the first step of a more elaborated and ambitious goal, which we have designed and that is currently under scrutiny in our laboratory (for preliminary reports, see refs. 27, 28). The system is composed by three integral membrane proteins/complexes (see Fig. 2a):

(a) the above mentioned RC, which transduces light energy in chemical energy (a proton gradient, alkaline inside the synthetic cell), producing a redox couple (oxidised cytochrome c_2 and quinol);
(b) the cytochrome bc_1 complex, which, starting from the redox couple generated by RC, reverts it back to the original compounds (reduced cytochrome c_2 and quinone), further strengthening the proton gradient in the same direction;
(c) the ATP synthase, which exploits the proton gradient generated by the light-driven RC/cytochrome bc_1 cycle, and produces ATP inside the synthetic cell, from ADP and inorganic phosphate.

(Note that by quinone/quinol we refer to ubiquinone/ubiquinol or to their mimics, like decylubiquinone/decylubiquinol or similar compounds).

Figure. 2b shows the system we have in mind. It consists in a physiological-like arrangement of these three proteins in GVs with the explicit goal of producing ATP inside the vesicles after actinic irradiation. Such ATP molecules would then fuel other biochemical processes occurring inside each single vesicle, thus contributing to construct an autonomous synthetic cell. It is important to recall that in order to achieve this goal, it is necessary to control in a detailed manner the insertion of these three proteins in the lipid bilayer. Several open questions are related to the successful realization of this multi-component system, which includes the orientation and the concerted activity of the three proteins. Moreover, it is of practical relevance the development of innovative and general experimental methods for its realization.

But there is also an important conceptual implication related to this project. A system capable of producing compartmentalized ATP under illumination, and use the *in situ* produced ATP for fuelling other reactions, is a light-driven far-from-equilibrium system. Complex patterns like the onset of metabolic cycles, the generation of genetic information, sustained protein coding, genetic oscillations, etc. are possible, ultimately, from this very peculiar thermodynamic state, which is typical of dissipative structures, as insightfully evidenced by Ilya Prigogine in his fundamental works on non-equilibrium thermodynamics.[29,30]

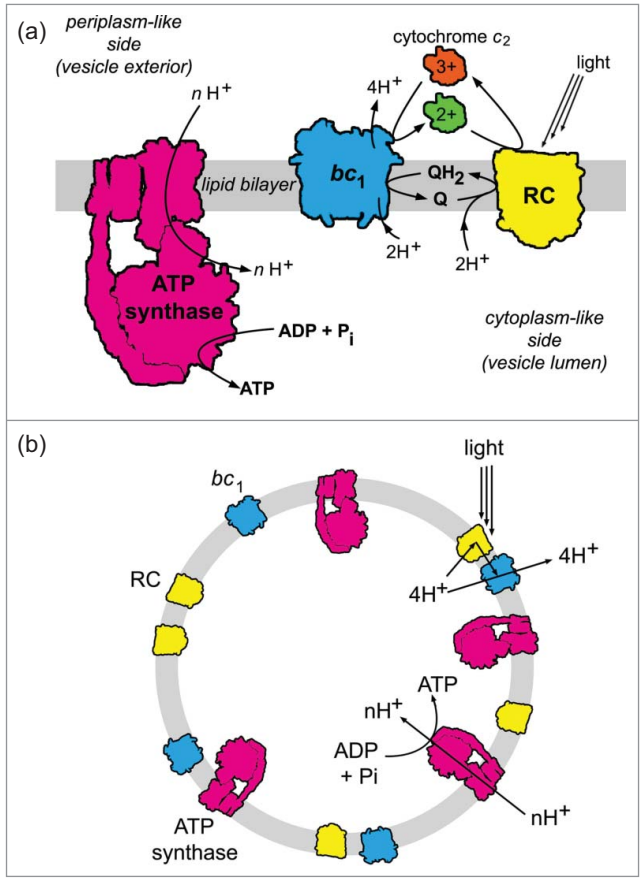

Figure 2. Light-transducing systems. (a) Expected location and orientation of the photosynthetic reaction centre (RC), cytochrome bc_1 complex (bc_1), and ATP synthase across the lipid bilayer (grey region). The reactions catalysed by these proteins are shown, based on current biochemical knowledge. In particular, RC converts – under irradiation – the quinone Q to quinol QH_2, abstracting two protons from the cytoplasm-like side and oxidising cytochrome c_2. In contrary, bc_1 catalyses the opposite reactions and further contributes to the proton gradient, which is acidic outside. ATP synthase exploits the proton gradient (dissipating it; note that n = number of c subunits of ATP synthase/3[51]) and catalyses the formation of ATP. (b) Ideal arrangement of RC, bc_1, and ATP synthase in an artificial vesicle, in order to function as transductor of light into *intra-vesicle* chemical energy, via generation of a proton gradient (acid outside). Such a system is currently under investigation in our laboratory. Note that although previous work has reconstituted ATP synthase in vesicles, the protein orientation is opposite, and produces ATP outside vesicles, with no utility for the compartment.

The droplet transfer method

Central to our approach is the so-called 'droplet transfer' method for the production of GVs. This method,

sometimes called 'emulsion inversion', was reported by Weitz and collaborators in 2003,[31] as well as by others,[32,33] originally aiming at the formation of asymmetric vesicles.[34] The method consists in the transformation of water-in-oil (w/o) droplets, coated by a lipid monolayer, into vesicles (Fig. 3). This is possible if the w/o droplets acquire a second lipid monolayer during transfer to an aqueous phase, so to form a bilayer. The lipid coating of the w/o droplets will constitute the inner membrane leaflet, whereas the lipids added during the w/o droplet transfer will constitute the outer membrane leaflet. Details of the method are described in the figure caption and elsewhere.[35] The important point is that it is quite easy to form solute-filled w/o droplets just by emulsifying a small volume of an aqueous solution in a lipid oil solution (lipids are necessary to stabilize the w/o droplet interface). The successive transfer of the w/o droplets in water occurs with variable efficiency, but it is generally possible to obtain solute-filled GVs quite easily. For this reason the droplet transfer method has been employed in bottom-up SB for constructing cell-like systems.

Noireaux and Libchaber used this method for expressing a protein inside GVs for a prolonged period of four days[36] (this was possible because of α-haemolysin pores, allowing nutrients enter the vesicles). Yomo and collaborators largely employed this method for assessing several important physico-chemical facets of transcription-translation reactions inside vesicles, often analysed by flow-cytometry.[37-39] Our optimisation[40,41] involves the use of a sugar density gradient (originally introduced by Hamada and coworkers, (see ref. 42 for details) for facilitating the droplet transfer, and the extension of the method to mixed phospholipid/fatty acid mixed GVs[43]], and the encapsulation of water-soluble compounds, included the transcription-translation system.[42-44]

However, the real novelty that we intend to comment here is the extension of the droplet transfer method to reconstitute membrane proteins (whereas the above

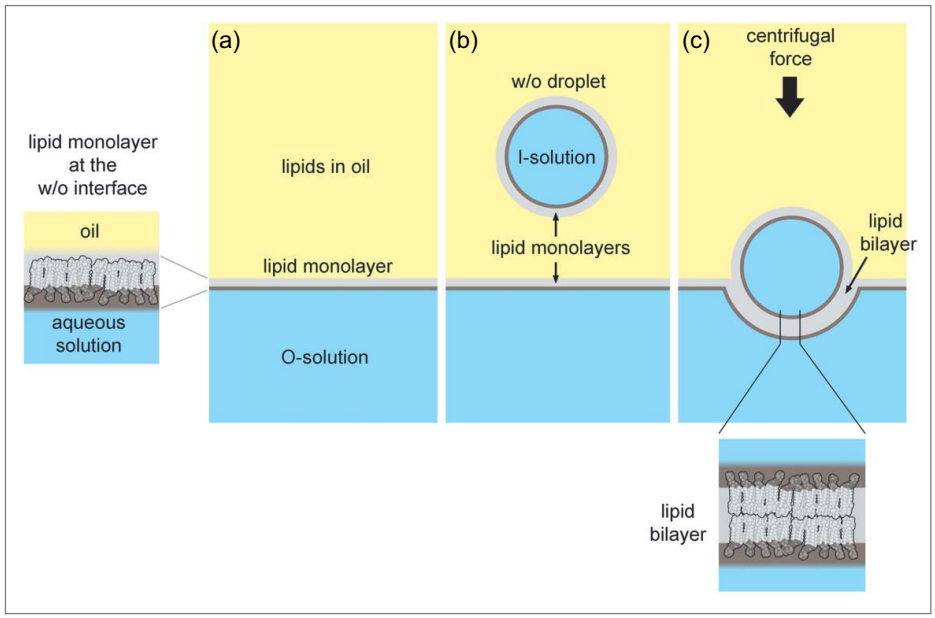

Figure 3. Preparation of GVs by the droplet transfer method. (a) A lipid-in-oil solution is gently stratified over an "outer" aqueous solution (O-solution) which will constitute the external phase of GVs. The lipid molecules will organize in form of a monolayer at the water/oil interface (w/o interface) as schematically shown in the zoom on the left. (b) In another vial, a small amount of "inner" aqueous solution (I-solution) is emulsified in a lipid-containing oil solution, so that millions of water-in-oil droplets (w/o droplets) are obtained. The resulting emulsion is gently poured over the system prepared in (a). For the sake of simplicity, only one w/o droplet is shown. It can be seen that the w/o interface and w/o droplet are coated (stabilized) by two oppositely oriented lipid monolayer, which could form a bilayer when in contact. (c) The system is then centrifuged so that the w/o droplets sediment at the w/o interface, and cross it, becoming covered by the second lipid monolayer, forming a lipid bilayer, as schematically shown in the zoom on the right. Consequently, w/o droplets become completely covered by a lipid bilayer when successful transferred in the O-solution (not shown). Note that w/o droplet sediment in the oil phase due to the density difference between oil and water, but in order to efficiently enter the aqueous phase, I-solution and O-solution, although isotonic, are prepared at two different densities (sucrose in I-solution, glucose in O-solution). Due to this density difference, GVs travel through the whole vial during centrifugation and are generally collected on the bottom of the vial. Note that, as the bilayer is stepwise constructed by juxtaposing two monolayers, these can be different, and this method can be adopted for the construction of *asymmetric* vesicles. The droplet transfer method is typically used to prepare solute-filled GVs as these kinds of solutes are easily entrapped in w/o droplets. The reconstitution of a membrane protein can occur – in principle – from the inside or from the outside (see text for details).

examples refer to the encapsulation of water-soluble compounds). As described in our work[26] and in a previous report,[47] the rationale is the following. The starting point of most membrane protein reconstitution procedure is a micellar solution, i.e., a solution made of a membrane protein surrounded by suitable micelle-forming surfactants (and possibly strongly bound lipids, residual from the extraction procedure). When applied to the droplet transfer, protein micelles are encapsulated inside w/o droplets aiming at the insertion of the protein in the vesicle bilayer. It is expected that micelles deliver the protein to the droplet monolayer or vesicle bilayer driven by hydrophobic interactions. However, the actual insertion of the protein is spontaneous and ultimately controlled by protein/lipids interactions. We reasoned that if a protein has an asymmetrical distribution of polar and non-polar regions, its insertion in the lipid monolayer or bilayer will follow a preferential route, leading to a selective orientation. This is well evident in the RC case, because this protein is composed by three subunits, two of which are hydrophobic and one hydrophilic. It is expected, therefore, that a vectorial insertion occurs, leading to high orientation (the polar side faces inside the vesicle).

Current observations do not allow to specify the exact moment of protein insertion. There are at least three working hypothesis, which might be called "early", "late" or "on the fly" insertion (Fig. 4). "Early" insertion foresees that the protein already inserts in the w/o droplet monolayer, with a specific orientation, guided by the nature of the water/lipid/oil interface, which is *per se* a strong vectorial microenvironment. The partially inserted proteins complete their insertion, spanning throughout the lipid bilayer, after the droplet transfer or while the droplet is moving across the oil/water interface. In contrary, "late" insertion would occur if the protein micelles insert in the vesicle bilayer, not in the w/o droplet monolayer. In other words, the reconstitution would occur only after the transformation of droplets into vesicles. The "on the fly" model would correspond to the insertion of a membrane protein in the nascent lipid bilayer *while* it is assembled (the lipid bilayer self-assembles in a zip-like mechanism[48]), namely, in the transient moment when the droplet is being transformed into a vesicle (Fig. 3, right side).

Elucidating these mechanistic aspects, and discovering the details of membrane protein reconstitution in GVs prepared by the droplet transfer method are some of the next questions to address. Another question focuses on testing the general applicability of this method for reconstituting the very different types of membrane proteins. Finally, in the case of membrane proteins that require an opposite orientation (the polar side facing outside

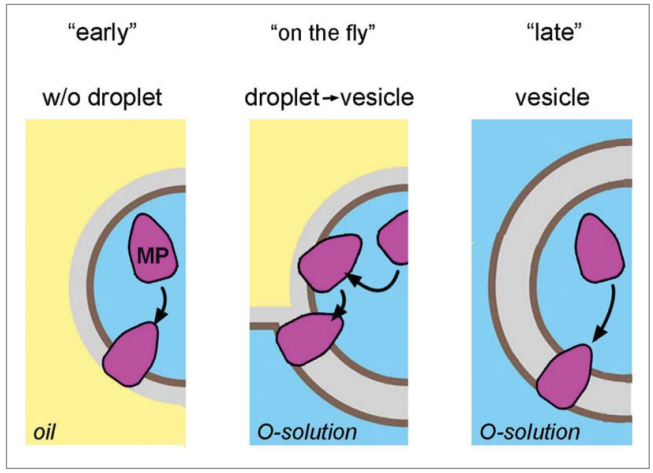

Figure 4. Hypothetical mechanisms of membrane protein insertion in the lipid monolayer or bilayer as resulting from the droplet transfer method. Membrane protein (MP) is shown in magenta, but for the sake of simplicity the surfactant layer around it (i.e., which stabilizes it and solubilizes it as a micelle) has not been drawn. "Early" insertion means that MP inserts (partially) in the lipid monolayer as soon as w/o droplets are prepared from an I-solution containing MP micelles. "On the fly" insertion means that MP inserts as soon as a lipid bilayer is being formed, i.e., in the exact moment of droplet-to-vesicle transformation, when the two oppositely facing lipid monolayers juxtapose to each other like in a zip mechanism. "Late" insertion means that MP micelles insert into the lipid bilayer only after it has been formed, i.e., in the vesicle membrane, from inside. See text for further details.

vesicles) it has been proposed,[28] but not yet tested, that the micellar solution of the protein of interest should be included in the O-solution and not in the I-solution (for explanation of these terms, see Fig. 3 caption). In this way, the insertion of a membrane protein from outside should be allowed, maybe according to some of the postulated mechanisms sketched above.

Concluding remarks

While the research on synthetic cell is well progressing, not much attention has been given, instead, neither to reaction occurring on the liposome membranes nor on the apparatus for chemical energy production from the resource present in the external surroundings. However membrane-bound proteins can exert this important biological function making synthetic cells able to self-sustenance by transducing light into chemical energy stored as high energy compounds. This represents a first step towards the implementation of real self-maintaining organisms, i.e. the artificial autopoiesis, and requires urgent and extensive investigations on such systems whose understanding will be functional to the future construction of synthetic cells.

Disclosure statement

No potential conflicts of interest were disclosed.

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