Extrinsic stochastic factors (solute partition) in gene expression inside lipid vesicles and lipid-stabilized water-in-oil droplets: a review

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Abstract

The encapsulation of transcription–translation (TX–TL) machinery inside lipid vesicles and water-in-oil droplets leads to the construction of cytomimetic systems (often called 'synthetic cells') for synthetic biology and origins-of-life research. A number of recent reports have shown that protein synthesis inside these microcompartments is highly diverse in terms of rate and amount of synthesized protein. Here, we discuss the role of extrinsic stochastic effects (i.e. solute partition phenomena) as relevant factors contributing to this pattern. We evidence and discuss cases where between-compartment diversity seems to exceed the expected theoretical values. The need of accurate determination of solute content inside individual vesicles or droplets is emphasized, aiming at validating or rejecting the predictions calculated from the standard fluctuations theory. At the same time, we promote the integration of experiments and stochastic modeling to reveal the details of solute encapsulation and intra-compartment reactions.

Key words: lipid vesicles (liposomes); water-in-oil droplets; cell-free protein synthesis; stochastic solute partition; synthetic cell

1. Introduction

Very probably, one of the most interesting research arenas in synthetic biology comprises the current attempts of assembling cell-like structures of non-trivial complexity. It was in the early 1990s that origins-of-life researchers started to fill liposomes and fatty acid vesicles with enzymes to construct primitive cell models (1–4). But few people recognized, at that time, the long-term potentiality of such approaches for basic science and biotechnology. Synthetic biology—as we intend it today—was not yet 'invented', and the art of combining complex *in vitro* enzymatic reactions with lipid compartments was probably considered an exotic exercise. Ten years later (ca. 2000s), however, the first collection of experimental results definitely started a field based on the combination of lipid microcompartments and cell-free systems (reviewed in Stano et al. (5)). The enterprise of '*Synthesizing Life*' from scratch by a synthetic or semi-synthetic approach had just begun (6).

Protein synthesis inside lipid vesicles is considered a fundamental technology for projects focused on the construction of the so-called minimal cells (7, 8). Enzyme reactions inside reverse micelles and liposomes are known since the 1970s (9–12),
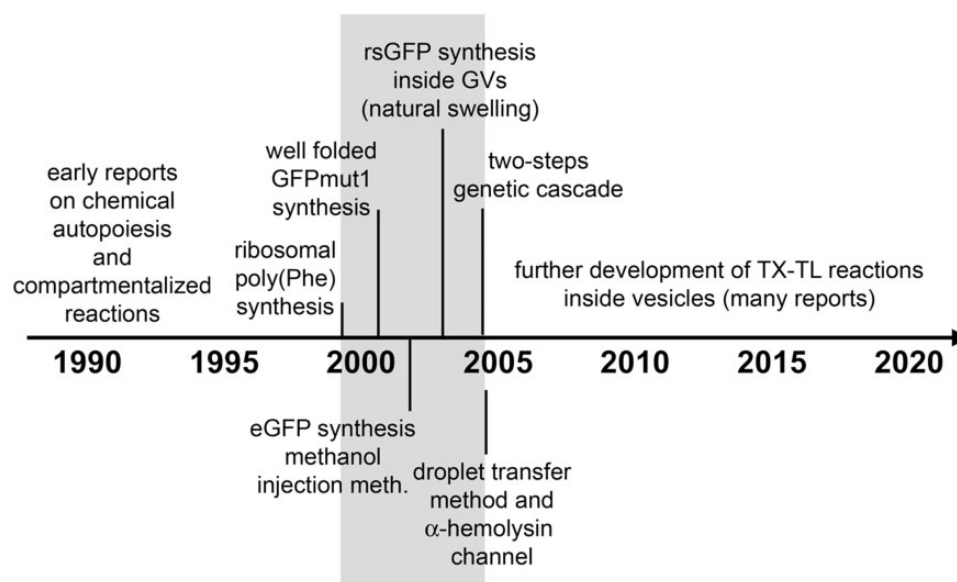
Figure 1. Schematic and simplified representation of the development of intra-vesicle reactions over time. 'Pioneer' phase (1989–1999); 'burst' phase (2001–2004); 'consolidation' phase (2005–today). Although most of the techniques for carrying out enzymatic reactions inside liposomes were known also before 1990s, key experiments specifically designed to construct cellular models were done in in the 'pioneer' phase, inspired by the theory of autopoiesis. The fundamental works on TX–TL reactions inside liposomes instead refer to the early 2000s (the 'burst' phase), in gray. Next, a 'consolidation' phase has followed, where gene expression inside liposomes (and other compartments as well) has been studied in great detail.

Table 1 Summary of early reactions in lipid- and fatty acid-vesicles (1991–1999)

Year	Achievement	Compartment	References
1991	Four-steps enzymatic lipid synthesis	Soybean PC vesicles	(3)
1994	PNPase-catalyzed ADP polymerization	'Static' DMPC vesicles or 'self-reproducing' oleic acid/oleate vesicles	(4, 13)
1995	Qβ-replicase-catalyzed RNA replication	'Self-reproducing' oleic acid/oleate vesicles	(14)
1995	PCR	'Static' POPC vesicles	(15)
1999	ribosomal poly(Phe) synthesis	'Static' POPC vesicles	(16)

More comments can be found in the Supplementary Data.

'Static' vesicles are those vesicles not undergoing any morphological transformation while internalized reactions occur. In contrary, 'self-reproducing' vesicles grow and divide upon the uptake of a membrane precursor (17) while, simultaneously, a reaction occurs in their aqueous lumen.

ADP, adenosine diphosphate; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine; PC, phosphatidylcholine; PCR, polymerase chain reaction; PNPase, polynucleotide phosphorylase; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine.

but when enzymes and nucleic acids were firstly encapsulated in lipid vesicles such a research on 'chemical autopoiesis' had quite a different flavor.

Figure 1 summarizes the time evolution of these studies. Three phases can be recognized: a 'pioneer' phase (1989–1999), when many relevant reactions were carried out inside fatty acid and phospholipid vesicles (summarized in Table 1), culminating with the report, in 1999, of the ribosomal poly(Phe) synthesis inside conventional phospholipid vesicles. These first studies were mainly due to the group of Pier Luigi Luisi at the ETH Zurich.

Next, it came a 'burst' phase (2001–2004), when major advancements in intra-liposomal protein synthesis have been reported. In particular, the first synthesis of a well-folded protein (a GFP mutant) was reported in 2001 (18), followed by a similar synthesis in sub-micron liposomes (19), and giant liposomes (20). The first two-steps genetic cascade based on TX–TL reaction was published in these years as well (21), and the prolongation of gene expression thanks to the intra-liposomal synthesis of a membrane pore (α-hemolysin) was another success (22). The latter work decisively innovated the field also thanks to the use of a novel method of giant liposome preparation (the droplet transfer method (23, 24), see below).

A 'consolidation' phase follows, dedicated to further developments, optimization, expansion, and full-characterization (2005–today). Protein synthesis inside microcompartments (e.g. liposomes, w/o droplets, polymersomes (25), coacervates (26)) has been studied, thus, at a great extent (5). Recent investigations have shown that not only soluble proteins but also membrane proteins can be produced inside liposomes (27–29). Performing DNA duplication (30), encapsulating responsive genetic networks (31), or producing therapeutic proteins (32) is now possible. Moreover, these sorts of 'synthetic cells' can communicate with bacteria (33, 34).

Next phase, probably, will foresee components 'standardization' and 'combination of modules' and will involve emerging technologies such as microfluidics, numerical modeling, genetic circuit design, leading to more complex bio-engineered synthetic cells. It should be recalled however, that up to now synthetic cells built by the bottom-up approach are not alive.

Why protein synthesis inside microcompartments? The production of a well-folded and thus active protein, being it an enzyme, a receptor, a transcription factor, a cytoskeleton element, or a transmembrane channel, allows a 'functionalization' and a progress toward cytomimetic, yet minimalistic, cell-like systems. It is therefore crucial a full understanding of it.

Protein synthesis inside lipid vesicles or lipid-stabilized water-in-oil (w/o) droplets is achieved by the co-encapsulation of all transcription–translation (TX–TL) components together with a DNA template. Home-made and commercial cellular extracts (mainly, but not always, from *Escherichia coli*), or fully reconstituted systems are used (35).

In this article, we would like to focus on a physical aspect referring to microcompartmentalized TX–TL reactions that has been only partially discussed so far. When a TX–TL 'kit', which is composed by several dozens of macromolecules and low-molecular weight (MW) compounds, is encapsulated inside microcompartments—whose dimension lies typically from 0.1 to 10 μm—the amount of synthesized protein (or synthesis rate) shows a quite high between-compartments variability, which can be measured from the between-compartment coefficient of variation (CV = standard deviation/mean). In some cases, such a 'diversity' appears to be too high when compared with the expectations, i.e. the diversity due to the expected stochastic partition of TX–TL components inside the compartments. Although many authors have reported this phenomenon, there is not yet a clear interpretation or explanation for it.

Here, we will look at published experimental evidences and interpret them as derived from the solute partition effects, also called 'extrinsic' stochastic effects. These have to be distinguished from the 'intrinsic' ones, which refer to the stochastic course of chemical reactions. Both effects are very relevant for any microcompartmentalized reactive system.

Extrinsic stochastic effects, thus, refer to stochastic solute partition, and define the composition of the reacting system. The number and the type of solutes contained inside the microcompartment vary according to random encapsulation events. In contrary, intrinsic stochastic effects depends on the discrete nature of molecules and on the intrinsic randomness of reacting events. Stochastic reactions can be simulated *in silico* by considering the number of molecules of a reacting system (e.g. the aqueous vesicle core), and calculating the probability that any possible reaction takes place accordingly (this is technically possible by introducing the so-called Master Equation, and solve it by Monte Carlo methods (36)). By solving the Master Equation, the average time evolution of the reacting system is obtained, *along with* the displacements from the average pattern due to random fluctuations that bring the system toward regimes unpredictable by the deterministic approach (37).

We will recapitulate and discuss extrinsic stochastic effects for TX–TL reactions in lipid vesicles [especially in giant ones, giant vesicle (GVs)] and w/o droplets. Our goal is to explicitly recognize the role of stochastic solute partition to shape the protein synthesis pattern in synthetic cell populations. We will first introduce an elementary discussion on solute encapsulation, and then present a collection of published data with detailed comments. This survey will let us discuss, although not in a conclusive way, the role of solute partition effects. Numerical stochastic modeling will be also presented, highlighting its usefulness when a careful analysis of microcompartment populations is under scrutiny; a significant example is included. We will shortly mention the option of producing solute-filled microcompartments by microfluidic devices in order to reduce the solute partition effects. How these approaches compare with the use of cell-like models in origins-of-life scenarios is also commented, because origins-of-life research shares with synthetic biology the so-called 'constructive paradigm' (38, 39).

Finally, a clarification on terminology used in this article is needed. We will use terms like 'between-compartments' or 'between-vesicles' or 'between-droplets' *variability* or *diversity* (40) to refer qualitatively to the central idea discussed in this article, i.e. the difference between individual compartments in terms of solute content and/or protein synthesis. On the other hand, when a quantitative measure of this variability is available, we will report it by means of standard statistical metrics (variance, standard deviation, and CV).

2. The stochastic nature of solute encapsulation

Working with cell-like microcompartments generated by batch methods frequently reveals that the solute content of such structures, even of equal size, differs in evident way within a population. The most intriguing fact is not the variability *per se*, which is somehow expected, but its quite large magnitude and, in some cases, the shape of the frequency distribution.

2.1 Single solute

Let us consider the formation of a vesicle in an aqueous solution by the simple lipid swelling (Figure 2a). The unperturbed hydration of lipid films stratified on the surface of the container gives GVs. Water-in-oil droplets are generated by emulsifying a small aqueous drop in an apolar solvent (Figure 2b). The aqueous drop can be fragmented into millions of microdroplets by shearing forces (stirring, pipetting, vortexing).

In both the cases, the simplest assumption is that the compartments capture (i.e. sample) the aqueous phase in the moment of their formation. Here, the 'null hypothesis' H_0 says that, despite the expected between-compartments variability, the average concentration of internalized solutes should corresponds to their bulk concentration in the 'mother' solution, i.e. $H_0: <c_k> = c_0$ (c_k is the solute molar concentration inside the k-th compartment, $<...>$ denotes the average value, and c_0 is the bulk molar concentration). Clearly, H_0 does not apply to special cases where strong forces exist between solutes and lipids (anionic DNA and cationic lipids, for instance).

In a scenario ruled by H_0, it is possible to calculate the deviations from the average by the fluctuation theory. The random encapsulation of a solute, whose bulk molar concentration is c_0, in a compartment of volume V, is expected to lead to an average number of entrapped solute molecules n_0 given by Equation (1).

$$n_0 = N_A c_0 V \tag{1}$$

N_A being the Avogadro number. Most compartments should contain n_0 solutes, but others will contain a different number n. It is then assumed that compartments containing n solute molecules, when n_0 is expected, occur with a frequency proportional to a probability function $p(n)$ – for example the Poisson distribution when n_0 is small (Equation 2)

$$p(n) = \frac{n_0^n}{n!} e^{-n_0} \tag{2}$$

or the normal distribution when n_0 is large. Let us postpone the discussion on the *shape* of the solute occupancy distribution to a short mention in Section 5 and focus instead on the order of magnitude of the fluctuations around n_0.

Because n_0 originates from random events, the fluctuations around n_0 are expected to be proportional to the square root of n_0 ($\delta n_0 \sim \sqrt{n_0}$), and therefore the CV should scale as $\delta n_0/n_0 = 1/\sqrt{n_0}$. Table 2 reports the values of $n_0 \pm \delta n_0$, and the corresponding CV
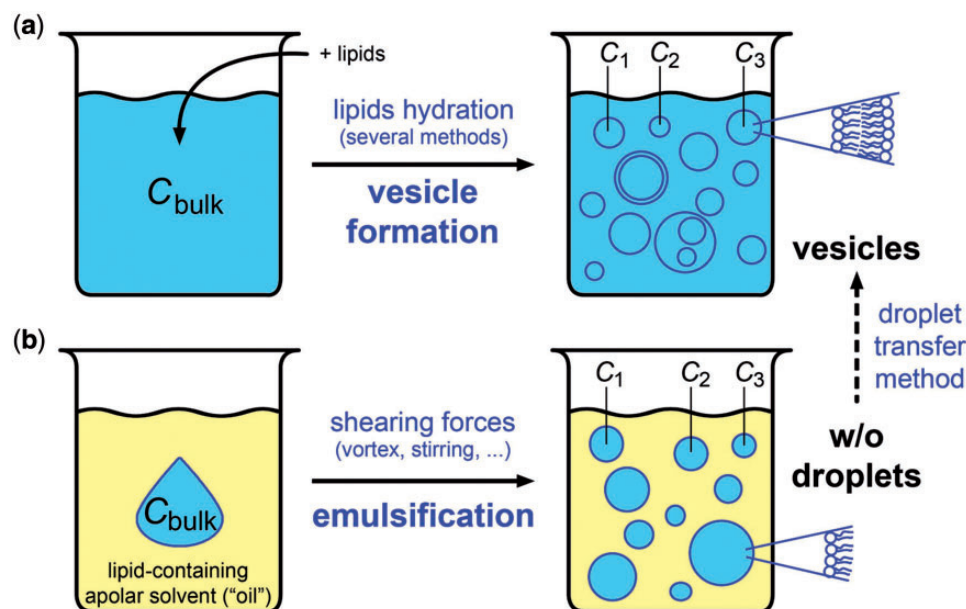
Figure 2. Schematic representation of bulk preparation methods of vesicles or w/o droplets. (a) An aqueous solution is employed to hydrate lipids, for example by the lipid film hydration method, allowing the lipid self-assembly in form of vesicles. Vesicles of various size and morphology are obtained. Each vesicle encapsulates part of the aqueous solution. If we denote with c_{bulk} (or C_{bulk}) the concentration of one (or more) solutes, each vesicle will have a specific intra-vesicle concentration $c_1, c_2, c_3, \dots$ ($C_1, C_2, C_3, \dots$). (b) The preparation of w/o droplets consists in the emulsification by application of shearing forces of an aqueous solution drop in an apolar solvent that contains lipids. As in happens in the case of vesicles, each w/o droplet has a specific internal concentration. Notably, w/o droplets can be converted into vesicles by the droplet transfer method. The drawings are not to scale. The blue boundary around the microcompartments represents a lipid bilayer (a) or a monolayer (b).

Table 2. Solute encapsulation inside a spherical compartment (droplet/vesicle) of a given diameter, formed from an aqueous solution of the indicated bulk concentration

Diameter (µm)	Volume (fL)	Bulk solute concentration (c_{bulk})			
		1 nM	10 nM	100 nM	1 µM
2	4.2	2.5 ± 1.6 (60%)	25 ± 5 (20%)	250 ± 16 (6%)	2500 ± 50 (2%)
5	∼65	39 ± 6 (20%)	390 ± 20 (5%)	3900 ± 63 (2%)	39 000 ± 200 (0.5%)
10	∼520	315 ± 18 (6%)	3150 ± 56 (2%)	31 500 ± 180 (0.6%)	315 000 ± 560 (0.2%)
20	∼4, 200	2500 ± 50 (2%)	25 000 ± 160 (0.6%)	250 000 ± 502 (0.2%)	2 500 000 ± 1600 (0.06%)

The values in the table refer to the expected mean number of solute molecules (n_0) ± standard deviation ($\delta n_0 = \sqrt{n_0}$). The percent coefficient of variation (CV = $100 \times \delta n_0/n_0$) is given in the square brackets. Note that, given a fixed volume V, the CV of solute concentration is equal to the CV of the number of captured solutes.

in the square brackets, for combinations of c_0 and V typically encountered when working on GVs and w/o droplets.

It can be seen that relevant stochastic fluctuations are expected only in the top-left corner of Table 2, corresponding to small compartment volumes *and* low solute concentrations. In all other cases, namely when the compartment diameter is ≥5 µm and the solute concentration is ≥10 nM, and also in cases of large volumes and small concentrations, or vice versa, the expected CV is experimentally negligible or around 5%—a figure that compares with the typically accepted variability in experimental sciences.

When contrasted with the expectations in Table 2, experiments with GVs and w/o droplets often show larger between-compartment variability. This can be observed even when a single compound is encapsulated. The mean concentration might still correspond to the expectations (H_0: $< c_k > = c_0$), but abnormally high variances have been sometimes measured, and the experimentally determined solute occupancy distributions might differ from the Poissonian or Gaussian ones (41, 42) (see Section 5). Clearly, solute encapsulation depends on the

mechanism of compartment formation, which in turn is determined by *local conditions* affecting each individual compartment. 'Sampling' the solution with very small 'containers', i.e. the vesicles/droplets, helps to reveal the microscopic granular nature of any solution. But the central question is whether the between-compartment variability lies within the realm of stochastic expectations or not. In this second case, this phenomenon can become an interesting topic for further investigation in order to reveal its generative mechanisms. Moreover, if fully understood, it might lead to technological processes where solute-rich compartments are formed for specific purposes.

2.2 Multiple solutes (TX–TL kits)

In the context of protein synthesis, it should be firstly considered that a TX–TL system requires several molecules to function. Minimal kits, assembled *in vitro* from purified components, allow us to estimate the minimal number of components required for synthesizing a protein from a DNA template. The PURE system (35) contains about 80 macromolecules,

including RNA polymerase, tRNAs, energy recycling enzymes and ribosomes (the ribosome is considered as an individual macromolecule), and about two dozens of low-MW compounds (amino acids, nucleotides, etc.), leading to about 100 different solutes.

The concentration range of these solutes is the following: (i) the DNA (or RNA) template is generally present at low concentration (10 nM and below), (ii) the concentrations of other macromolecules are in the 10 nM–1 μM range (35, 43–45), and (iii) low-MW compounds, such as amino acids, nucleotides, and other chemicals have concentrations in the mM range. The latter do not pose any issue about stochastic partition, even when encapsulated inside very small compartments. On the contrary, macromolecules such as DNA, tRNAs, enzymes, ribosomes have concentrations in the μM range and below. From Table 2, we learn that large random fluctuations are expected when 1 μM solutes are captured inside submicron compartments (diameter < 1 μm), or when 10 nM are captured in smallest micrometer-sized compartments (diameter ∼ 2 μm). Therefore, if we focus on GVs and w/o droplets (discarding for the moment sub-micrometer compartments), it results that the stochastic partitioning of DNA template is expected to be the main source of between-compartment diversity.

It should be noted that working at low DNA concentration is a way of achieving $n_0 < 1$ in Equation (1), so that most of compartments have either $n = 0$ or $n = 1$, and only few have $n \geq 2$, according to a Poissonian distribution, Equation (2). Such a strategy allows running experiments with single copies of template. In these conditions, a rigorous approach to the numerical modeling of intra-compartment reactions must take into account also intrinsic stochasticity (46, 47), but this is outside the scope of this article.

The problem of stochastic entrapment of TX–TL kits leads to three considerations. First, the correct multi-solute entrapment model should be identified and evaluated. In the simplest model, the entrapment of each solute is considered independent from the entrapment of other solutes. This gives an overall entrapment probability that is simply the product of j independent probabilities (44, 45, 48), i.e.

$$P = \prod_j p_j \qquad \text{(Equation 3)}$$

where P is the overall co-entrapment probability and p_j is the entrapment probability of the jth species. Such an approach brings about to easy-to-compute P-values, but does not consider interaction between solutes. Despite its simplicity this model turns to be useful because it is a benchmark for experiments versus prediction comparisons (44, 45).

A detailed experimental verification of Equation (3) is very complicated. To our best knowledge, no attempts have been done to quantify the entrapment of each solute of a TX–TL mixture, or even of mixtures of a dozen of solutes. Such a quantitation would allow the determination, for each compartment, of the solute concentration vector $C_k = \{c_{1k}, c_{2k}, \ldots c_{jk}\}$, where c_{1k}, $c_{2k}, \ldots c_{jk}$ are the concentration of the 1st, 2nd, ... jth TX–TL component inside the kth compartment. The future development of techniques for determining C_k is of great importance in the field. Single-cell proteomics methods (49) could be perhaps applied to face this issue in the near future. The use of multiple macromolecular fluorescent probes, each emitting in a specific spectral region, could be another way to gather information on the statistics of co-encapsulation (for example by confocal microscopy, flow cytometry, fluorescence correlation spectroscopy).

Because the TX–TL reactions produce a protein, which can be easily quantified if fluorescent, the production rate or the amount of synthesized protein can be used as a proxy for C_k. Measuring how much, or how fast, protein is synthesized inside a population of compartments can reveal how compartments have encapsulated the TX–TL components (mean value, spread of the distribution, extreme values, and so on).

This leads us to discuss the second problem related to microcompartmentation of TX–TL reactions. It originates from the non-linearity of TX–TL mechanism. Can the between-compartment variability of protein production be comfortably used as a proxy for C_k variability? The problem is that protein synthesis in an observable that is linked to C_k by a complex non-linear relationship.

Fortunately, for practical purposes it is often possible to reduce the TX–TL mechanism to only one or few rate-limiting step(s) that determine the whole process. For the sake of exemplification, consider a Michaelis–Menten-like kinetic expression with Hill exponent h ($h \geq 1$). It can be applied to model the protein synthesis rate r:

$$r = k[\text{Enz}] \frac{[\text{sub}]^h}{K^h + [\text{sub}]^h} \qquad (4)$$

Let us imagine that this rate law refers to the rate-limiting step of the TX–TL reactions (e.g. translation), and that 'Sub' and 'Enz' are both macromolecules whose intra-compartment concentration fluctuates according to the values of Table 2.

For example, if the CV of [Enz] and [Sub] is 2%, the CV of r becomes 3% when $h = 1$, and 6% when $h = 4$ (these calculations have been carried out considering [Sub] $\approx K$; if [Sub] = 10 K the two values become 2% and 2%, respectively, whereas if [Sub] = 0.1 K, they are 4% and 10%, respectively; see Supplementary Figure S1).

When two or more terms of this type are combined (i.e. when it is not possible to define a single rate determining step), an increase of the rate CV is expected, but, depending on the numerical values of the parameters, such an increase can still lie within the order of magnitude of reactants concentration CV.

Therefore, when indirectly measured from the rate of protein synthesis or from the amount of synthesized protein, the between-compartment CV can be broader than the CV of C_k, due to the underlying non-linear complex mechanism, but with a certain confidence it can be assessed that rarely it can become one order of magnitude larger. This statement is not general; it is valid when extreme cases are excluded, namely, those with small V and low c_0 (Table 2). Therefore the question is complicated because one should know which reactants mainly contribute to the rate-limiting step(s), and this in turn can depend on the molecular details (e.g. the nature of the promoter, the type of polymerase, the degree of 'coupling' between TX and TL, the effective concentration of active species, the environmental tuning of reaction rates, and so on).

The third consideration focuses on a more fundamental theme, namely, the effect of confinement on TX–TL reactions. When protein synthesis is used as a proxy for C_k, it should be considered the possibility that the course of TX–TL reaction inside the microcompartments might differ from the bulk reaction due to confinement effects. Protein synthesis might vary in unexpected manner depending on the nature of the lipid boundary, compartment size, concentration, and ratio among
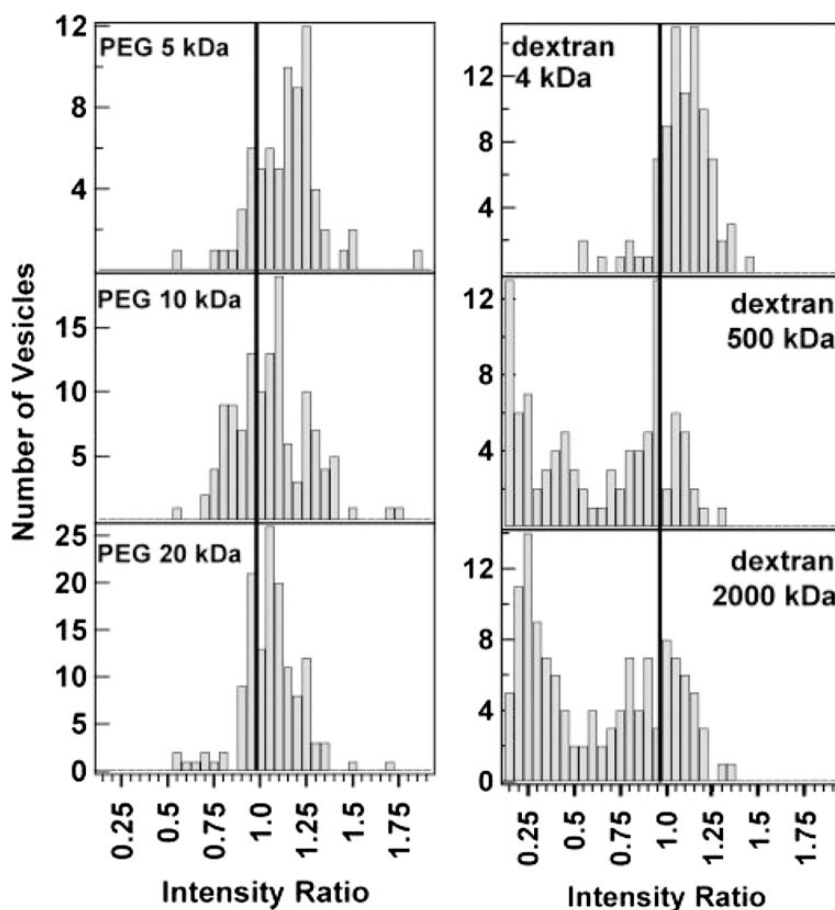
Figure 3. Encapsulation of individual solutes inside GVs. Solute occupancy distribution of fluorescently-labelled PEG (5–20 kDa) or dextran (4–2000 kDa) inside GVs. The vertical line indicates that intra-vesicle concentration is equal to expected bulk concentration. Reprinted with permission from (57). ©2007 American Chemical Society.

the solutes (i.e. C_k itself), crowding and depletion forces (50–56). All these effects can vary non-linearly with the solutes concentration. TX–TL components could not function (or function with low efficiency) due to full (or partial) denaturation, which in turn might depend, locally, on protein/lipid interactions and protein–protein ones.

It is easy to grasp that it is very difficult to disentangle the partition effects from the confinement ones. Paradoxically, in order to experimentally verify possible confinement effects, the TX–TL system must be microcompartmentalized, but, consequently, stochastic partition effects will produce unknown C_k. As mentioned above, experimental techniques for measuring C_k can help considerably.

By keeping in mind that available experimental data deal primarily with the end product of TX–TL reaction, and not with C_k, we can ask: what are the experimental evidences of between-compartment diversity? Is the measured variance explainable on the basis of expected stochastic partition or not? If not, is this due to a sort of amplification of C_k diversity due to the mechanism of protein synthesis, or due to larger-than-expected variance of solute partition? In the latter event, what additional mechanisms contribute to it?

This review will not give definitive answers to these questions. Rather, it aims to highlight these aspects and to stimulate further studies.

3. A survey of published results

In this section, we are going to comment on published experimental results reporting between-compartment variability in solute encapsulation or protein production. Our focus is the discussion of this variability within a population of compartment of a given experiment, whereas it is difficult to compare the between-laboratory variability. The latter depends on many experimental variables whose discussion lies outside the scope of this article.

3.1. Single solute encapsulation inside GVs prepared by the film hydration method

It is interesting to comment on the formation of large GVs, by the thin lipid film gentle hydration, in the presence of aqueous phases containing carboxyfluorescein, Alexa-Fluor-488-PEG (from 5 to 20 kDa) and FITC-dextran (from 4 to 2000 kDa) (57). It should be noted that the 'natural swelling' of thin lipid films, especially when neutral (zwitterionic) lipids are employed, gives best results at low ionic strength (58). The typical GVs diameter is ca. 20 μm, while the solute bulk concentration ranges from 0.1 to 100 μM, and from Figure 3, CV is ∼15% (but the theoretical CV is around and below 0.2%). Similar conclusions are obtained

when large GVs (>20 μm) are formed by electroswelling in the presence of 0.5 μM FITC-dextran (70 kDa) (59).

On the other hand, when 350 nm polystyrene latex nano-beads or 32 000 kDa λDNA are encapsulated at very low concentration (c_0 = 4–35 pM) inside 2.7–7.3 μm GVs prepared by the film hydration method (and droplet transfer method as well), the observed CV, obtained from flow cytometry, fits with Poissonian expectations (60).

3.2. TX–TL reactions inside GVs prepared by different methods

In many of the published reports, the issue of large between-vesicles variance is explicitly shown or at least mentioned. It is convenient to distinguish among GVs prepared by swelling methods (either starting from lipid films or from freeze-dried vesicles) and those based on the droplet transfer method (22–24, 61, 62).

Swelling methods. By using giant multi-vesicular vesicles, whose typical size is 1–100 fL (diameter 1.2–12.4 μm), it was shown by flow cytometry that the diversity among protein production inside GVs, spans of about 1–1.5 orders of magnitude (44, 63). In these studies, c_{DNA} = 0.021, 0.21, and 2.1 nM (Figure 4a).

GVs can be also prepared by the gentle swelling of lipids stratified over glass beads (64). High variability among vesicles (CV ~ 50-80%) has been observed by confocal microscopy (Figure 4b). In a follow-up study, the correlation between GVs size, DNA content, and produced protein was investigated. The distribution of DNA concentration inside vesicles was characterized (CV ~ 30%), resulting in agreement with a Poissonian DNA partition (c_{DNA} = 1.34 nM, typical vesicle diameter 4 μm). But the yield of synthesized protein (CFP) was poorly correlated to the intra-vesicle gene concentration (and to vesicle size as well), suggesting that the stochastic partition of other TX–TL components plays a role in determining the protein synthesis, and that between-vesicle diversity does not seem to decrease when vesicle size increases (opposite to the theoretical expectations). Similarly, when mRNA and produced protein (YFP) were simultaneously determined for intra-GVs TX–TL reactions (c_{DNA} 30 nM, typical vesicle diameter 4 μm), high variability of these two concentrations was found (~1 order of magnitude for mRNA, and 2 orders of magnitude for protein), and almost no correlation between these two concentrations (68).

Droplet transfer method. This novel GVs production method is successfully applied to the encapsulation of physiological solutions of macromolecules, and it is therefore especially useful for constructing cytomimetic systems. The seminal paper from (22) has shown the potentiality of the method, whose usage is growing constantly (for a review, see (58)). The method consists in the transfer of previously formed lipid-stabilized w/o droplets from the 'oil' phase to an aqueous phase, so obtaining GVs (Figure 5). Important features of the droplet transfer method are highlighted in Figure 5 caption.

'Hanging' GVs, attached to the water–oil interface, can be obtained if the droplet transfer occurs spontaneously and gently, just by gravity. The GVs (size 1–100 μm), encapsulating a TX–TL mixture, were monitored in time via confocal microscopy (Figure 4c). The amount of produced GFP was variable (CV ~ 35%), irrespective from the nature and concentration of the template molecule (c_{DNA} from 3 pM to 30 nM, c_{RNA} from 25 nM to 1 μM) (65, 70).

More results have been reported by the Yomo group (66, 71). Given the GVs volume of 1–100 fL, the distribution of the product

concentration measured by flow cytometry was about 1–1.5 orders of magnitude broad, partially dependent from GVs size (as vesicle size increases, the width decreases); c_{DNA} = 0.1 nM (Figure 4d). When two proteins (YFP, BFP) where simultaneously synthesized from two identical but physically separated T7 promoters on the same plasmid (c_{DNA} = 0.33–9 nM), the spread of protein concentration, given a certain vesicle size, was similarly extended (72). When c_{DNA} was increased, the between-vesicle diversity decreased.

Polymersomes and microchambers. Small between-compartment CV values (ca. 12%, Figure 4e) of synthesized protein concentration have been reported for polymer vesicles prepared by microfluidic methods (25). Considering that the vesicles were quite large (diameter ca. 120 μm), and c_{DNA} ca. 4 nM, the reported value largely exceeds the expectations (0.06% calculated just on c_{DNA}). On the other hand, when glass (40 fL–7 pL) or PDMS (15–400 fL) microchambers have been used as compartments for the TX–TL reactions, contrasting results have been reported, including small (c_{DNA} 30 nM, CV ~ 6% (73);) and large (c_{DNA} ~ 5 nM, CV ~ 30%; (67, 74)) between-chamber variability (Figure 4f). The use of 40 fL microchamber has allowed the confirmation that at low c_{DNA} (17 pM), Poisson distribution rules the protein production distribution (73).

From this brief summary, it is evident that experimental results show large between-vesicle variability, very often larger-than-expected, at least when the CV is estimated from c_{DNA} and vesicle volume only. In other cases, harmony between observed distributions and Poisson law has been reported, especially for very low c_{DNA} (~1 nM and below). The DNA template is among the components with lowest concentration, and therefore it becomes pattern-determining. But some studies suggest that other components could also affect the observed between-compartment variance. As mentioned before, understanding whether large protein synthesis fluctuations are due to expected (or non-expected) C_k diversity or to confinement effects is not easily done, and, moreover, the two effects do not necessarily exclude each other.

However, it appears useful to gain more information on C_k diversity and on its consequence on the protein synthesis, before exploring potential confinement effects. At this aim, a full understanding of the vesicles and droplets formation mechanism is essential. The mechanisms of vesicle formation in the film hydration method and in the droplet transfer methods are very different. They share only a crucial moment, namely, the self-closure (the self-sealing) of a lipid boundary around a portion of aqueous phase.

What do we really know about these mechanisms? The pictorial explanation of GVs formation from the lipid film swelling looks intuitively clear, but details of bilayer swelling, penetration of the aqueous solution among the layered lipids, budding, vesicle swelling, detachment, sealing, are all very complicated. GVs prepared by the droplet transfer method are also broadly diverse. The method is based on the conversion of pre-formed w/o droplets to GVs, by a zip-like mechanism (75) occurring between two lipid monolayers in the moment of the droplet transfer (Figure 5). The diversity of the resulting GVs actually comes from the diversity of their precursor, i.e. the w/o droplets. It is therefore interesting to report what is known about w/o droplets.

3.3. A closer look to w/o emulsion droplets

Water-in-oil droplets can be studied as simple cellular models, or can be used as precursors of GVs (Figures 2 and 5). They can
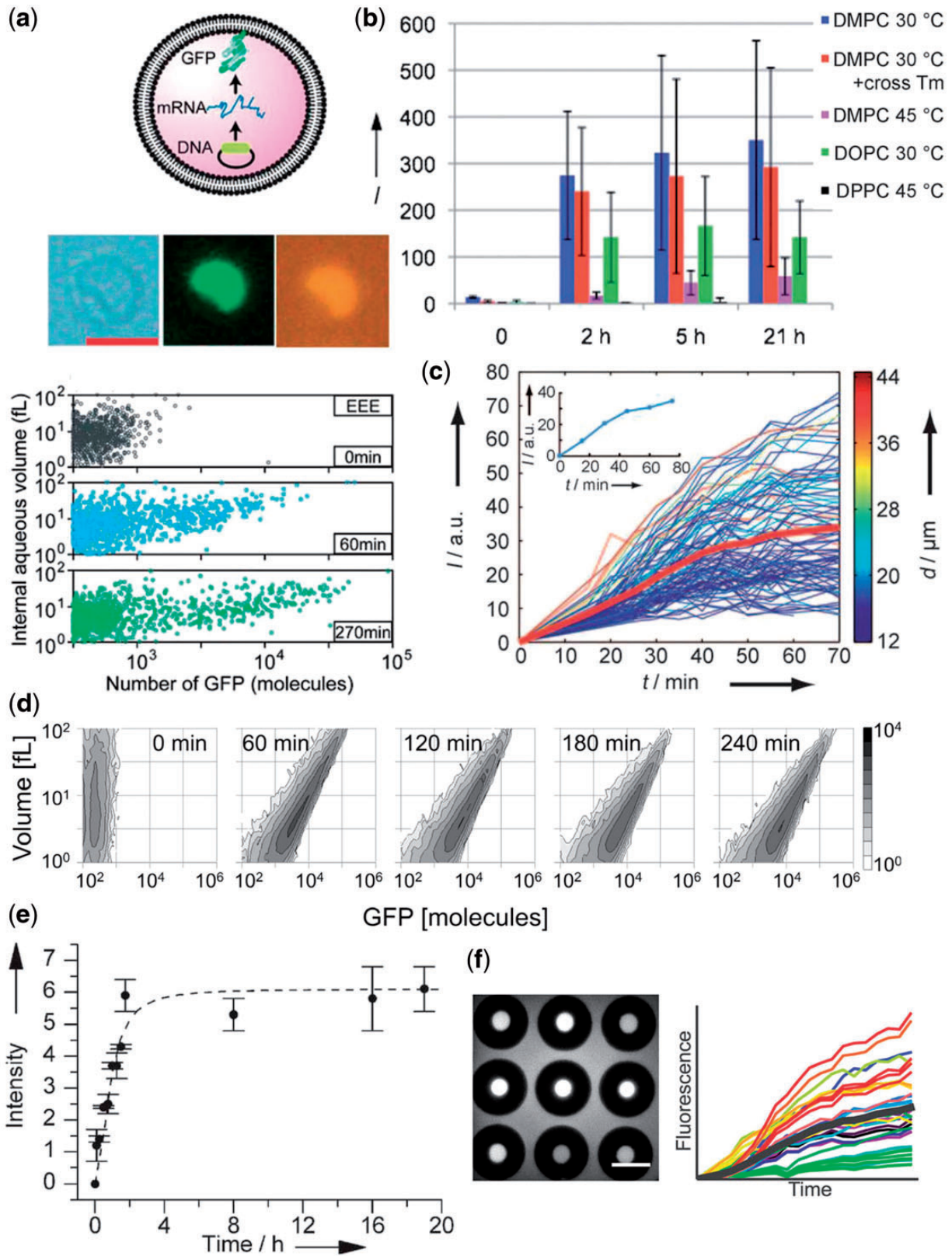
Figure 4. Experimentally determined between-compartment variations referring to TX–TL reactions. Note the width of protein synthesis distribution measured by flow cytometry or microscopy. See details in the text. (**a**) Reprinted with permission from (63) ©2010 American Chemical Society. (**b**) Reprinted with permission from (64) Copyright © 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim (**c**) Reprinted with permission from (65), Copyright © 2009 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. (**d**) Reprinted with permission from (66) ©2012 American Chemical Society. (**e**) Reprinted with permission from (25) Copyright © 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. (**f**) Reprinted with permission from (67), ©2017 American Chemical Society.
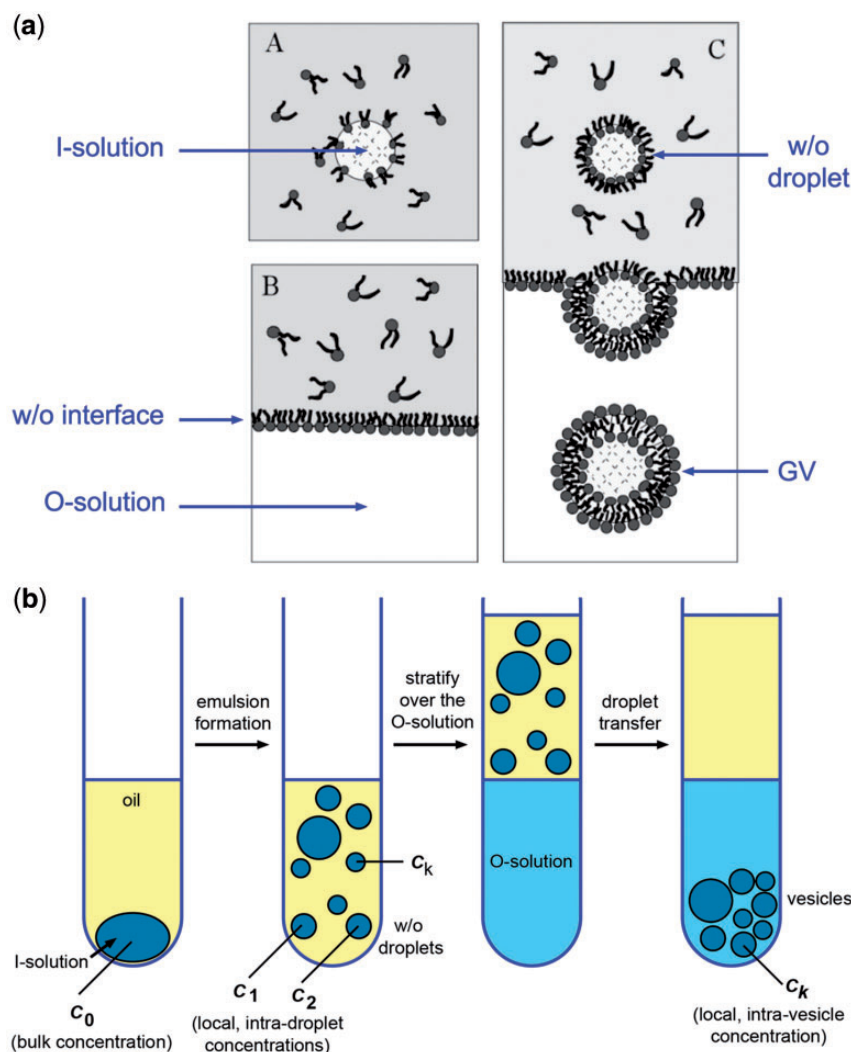
Figure 5. The droplet transfer method. (**a**) Details of the droplet transfer: w/o droplets are formed by the emulsification of an aqueous I-solution in a lipid-containing oil solution (A). Lipid molecules stabilize the droplet interface. Separately, a flat lipid-covered w/o interface is created by stratification of a second lipid-containing oil solution over an aqueous phase (O-solution) (B). Finally, the w/o droplets are placed over the flat interface and cross them by gravity (or by centrifugation) (C). During the transfer, a lipid bilayer forms around the droplet—so to have a vesicle. Note that the inner and the outer membrane leaflets can be different (asymmetric vesicles will be produced). Reprinted with permission from (24) ©2003 American Chemical Society. (**b**) Schematic illustration of the droplet transfer method; note that millions of microscopic w/o droplets (whose inner solute concentration is $c_1, c_2, \ldots c_k$) arise from the fragmentation of the macroscopic droplet. After droplet transfer, this between-droplets diversity translates into a between-vesicles diversity. *Historical comment.* Note that the droplet transfer method (also called 'emulsion inversion' method) is probably the most relevant novelty in liposome technology of the past 15 years, certainly a very innovative procedure for producing GVs. It was discovered—or better, re-discovered (69)—by D. A. Weitz in 2003, when he published two papers on this method, with emphasis on membrane asymmetry rather than on the GVs production (23, 24). It was used for assembling GVs that encapsulated the TX–TL kit, for the first time, in 2004 (22). It performs well also in the presence of high salt concentration, in contrary to other GVs preparation methods (58). Several groups applied and optimized this method for intra-vesicle protein expression. A mention should be done to the Yomo's group who intensively utilized the method, often in conjunction with flow cytometry detection of the produced protein. A detailed protocol is now available (62).

be prepared by emulsifying a macroscopic aqueous drop (e.g. 5 μL), in a lipid-containing hydrocarbon solvent (the 'oil'). Dispersion is usually attained by pipetting, vortexing, sonication. The initial drop splits into millions of w/o microdroplets whose volume lies in the 1–100 fL range. This fragmentation process is counteracted by droplet coalescence; the latter depends on droplet stability, and therefore on the chemical nature of the oil/lipid/aqueous interface. The fact that w/o droplets, under the input of mechanical shearing forces, undergo fragmentation-coalescence is evident from the observation that if different groups of TX–TL components are emulsified separately and next the different w/o emulsions are mixed together, the droplets are still able to produce a protein (76–78). Note that

fragmentation-coalescence do not occur in typical phospholipid vesicle systems.

Stochastic partition of TX–TL solutes among the droplets occurs during fragmentation-coalescence. In this system, H_0 still holds (Equation 1 and Table 2); c_0 becomes, in this scenario, the concentration of a solute in the macroscopic aqueous drop that generates the microemulsion (Figure 2b).

Several studies report wide between-droplets variance for TX–TL reactions. It was found that the CV between TX–TL reactions inside droplets with radius of 5–10 μm was around 10% (76, 77). As c_{DNA} was 38 nM, its stochastic partition would lead to CV 0.3–1%
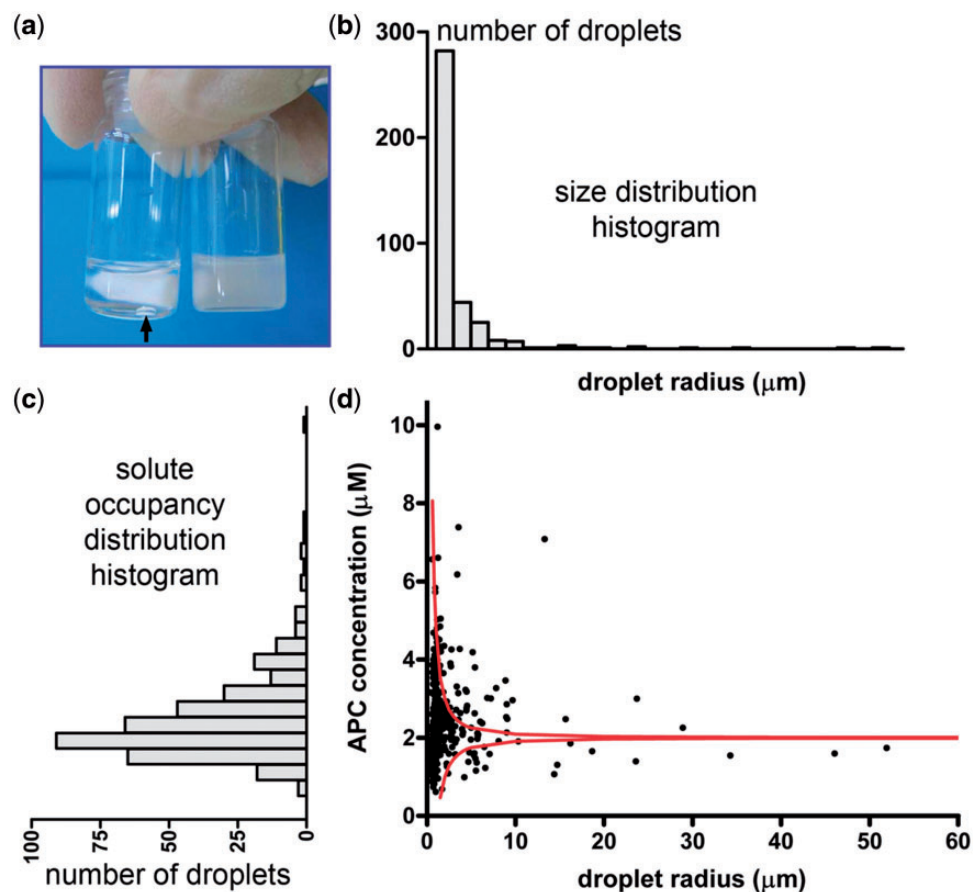
Figure 6. Allophycocyanin (APC)-containing w/o emulsion droplets. (**a**) Photograph of a water drop before (left) and after (right) emulsification, compare with Figure 2b. An arrow indicates the drop. (**b**) APC-containing droplet size distribution. (**c**) APC occupancy distribution inside the droplets. (**d**) Dot-plot revealing the variation of APC intra-droplet concentration with droplet size. Largest droplets have internal APC concentration somehow similar to the expectation ($2 \mu M$). Most points lies within $\pm$CV expectations (red lines), but other appear to deviate from them. The panel (d) has been re-drawn from a published dataset (40) with the addition of panels (a–c) for illustrative purposes. Similar results have been obtained with other macromolecular solutes.

Tsuji and Yoshikawa (79) reported unexpectedly high TX diversity (c_{DNA} 1.5 nM, $V \sim 30$ pL).

When a synthetic transcriptional oscillator was encapsulated inside w/o droplets (radius 2–$10 \mu m$) (80), reported large between-droplets diversity (CV ~ 25%) that could not be quantitatively explained by Poisson stochastic partition, even for large droplets.

Water-in-oil droplets prepared by microfluidic technology were capable of synthesizing GFP but a CV of about 20% have been observed, while, only based on DNA stochastic partition, 6% is expected (c_{DNA} 30 pM, droplet radius $15 \mu m$) (81).

We have investigated the distribution of macromolecular solutes inside soybean lecithin-stabilized w/o droplets (40). In particular, we have shown that between-droplet diversity increases, as expected, when droplet size decreases, but the magnitude of this diversity is often higher than expected. In particular, experiments were carried out with several fluorescent macromolecular solutes (allophycocyanin, albumin-FITC, dextran-FITC, phycoerythrin) and the intra-droplet concentration was measured for each droplet, together with the droplet size. This led to concentration versus size dot-plots (as the one shown in Figure 6). By overlapping the theoretical curves (calculations as in Table 2), it is possible to see which droplets lie outside the $\pm$standard deviation band.

Moving to the case of TX–TL reactions inside w/o droplets, interesting experiments have been presented by (53), who revealed inverse relationship between the protein production rate (or protein amount) and the droplets size (radius 15–$100 \mu m$). Protein synthesis inside small droplets resulted to be more efficient than larger ones.

4. Variations of the PURE system composition

For investigating how different TX–TL compositions affect protein synthesis, one can refer to a very interesting dataset published by Yomo and collaborators (82). This study was focused on *epistatic interactions* (non-additive effects) among PURE system components. In particular, the investigation refers to the quantification of produced protein by the PURE TX–TL system when different 'modules' were combinatorially mixed (each module is a group of PURE system component, see Figures 7 and Supplementary Figure S2). Experiments show that the amount of produced protein is highly variable (up to ca. 1 order of magnitude), depending on the concentration of the modules (not including template DNA) that were selected for variation.

These evidences, when applied to the stochastic partition scenario, reveals that stochastic partition of solutes or group of solutes, even if unexpected from the statistical viewpoint, can
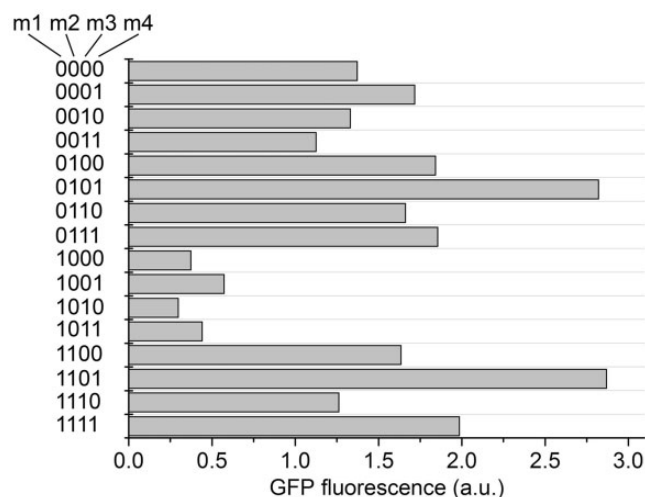
Figure 7. Variation of GFP production as function of PURE system composition (82). The PURE system components were grouped in four modules (m1: small molecules; m2: translation factors; m3: amino acyl-tRNA synthetase and other enzymes; m4: ribosomes). The concentration of all species was either kept equal to the standard concentration (label '0'), either changed (label '1'). Note that the GFP production, as revealed by fluorescence measurements, varies from about 0.3 (mixture '1010') to 2.9 a.u (mixture '1101'). Additional data are presented in Supplementary Figure S2. The actual concentrations of PURE system species are available in the original paper.

justify the observed large inter-compartment variance in terms of protein production.

5. Numerical simulations: what can we learn?

Together with experiments, bottom-up synthetic biology approaches heavily rely on numerical simulations, especially when they can be predictive. The numerical models referring to TX–TL reactions are of special interest, and have been developed by several groups (83–86).

We have recently reported a deterministic (non-stochastic) model for the PURE system based on 6 reactions, 19 kinetic constants, and 17 chemical species (Supplementary Figures S3 and S4), and on Michaelis-Menten-like kinetic rates for all steps (87). Despite its simplicity, the model reproduces the essential kinetic features of TX–TL reactions and is capable of accounting for resource consumption and responsiveness to reactant concentration variations.

A useful use of TX–TL predictive numerical models could be hypothesis testing about solute partition. We have applied such an approach to decipher a series of experimental results based on the encapsulation of the *diluted* PURE system in small GVs (radius 1 μm) (88). A second target is the encapsulation of the PURE system in conventional sub-micron vesicles (radius 0.10–0.15 μm) (45). In both examples, the co-entrapment probability P (Equation 2) is very low (i.e. n_0 for each chemical species is a small number) but for two different reasons. In the first case, n_0 is small because c_0 is small; in the second case, n_0 is small because V is small.

The second piece of information derives from experimental observations on the partition of macromolecules inside vesicles prepared with different methods (89). When ferritin (41) or ribosomes (90) have been encapsulated inside sub-micron vesicles, non-Poissonian solute occupancy distributions have been measured. In particular, a power-law distribution has been suggested. Indications of a similar pattern have been observed also for ribo-peptidic complexes (91), and several types of macromolecules (proteins, dextrans) inside small GVs (92). To date, there is no clear explanation of why a power-law distribution should describe the encapsulation of these molecules inside

conventional lipid vesicles. It has been proposed that the rate of bilayer fragment closure plays a central role. The rate of vesicle formation could be changed by the presence of macromolecules (41, 90). Coarse-grained simulations have not supported this hypothesis (93). On the other hand, a model based on the Cox theory of renewal point processes explains the experimental pattern (whereby it is supposed a cooperative interaction between the solute molecules and the forming liposomes) (94).

The outcome of the two scenarios (Poissonian/Gaussian versus non-Poissonian/non-Gaussian stochastic partition of TX–TL components) can be simulated by the help of a mathematical model (Figure 8). The number of encapsulated reactants and the width of their distribution can be estimated according to the two cases, and the resulting protein production can be computed for a high number of concentration combinations, each corresponding to the lumen composition of one simulated vesicles. Following this approach, we showed that for small GVs (radius 1 μm) the two encapsulation models result in very different protein production (different mean value and different distribution) (42). As shown in Figure 9, the kinetic profiles of 5000 *in silico* GVs were simulated. Each GVs was filled by a stochastically generated mixture of species (referring to the model, see Supplementary Figures S3 and S4), obeying to Poissonian/ Gaussian or power-law partition. The distributions of produced protein inside the vesicles differ strongly. The amount of vesicles that produces protein above a threshold (>0.2 μM) was 0.0% and 0.8%, respectively. Because the simulation was carried out on a diluted PURE system, the simulations support the hypothesis that extrinsic stochastic effects based on non-Poissonian encapsulation can explain the protein synthesis pattern. In particular, power-law partition allows the formation of vesicles that contain all PURE system components, and in sufficient copies, to produce proteins. In contrary, the probability that such protein-producing vesicles originate according to a Poissonian/Gaussian solute encapsulation is negligible.

6. Conclusions

The discussion and the data presented in this work aim at evidencing an important aspect of microcompartmentalized
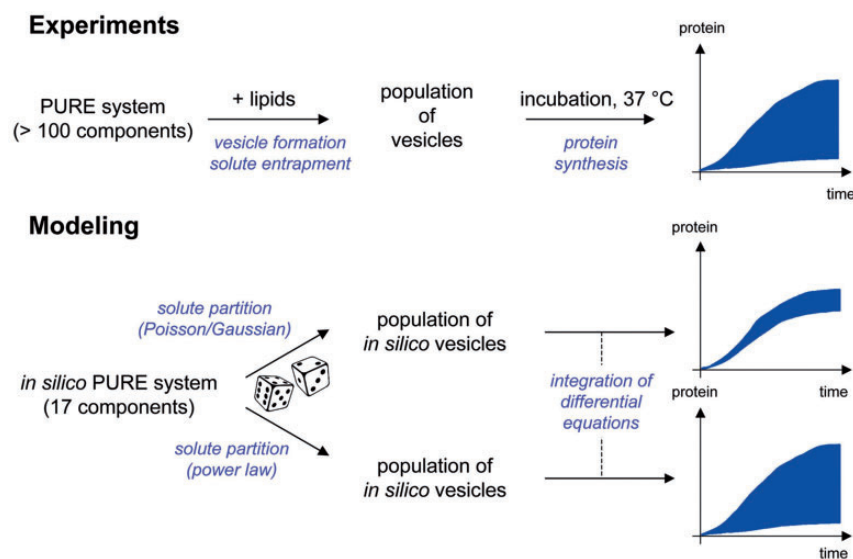
Figure 8. Using an *in silico* TX–TL model for investigating solute partition inside microcompartments. A simplified model for the PURE system operation is firstly developed ([87]), based on 17 components. These components are then stochastically partitioned inside *in silico* compartments (a large population is generated). For each compartment, the time course of protein synthesis is simulated (by numerical integration of kinetic rate differential equations), and the resulting population pattern is compared with the experimentally determined one.

reactions. Random events at the molecular and colloid scale generate a 'diversity' that becomes well evident in these systems. Overlaid to the expected variations, other phenomena can play a role, either by themselves, either mediated by the mechanisms of vesicle or droplet formation. As commented by ([80]), the source of additional variability can be manifold, such as partial enzyme inactivation ('effective' concentration reduction), incomplete solution mixing (lack of spatial homogeneity of the solution), protein multimerization/aggregation (reduction of the effective concentration, multiple forms) or solutes–lipids interaction (preferential encapsulation or exclusion). All these effects add to the already investigated low-c_{DNA} effect, but still lack of experimental verification. Moreover, we believe that especially for fundamental biophysical studies, a full understanding of vesicles and droplets formation mechanisms is essential to unveil solute encapsulation details. Short-range and long-range solvent mediated boundary/solute interactions, depletion forces, and local crowding (without aggregation), or formation of functioning-dependent hyperstructures ([95]) all change the idealized 'aqueous solution' picture we might have in mind. In the moment and in the microscopic region when/where encapsulation occurs, the system is lipid-rich, biphasic, vectorial—these are conditions that might promote long-range order and solute-solvent structuring.

In this article, we have highlighted a fascinating question related to TX–TL reactions inside compartments. It seems premature to draw a conclusion, except the suggestion that such phenomena require more detailed experimental and modeling studies. In particular, we have emphasized the need of powerful experimental technique for the determination of single-compartment composition, and the search for more detailed investigation on the mechanisms of vesicle/droplet formation—in connection with solute capture. And finally, for theorists, what are the conditions that might lead to a violation of the Poissonian model?

The microfluidic perspective. We were among the first enthusiastic supporters of GVs microfluidic generation ([5]). Inspired by

few early papers ([96–98]) we called for the development of an on-chip technology for the production of solute-filled GVs. In recent years, numerous advancements have been reported, reviewed by ([99], [100]). The synthesis of MreB protein inside polymer vesicles ([25]), and MscL protein inside phospholipid/cholesterol/fluorinated surfactants vesicles ([101]) are two examples of the current research on complex reactions inside microfluidic-generated compartments. The research on microfluidic devices is progressing rapidly, see recent reports ([102], [103]). Ideally, microfluidic devices should be capable of assembling vesicles from one or more phospholipid, with a control on vesicle size and content. This will allow the production of solute-filled compartments with small between-compartment variance, so to have homogeneous and easy-to-study synthetic cell populations. Moreover, this will later trig biotechnological usage. The long-term objective is the automatic production of 'artificial cells' for nanomedicine ([32], [104]), with the caveat of being able of control their size for *in vivo* applications.

The relevance of TX–TL reactions inside lipid vesicles in origins of life studies. If, from one side, the beauty of a homogeneous vesicle population generated by microfluidics is attractive because it eliminates almost completely odd uncontrolled variations, when the TX–TL components are encapsulated inside vesicles intended as primitive cell models, the reproducibility of the microfluidic 'assembly line' might hide something. Working with vesicle populations which are homogeneous in terms of size, lamellarity, and content is a plus when the properties of internal reactions must be carefully studied. But when the spontaneous onset of protocellular structures has to be investigated, the combination of compartment formation and solute encapsulation turns more informative. Here, the added value is the diversity among compartments, which leads either to the concept of 'individuality' of a protobiological entity, either to the concept of population, in biological sense, i.e. which includes extreme individuals. Important concepts stems from this view, such as selection, competition, coexistence, cooperation, are a product of the individual/population duality.
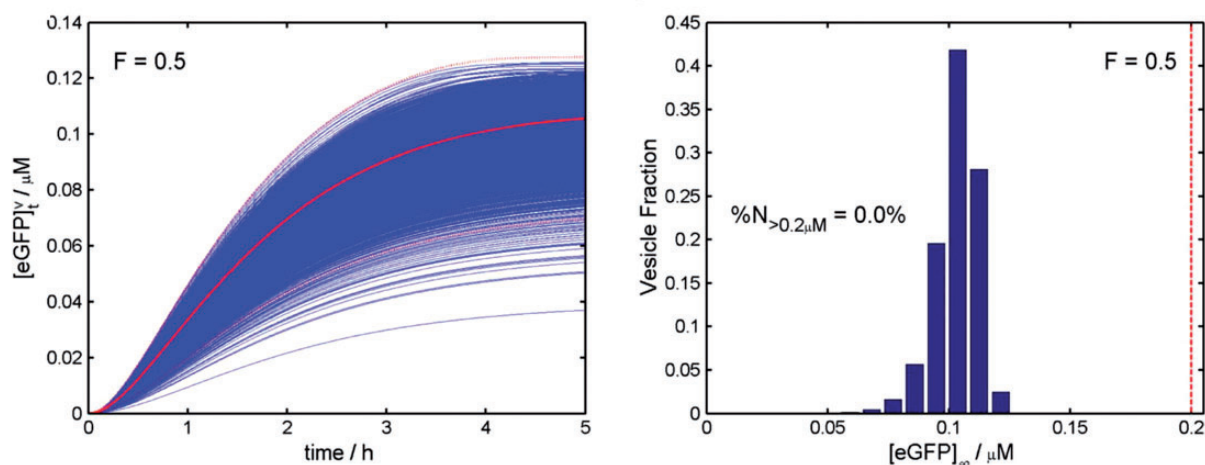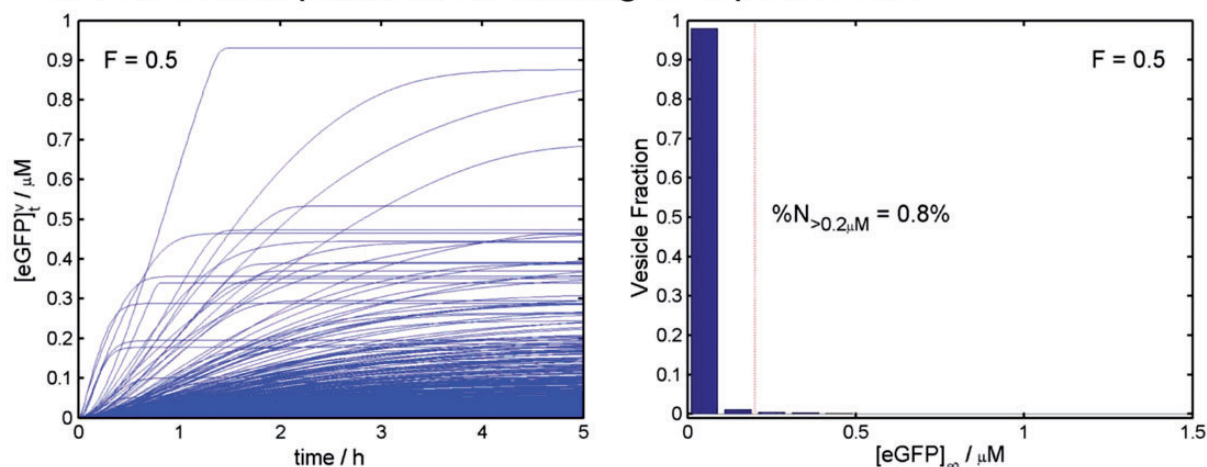
Figure 9. Simulated protein expression in a population of 5000 vesicles with solute distributed according to (**a**) Gaussian or (**b**) power-law distributions. In both the cases, simulation are run on a 2-fold diluted *in silico* PURE system. On the left, time courses of the internal protein production of each single vesicle (blue curves); note the different scale on the *y*-axes. On the right, statistics of the protein yield (eGFP) (histograms). The dashed red lines on the right plots indicate a threshold value (0.2 μM). Reprinted from (42) (Fall 2016) © 2015 by the Massachusetts Institute of Technology. Reprinted by permission of the MIT Press.

With respect to between-compartment diversity, beyond-expectations large stochastic extrinsic effects lead to the formation of protein-producing vesicles even when a diluted (and thus non-productive) PURE system was used (88). This suggests that the mechanism of vesicle formation/solute encapsulation can trigger the onset of cellular systems, not only due to confinement-protection but also by favoring chemical reactions in the sense that it made them possible when the opposite was expected. Although occurring in rare and unfortunately still unknown local conditions, self-organization in this case is a for-free exergonic route to the emergence of protocells.

The authors that started the study biochemical reactions inside vesicles, including protein synthesis, aimed at understanding the interplay between vesicle biophysics, reaction confinement, and self-production (autopoiesis) of protocellular components (14, 105). Now that this research spreads among the community of synthetic biology, the 'bottom-up' approaches to synthetic cells are now strongly emerging (106). TX–TL reactions have been, and are, the keystone of these efforts.

SUPPLEMENTARY DATA

Supplementary Data are available at SYNBIO Online.

Acknowledgements

References

1. Deamer,D.W. and Oró,J. (1980) Role of lipids in prebiotic structures. *BioSystems*, 12, 167–175.
2. Morowitz,H.J., Heinz,B. and Deamer,D.W. (1988) The chemical logic of a minimum protocell. *Orig. Life Evol. Biosph.*, 18, 281–287.
3. Schmidli,P.K., Schurtenberger,P. and Luisi,P.L. (1991) Liposome-mediated enzymatic synthesis of

phosphatidylcholine as an approach to self-replicating lipo-somes. *J. Am. Chem. Soc.*, 113, 8127–8130.

4. Walde,P., Goto,A., Monnard,P., Wessicken,M. and Luisi,P. (1994) Oparins reactions revisited - enzymatic-synthesis of poly(adenylic acid). *J. Am. Chem. Soc.*, 116, 7541–7547.

5. Stano,P., Carrara,P., Kuruma,Y., Souza,T.P. and Luisi,P.L. (2011) Compartmentalized reactions as a case of soft-matter biotechnology: synthesis of proteins and nucleic acids in-side lipid vesicles. *J. Mater. Chem.*, 21, 18887–18902.

6. Szostak,J.W., Bartel,D.P. and Luisi,P.L. (2001) Synthesizing life. *Nature*, 409, 387–390.

7. Luisi,P.L. (2002) Toward the engineering of minimal living cells. *Anat. Rec.*, 268, 208–214.

8. ——, Ferri,F. and Stano,P. (2006) Approaches to semi-synthetic minimal cells: a review. *Naturwissenschaften*, 93, 1–13.

9. Sessa,G. and Weissmann,G. (1970) Incorporation of lyso-zyme into liposomes. A model for structure-linked latency. *J. Biol. Chem.*, 245, 3295–3301.

10. Gregoriadis,G., Leathwood,P.D. and Ryman,B.E. (1971) Enzyme entrapment in liposomes. *FEBS Lett.*, 14, 95–99.

11. Martinek,K., Levashov,A.V., Khmelnitsky,Y.L., Klyachko,N.L. and Berezin,I.V. (1982) Colloidal solution of water in organic solvents: a microheterogeneous medium for enzymatic reactions. *Science*, 218, 889–891.

12. Luisi,P. (1985) Enzymes hosted in reverse micelles in hydro-carbon solution. *Angew. Chem. Int. Ed. Engl.*, 24, 439–450.

13. Chakrabarti,A., Breaker,R., Joyce,G. and Deamer,D. (1994) Production of RNA by a polymerase protein encapsulated within phospholipid-vesicles. *J. Mol. Evol.*, 39, 555–559.

14. Oberholzer,T., Wick,R., Luisi,P.L and Biebricher,C.K. (1995) Enzymatic RNA replication in self- reproducing vesicles: an approach to a minimal cell. *Biochem. Biophys. Res. Comm.* 207, 250–257.

15. ——, Albrizio,M. and Luisi,P.L. (1995) Polymerase chain re-action in liposomes. *Chem. Biol.*, 2, 677–682.

16. ——, Nierhaus,K.H. and —— (1999) Protein expression in liposomes. *Biochem. Biophys. Res. Commun.*, 261, 238–241.

17. Walde,P., Wick,R., Fresta,M., Mangone,A. and Luisi,P. (1994) Autopoietic self-reproduction of fatty-acid vesicles. *J. Am. Chem. Soc.*, 116, 11649–11654.

18. Yu,W., Sato,K., Wakabayashi,M., Nakaishi,T., Ko-Mitamura,E.P., Shima,Y., Urabe,I. and Yomo,T. (2001) Synthesis of functional protein in liposome. *J. Biosci. Bioeng.*, 92, 590–593.

19. Oberholzer,T. and Luisi,P.L. (2002) The use of liposomes for constructing cell models. *J. Biol. Phys.*, 28, 733–744.

20. Nomura,S., Tsumoto,K., Hamada,T., Akiyoshi,K., Nakatani,Y. and Yoshikawa,K. (2003) Gene expression within cell-sized lipid vesicles. *Chembiochem*, 4, 1172–1175.

21. Ishikawa,K., Sato,K., Shima,Y., Urabe,I. and Yomo,T. (2004) Expression of a cascading genetic network within lipo-somes. *FEBS Lett.*, 576, 387–390.

22. Noireaux,V. and Libchaber,A. (2004) A vesicle bioreactor as a step toward an artificial cell assembly. *Proc. Natl. Acad. Sci. USA*, 101, 17669–17674.

23. Pautot,S., Frisken,B.J. and Weitz,D.A. (2003) Engineering asymmetric vesicles. *Proc. Natl. Acad. Sci. U.S.A.*, 100, 10718–10721.

24. ——, —— and —— (2003) Production of unilamellar vesicles using an inverted emulsion. *Langmuir*, 19, 2870–2879.

25. Martino,C., Kim,S.-H., Horsfall,L., Abbaspourrad,A., Rosser,S.J., Cooper,J. and Weitz,D.A. (2012) Protein expression, aggregation, and triggered release from poly-mersomes as artificial cell-like structures. *Angew. Chem. Int. Ed. Engl.*, 51, 6416–6420.

26. Dora Tang,T.-Y., van Swaay,D., deMello,A., Ross Anderson,J.L. and Mann,S. (2015) In vitro gene expression within membrane-free coacervate protocells. *Chem. Commun.*, 51, 11429–11432.

27. Kuruma,Y., Stano,P., Ueda,T. and Luisi,P.L. (2009) A syn-thetic biology approach to the construction of membrane proteins in semi-synthetic minimal cells. *Biochim. Biophys. Acta*, 1788, 567–574.

28. Hamada,S., Tabuchi,M., Toyota,T., Sakurai,T., Hosoi,T., Nomoto,T., Nakatani,K., Fujinami,M. and Kanzaki,R. (2014) Giant vesicles functionally expressing membrane receptors for an insect pheromone. *Chem. Commun.*, 50, 2958–2961.

29. Soga,H., Fujii,S., Yomo,T., Kato,Y., Watanabe,H. and Matsuura,T. (2014) In vitro membrane protein synthesis in-side cell-sized vesicles reveals the dependence of mem-brane protein integration on vesicle volume. *ACS Synth. Biol*, 3, 372–379.

30. van Nies,P., Westerlaken,I., Blanken,D., Salas,M., Mencía,M. and Danelon,C. (2018) Self-replication of DNA by its encoded proteins in liposome-based synthetic cells. *Nat. Commun.*, 9, 1583.

31. Shin,J. and Noireaux,V. (2012) An *E. coli* cell-free expression toolbox: application to synthetic gene circuits and artificial cells. *ACS Synth. Biol.*, 1, 29–41.

32. Krinsky,N., Kaduri,M., Zinger,A., Shainsky-Roitman,J., Goldfeder,M., Benhar,I., Hershkovitz,D. and Schroeder,A. (2018) Synthetic cells synthesize therapeutic proteins inside tumors. *Adv. Healthc. Mater.*, 7, 1701163.

33. Lentini,R., Martín,N.Y., Forlin,M., Belmonte,L., Fontana,J., Cornella,M., Martini,L., Tamburini,S., Bentley,W.E., Jousson,O. et al. (2017) Two-way chemical communication between artificial and natural cells. *ACS Central Sci.*, 3, 117–123.

34. Rampioni,G., D'Angelo,F., Messina,M., Zennaro,A., Kuruma,Y., Tofani,D., Leoni,L. and Stano,P. (2018) Synthetic cells produce a quorum sensing chemical signal perceived by *Pseudomonas aeruginosa*. *Chem. Commun.*, 54, 2090–2093.

35. Shimizu,Y., Inoue,A., Tomari,Y., Suzuki,T., Yokogawa,T., Nishikawa,K. and Ueda,T. (2001) Cell-free translation recon-stituted with purified components. *Nat. Biotechnol.*, 19, 751–755.

36. Gillespie,D.T. (2007) Stochastic simulations of chemical ki-netics. *Annu. Rev. Phys. Chem.*, 58, 35–55.

37. Mavelli,F. and Piotto,S. (2006) Stochastic simulations of ho-mogeneous chemically reacting systems. *J. Mol. Struct.*, 771, 55–64.

38. Kaneko,K. (2006) *Life: An Introduction to Complex Systems Biology; Understanding Complex Systems*. Springer, Berlin.

39. Damiano,L., Hiolle,A. and Cañamero,L. (2011) Grounding synthetic knowledge. In: T Lenaerts, M Giacobini, H Bersini, P Bourgine, R Doursat (eds). *Advances in Artificial Life, ECAL 2011*. MIT Press, Boston, pp. 200–207

40. Stano,P., Souza,T.P., Carrara,P., Altamura,E., D'Aguanno,E., Caputo,M., Luisi,P.L. and Mavelli,F. (2015) Recent biophysical issues about the preparation of solute-filled lipid vesicles. *Mech. Adv. Mater. Struct.*, 22, 748–759.

41. Luisi,P.L., Allegretti,M., Souza,T.P., Steiniger,F., Fahr,A. and Stano,P. (2010) Spontaneous protein crowding in liposomes: a new vista for the origin of cellular metabolism. *ChemBioChem*, 11, 1989–1992.

42. Mavelli,F. and Stano,P. (2015) Experiments on and numerical modeling of the capture and concentration of transcription-translation machinery inside vesicles. *Artif. Life*, 21, 445–463.

43. Shimizu,Y., Kanamori,T. and Ueda,T. (2005) Protein synthesis by pure translation systems. *Methods*, 36, 299–304.

44. Hosoda,K., Sunami,T., Kazuta,Y., Matsuura,T., Suzuki,H. and Yomo,T. (2008) Quantitative study of the structure of multi-lamellar giant liposomes as a container of protein synthesis reaction. *Langmuir*, 24, 13540–13548.

45. Souza,T.P., Stano,P. and Luisi,P.L. (2009) The minimal size of liposome-based model cells brings about a remarkably enhanced entrapment and protein synthesis. *Chembiochem*, 10, 1056–1063.

46. Mavelli,F. and —— (2010) Kinetic models for autopoietic chemical systems: the role of fluctuations in a homeostatic regime. *Phys. Biol.*, 7, 16010.

47. ——, Altamura,E., Cassidei,L. and Stano,P. (2014) Recent theoretical approaches to minimal artificial cells. *Entropy*, 16, 2488–2511.

48. Kita,H., Matsuura,T., Sunami,T., Hosoda,K., Ichihashi,N., Tsukada,K., Urabe,I. and Yomo,T. (2008) Replication of genetic information with self-encoded replicase in liposomes. *ChemBioChem*, 9, 2403–2410.

49. Su,Y., Shi,Q. and Wei,W. (2017) Single cell proteomics in bio-medicine: high-dimensional data acquisition, visualization, and analysis. *Proteomics*, 17, 1600267.

50. Bui,H.T., Umakoshi,H., Ngo,K.X., Nishida,M., Shimanouchi,T. and Kuboi,R. (2008) Liposome membrane itself can affect gene expression in the *Escherichia coli* cell-free translation system. *Langmuir*, 24, 10537–10542.

51. Zhou,H.-X., Rivas,G. and Minton,A.P. (2008) Macromolecular crowding and confinement: biochemical, biophysical, and potential physiological consequences. *Annu. Rev. Biophys.*, 37, 375–397.

52. Ge,X., Luo,D. and Xu,J. (2011) Cell-free protein expression under macromolecular crowding conditions. *PLoS One*, 6, e28707.

53. Kato,A., Yanagisawa,M., Sato,Y.T., Fujiwara,K. and Yoshikawa,K. (2012) Cell-sized confinement in microspheres accelerates the reaction of gene expression. *Sci. Rep.*, 2, 283.

54. Fujiwara,K. and Nomura,S.M. (2013) Condensation of an additive-free cell extract to mimic the conditions of live cells. *PLoS One*, 8, e54155.

55. Tan,C., Saurabh,S., Bruchez,M.P., Schwartz,R. and Leduc,P. (2013) Molecular crowding shapes gene expression in synthetic cellular nanosystems. *Nat. Nanotechnol.*, 8, 602–608.

56. Hansen,M.M.K., Meijer,L.H.H., Spruijt,E., Maas,R.J.M., Rosquelles,M.V., Groen,J., Heus,H.A. and Huck,W.T.S. (2016) Macromolecular crowding creates heterogeneous environments of gene expression in picolitre droplets. *Nat. Nanotechnol,*. 11, 191–197.

57. Dominak,L.M. and Keating,C.D. (2007) Polymer encapsulation within giant lipid vesicles. *Langmuir*, 23, 7148–7154.

58. Walde,P., Cosentino,K., Engel,H. and Stano,P. (2010) Giant vesicles: preparations and applications. *Chembiochem*, 11, 848–865.

59. Estes,D.J. and Mayer,M. (2005) Giant liposomes in physiological buffer using electroformation in a flow chamber. *Biochim. Biophys. Acta*, 1712, 152–160.

60. Sakakura,T., Nishimura,K., Suzuki,H. and Yomo,T. (2012) Statistical analysis of discrete encapsulation of nanomaterials in colloidal capsules. *Anal. Methods*, 4, 1648–1655.

61. Hamada,T., Miura,Y., Komatsu,Y., Kishimoto,Y., Vestergaard,M. and Takagi,M. (2008) Construction of asymmetric cell-sized lipid vesicles from lipid-coated water-in-oil microdroplets. *J. Phys. Chem. B*, 112, 14678–14681.

62. Fujii,S., Matsuura,T., Sunami,T., Nishikawa,T., Kazuta,Y. and Yomo,T. (2014) Liposome display for in vitro selection and evolution of membrane proteins. *Nat. Protoc.*, 9, 1578–1591.

63. Sunami,T., Hosoda,K., Suzuki,H., Matsuura,T. and Yomo,T. (2010) Cellular compartment model for exploring the effect of the lipidic membrane on the kinetics of encapsulated biochemical reactions. *Langmuir*, 26, 8544–8551.

64. Nourian,Z., Roelofsen,W. and Danelon,C. (2012) Triggered gene expression in fed-vesicle microreactors with a multifunctional membrane. *Angew. Chem. Int. Ed. Engl.*, 51, 3114–3118.

65. Saito,H., Kato,Y., Le Berre,M., Yamada,A., Inoue,T., Yosikawa,K. and Baigl,D. (2009) Time-resolved tracking of a minimum gene expression system reconstituted in giant liposomes. *ChemBioChem*, 10, 1640–1643.

66. Nishimura,K., Matsuura,T., Nishimura,K., Sunami,T., Suzuki,H. and Yomo,T. (2012) Cell-free protein synthesis inside giant unilamellar vesicles analyzed by flow cytometry. *Langmuir*, 28, 8426–8432.

67. Caveney,P.M., Norred,S.E., Chin,C.W., Boreyko,J.B., Razooky,B.S., Retterer,S.T., Collier,C.P. and Simpson,M.L. (2017) Resource sharing controls gene expression bursting. *ACS Synth. Biol.*, 6, 334–343.

68. van Nies,P., Nourian,Z., Kok,M., van Wijk,R., Moeskops,J., Westerlaken,I., Poolman,J.M., Eelkema,R., van Esch,J.H., Kuruma,Y. et al. (2013) Unbiased tracking of the progression of MRNA and protein synthesis in bulk and in liposome-confined reactions. *Chembiochem*, 14, 1963–1966.

69. Szoka,F. and Papahadjopoulos,D. (1980) Comparative properties and methods of preparation of lipid vesicles (liposomes). *Annu. Rev. Biophys. Bioeng.*, 9, 467–508.

70. Yamaji,K., Kanai,T., Nomura,S.-I.M., Akiyoshi,K., Negishi,M., Chen,Y., Atomi,H., Yoshikawa,K. and Imanaka,T. (2009) Protein synthesis in giant liposomes using the in vitro translation system of thermococcus kodakaraensis. *IEEE Trans. Nanobiosci.*, 8, 325–331.

71. Nishikawa,T., Sunami,T., Matsuura,T., Ichihashi,N. and Yomo,T. (2012) Construction of a gene screening system using giant unilamellar liposomes and a fluorescence-activated cell sorter. *Anal. Chem.*, 84, 5017–5024.

72. Nishimura,K., Tsuru,S., Suzuki,H. and Yomo,T. (2015) Stochasticity in gene expression in a cell-sized compartment. *ACS Synth. Biol.*, 4, 566–576.

73. Okano,T., Matsuura,T., —— and Yomo,T. (2014) Cell-free protein synthesis in a microchamber revealed the presence of an optimum compartment volume for high-order reactions. *ACS Synth. Biol.*, 3, 347–352.

74. Karig,D.K., Jung,S.-Y., Srijanto,B., Collier,C.P. and Simpson,M.L. (2013) Probing cell-free gene expression noise in femtoliter volumes. *ACS Synth. Biol.*, 2, 497–505.

75. Ito,H., Yamanaka,T., Kato,S., Hamada,T., Takagi,M., Ichikawa,M. and Yoshikawa,K. (2013) Dynamical formation of lipid bilayer vesicles from lipid -coated droplets across a planar monolayer at an oil/water interface. *Soft Matter*, 9, 9539–9547.

76. Pietrini,A.V. and Luisi,P.L. (2004) Cell-free protein synthesis through solubilisate exchange in water/oil emulsion compartments. *Chembiochem*, 5, 1055–1062.

77. Fiordemondo,D. and Stano,P. (2007) Lecithin-based water-in-oil compartments as dividing bioreactors. *Chembiochem*, 8, 1965–1973.
78. Gallo,V., ⸺ and Luisi,P.L. (2015) Protein synthesis in sub-micrometer water-in-oil droplets. *Chembiochem*, 16, 2073–2079.
79. Tsuji,A. and Yoshikawa,K. (2010) Real-time monitoring of RNA synthesis in a phospholipid-coated microdroplet as a live-cell model. *Chembiochem*, 11, 351–357.
80. Weitz,M., Kim,J., Kapsner,K., Winfree,E., Franco,E. and Simmel,F.C. (2014) Diversity in the dynamical behaviour of a compartmentalized programmable biochemical oscillator. *Nat. Chem.*, 6, 295–302.
81. Courtois,F., Olguin,L.F., Whyte,G., Bratton,D., Huck,W.T.S., Abell,C. and Hollfelder,F. (2008) An integrated device for monitoring time-dependent in vitro expression from single genes in picolitre droplets. *Chembiochem*, 9, 439–446.
82. Matsuura,T., Kazuta,Y., Aita,T., Adachi,J. and Yomo,T. (2009) Quantifying epistatic interactions among the components constituting the protein translation system. *Mol. Syst. Biol.*, 5, 297.
83. Frazier,J.M., Chushak,Y. and Foy,B. (2009) Stochastic simulation and analysis of biomolecular reaction networks. *BMC Syst. Biol.*, 3, 64.
84. Karzbrun,E., Shin,J., Bar-Ziv,R.H. and Noireaux,V. (2011) Coarse-grained dynamics of protein synthesis in a cell-free system. *Phys. Rev. Lett.*, 106, 048104.
85. Stögbauer,T., Windhager,L., Zimmer,R. and Rädler,J.O. (2012) Experiment and mathematical modeling of gene expression dynamics in a cell-free system. *Integr. Biol.*, 4, 494–501.
86. Matsuura,T., Tanimura,N., Hosoda,K., Yomo,T. and Shimizu,Y. (2017) Reaction dynamics analysis of a reconstituted *Escherichia coli* protein translation system by computational modeling. *Proc. Natl. Acad. Sci. USA*, 114, E1336–E1344.
87. Mavelli,F., Marangoni,R. and Stano,P. (2015) A simple protein synthesis model for the PURE system operation. *Bull. Math. Biol.*, 77, 1185–1212.
88. Stano,P., D'Aguanno,E., Bolz,J., Fahr,A. and Luisi,P.L. (2013) A remarkable self-organization process as the origin of primitive functional cells. *Angew. Chem. Int. Ed. Engl.*, 52, 13397–13400.
89. Souza,T.P., Fahr,A., Luisi,P.L. and Stano,P. (2014) Spontaneous encapsulation and concentration of biological macromolecules in liposomes: an intriguing phenomenon and its relevance in origins of life. *J. Mol. Evol.*, 79, 179–192.
90. ⸺, Steiniger,F., Stano,P., Fahr,A. and Luisi,P.L. (2011) Spontaneous crowding of ribosomes and proteins inside vesicles: a possible mechanism for the origin of cell metabolism. *ChemBioChem*, 12, 2325–2330.
91. ⸺, Stano,P., Steiniger,F., D'Aguanno,E., Altamura,E., Fahr,A. and Luisi,P.L. (2012) Encapsulation of ferritin, ribosomes, and ribo-peptidic complexes inside liposomes: insights into the origin of metabolism. *Orig. Life Evol. Biosph.*, 42, 421–428.
92. D'Aguanno,E., Altamura,E., Mavelli,F., Fahr,A., Stano,P. and Luisi,P. (2015) Physical routes to primitive cells: an experimental model based on the spontaneous entrapment of enzymes inside micrometer-sized liposomes. *Life*, 5, 969–996.
93. van Hoof,B., Markvoort,A.J., van Santen,R.A. and Hilbers,P.A.J. (2012) On protein crowding and bilayer bulging in spontaneous vesicle formation. *J. Phys. Chem. B*, 116, 12677–12683.
94. Paradisi,P., Allegrini,P. and Chiarugi,D. (2015) A renewal model for the emergence of anomalous solute crowding in liposomes. *BMC Syst. Biol.*, 9, S7.
95. Thellier,M., Legent,G., Norris,V., Baron,C. and Ripoll,C. (2004) Introduction to the concept of functioning-dependent structures in living cells. *C. R. Biol.*, 327, 1017–1024.
96. Sugiura,S., Kuroiwa,T., Kagota,T., Nakajima,M., Sato,S., Mukataka,S., Walde,P. and Ichikawa,S. (2008) Novel method for obtaining homogeneous giant vesicles from a monodisperse water-in-oil emulsion prepared with a microfluidic device. *Langmuir*, 24, 4581–4588.
97. Ota,S., Yoshizawa,S. and Takeuchi,S. (2009) Microfluidic formation of monodisperse, cell-sized, and unilamellar vesicles. *Angew. Chem. Int. Ed. Engl.*, 48, 6533–6537.
98. Matosevic,S. and Paegel,B.M. (2011) Stepwise synthesis of giant unilamellar vesicles on a microfluidic assembly line. *J. Am. Chem. Soc.*, 133, 2798–2800.
99. Martino,C. and de Mello,A.J. (2016) Droplet-based microfluidics for artificial cell generation: a brief review. *Interface Focus*, 6, 20160011.
100. Elani,Y. (2016) Construction of membrane-bound artificial cells using microfluidics: a new frontier in bottom-up synthetic biology. *Biochem. Soc. Trans.*, 44, 723–730.
101. Ho,K.K.Y., Lee,J.W., Durand,G., Majumder,S. and Liu,A.P. (2017) Protein aggregation with poly(vinyl) alcohol surfactant reduces double emulsion-encapsulated mammalian cell-free expression. *PLoS One*, 12, e0174689.
102. Deshpande,S. and Dekker,C. (2018) On-chip microfluidic production of cell-sized liposomes. *Nat. Protoc.*, 13, 856–874.
103. ⸺, Spoelstra,W.K., van Doorn,M., Kerssemakers,J. and Dekker,C. (2018) Mechanical division of cell-sized liposomes. *ACS Nano*, 12, 2560–2568.
104. Leduc,P.R., Wong,M.S., Ferreira,P.M., Groff,R.E., Haslinger,K., Koonce,M.P., Lee,W.Y., Love,J.C., McCammon,J.A., Monteiro-Riviere,N.A. et al. (2007) Towards an in vivo biologically inspired nanofactory. *Nat. Nanotechnol.*, 2, 3–7.
105. Luisi,P.L., Walde,P. and Oberholzer,T. (1999) Lipid vesicles as possible intermediates in the origin of life. *Curr. Opin. Colloid Interface Sci.* 4, 33–39.
106. Jia,H., Heymann,M., Bernhard,F., Schwille,P. and Kai,L. (2017) Cell-free protein synthesis in micro compartments: building a minimal cell from biobricks. *New Biotechnol.*, 39, 199–205.