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The Minimal Size of Liposome-Based Model Cells Brings about a Remarkably Enhanced Entrapment and Protein Synthesis

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The question of the minimal size of a cell that is still capable of endorsing life has been discussed extensively in the literature, but it has not been tackled experimentally by a synthetic-biology approach. This is the aim of the present work; in particular, we examined the question of the minimal physical size of cells using liposomes that entrapped the complete ribosomal machinery for expression of enhanced green fluorescence protein, and we made the assumption that this size would also correspond to a full fledged cell. We found that liposomes with a radius of about 100 nm, which is the smallest size ever considered in the literature for protein expression, are still capable of protein expression, and surprisingly, the average yield of fluorescent protein in the liposomes was 6.1-times higher than in bulk water. This factor would become even larger if one would refer only to the fraction of liposomes that are fully viable, which are those that contain all the molecular components (about 80). The observation of viable liposomes, which must contain all macromolecular components, indeed represents a conundrum. In fact, classic statistical analysis would give zero or negligible probability for the simultaneous entrapment of so many different molecular components in one single 100 nm radius spherical compartment at the given bulk concentration. The agreement between theoretical statistical predictions and experimental data is possible with the assumption that the concentration of solutes in the liposomes becomes larger by at least a factor twenty. Further investigation is required to understand the over-concentration mechanism, and to identify the several biophysical factors that could play a role in the observed activity enhancement. We conclude by suggesting that these entrapment effects in small-sized compartments, once validated, might be very relevant in the origin-of-life scenario.

Introduction

In recent years the notion of the "minimal cell", as a form of minimal life, has gained considerable attention both from the theoretical and experimental point of views.[1–5] This concept is important for assessing the minimal and sufficient conditions for cellular life, and also for gaining insight about the early cells, which were conceivably much simpler than the modern cells. Moreover, in addition to basic aspects, the research on minimal cells could be useful for developing simple cellular reactors for biotechnological applications.[6–8]

There are two sides to the notion of the minimal cell. One is the question of the minimal genome, namely the smallest number of expressed genes that permit cellular life, and is usually seen in terms of the triad self-maintenance, reproduction and evolvability.[9] The other concerns the physical dimension of the cell and the question, namely, about the smallest size that permits cellular life. The two aspects are to some extent related. The issue of size limits of very small microorganisms has been discussed in the literature by focusing on the complexity of modern and early cells, its relation to viability, biochemical requirements, as well as physical and evolutionary constrains.[10] Microorganisms that receive many basic nutrients and metabolites from their "environment" need fewer genes, as observed with intracellular mutualists, such as *Buchnera aphidicola* str. Cc (357 genes), or host-associated parasites, such as *Mycoplasma genitalium* (482 genes). In contrast, free-living prokaryotes, such as *Bacillus subtilis* or *Escherichia coli*, the metabolism of which needs to produce all kind of low molecular weight compounds, exceed 4000 genes (4099 and 4289, respectively).[11] The dimensions of such microorganisms range from about 0.3 to 1.5 μm, which correspond to a volume of about 0.013 and 1.6 μm^3 for *M. genitalium* and *E. coli*, respectively.[12] Together with a reduction of the number of molecular species, a reduction in size is possible only if the number of copies of each species is reduced. It follows that the efficiency and the reproduction rate of hypothetical small cells should decrease. According to a stringent assumption (e.g., 1 ribosome, 1 tRNA set, 1 mRNA for each of 100 nonribosomal proteins, each present in ten copies) the diameter of a spherical cell compatible with a modern system of genome expression would be between 200 and 300 nm.[10] The reduction in size is possible only when accompanied by a parallel reduction in gene number, as evident by the fact that long double-stranded DNA cannot be packed in small compartments—an argument that suggests the use of RNA, or of single-stranded, flexible DNA, by hypothetical small cells.[13] Small cells, on the other

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hand, might be favoured under diffusion-limited growth conditions, thanks to their high surface-to-volume ratio, and also because of the fact that at a given membrane composition small cells can sustain typical values of bacterial osmotic pressure, and therefore, avoid the construction of cell walls; in turn the amount of DNA as the part devoted to the construction of cell walls would not be required.[13] In addition to these theoretical considerations, we should mention the controversial reports on "nanobacteria",[14] which are particles found in human and cow blood with dimensions of around 200 nm (diameter). It has been pointed out that the bacterial status of nanobacteria still lacks satisfactory evidence, and the term "calcifying nanoparticles" has recently been adopted to describe such bodies.[15] Other authors have reported similar tiny corpuscles, but generally it is not clear whether they are "living" in the common sense of bacterial life.[16]

It is clear from this analysis that the question about the minimal size of a cell is still open and that it is a relevant question. The clarification of such a question would be important in the field of the origin of life, as it is conceivable that the origin of cells started with minimal protocells (possibly with a radius of <100 nm) along the pathway of evolution, and it is then interesting to assess whether and to what extent small compartments can permit life. The question becomes particularly timely in the present era of synthetic biology, as it has become possible to construct in the laboratory molecular *systems* that display living (or living-like) properties, such as in the case of the semisynthetic minimal cells.[4]

The present work tackles the question of minimal-cell size; our approach is based on the work that our group[17–19] and several others are pursuing[20–24] on lipid vesicles (liposomes) as models for minimal biological cells. In particular, we will use vesicles that entrap the transcription and translation macromolecular machinery. Moreover, it is based on the argument that the minimal size of such vesicles, which are able to display protein synthesis, could also characterize the minimal physical size for a viable cell. Protein biosynthesis—although it represents only a subset of cellular minimal metabolism (~65%)—is already a process of considerable complexity, and moreover, is to date the only experimentally feasible synthetic model.

Results and Discussion

Choice of the operational conditions

The first point to clarify was the minimal dimension of the lipid vesicles we had to work with. Previous work on protein expression in lipid vesicles has been carried out with vesicles in the micrometric range,[19,20,23,24] or in giant vesicles (larger than 10 μm).[21,22] In this study, however, we used vesicles with 200 nm diameter. From the biological point of view, a diameter of around 200–300 nm would be compatible with cells that have between 250 and 450 protein-coding genes.[10] The preparation of 200 nm vesicles is possible by two classical methods: 1) extrusion[25] of larger vesicles through polycarbonate membranes with 200 nm pores, and 2) spontaneous vesicle formation by injection.[26] As our in vitro protein expression kit we used: 1) commercial *E. coli* cell extracts, which are not well defined in terms of number and concentration of components; and 2) a reconstructed transcription–translation kit from purified components. The latter system, called the PURE (protein synthesis using recombinant elements) system, has been developed by Ueda's group in Tokyo.[27] It consists of a well-defined mixture of enzymes, ribosomes, tRNAs and low molecular-weight molecules, and has already been used to accomplish compartmentalized protein synthesis in vesicles.[19,24] The composition of the PURE system used in this study, which consists of 35 enzymes plus the ribosomes and tRNA mixture, collectively comprises 83 macromolecular elements (see Table S1 in the Supporting Information). Note that the PURE system also represents the *minimal* protein-expressing system, and that further removal of its components leads to an abrupt decrease in protein biosynthesis.[27] As in previous approaches,[18–24] in order to readily follow protein expression we introduced the *egfp* gene under the control of the T7 promoter into the translation system, and focused on the expression of enhanced green fluorescent protein (EGFP).

Protein expression in small liposomes

In order to construct microcompartments able to host the coupled transcription and translation reactions, we formed lipid vesicles, in situ, in a solution containing all the molecular components needed to perform the reaction. This was accomplished by injecting a concentrated solution of POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine) in ethanol or methanol into an aqueous phase.[26]

The formation of liposomes was immediate and their size and morphology was depended on the concentration of POPC in the alcohol solution. As shown qualitatively in Figure 1A, when concentrated POPC was used, large vesicles (multilamellar, multivesicular) and often vesicle clusters were formed, which are heterogeneous in terms of lamellarity and size/shape (here called V1). This heterogeneous population of vesicles can be transformed into a homogeneous population of small vesicles (V2) by extrusion, which is a mechanical reduction of vesicle size. As an alternative procedure, injection was carried out at a lower POPC concentration. It was then possible to produce a homogeneous population of small spherical vesicles (V3).[28] We preliminarily optimised the nature of the solvent (ethanol or methanol), POPC concentration, and the injection volume, in order to get the smallest lipid vesicles, at the highest lipid concentration and the lowest alcohol content in the samples. It follows that injection of 500 or 150 mM POPC in ethanol represents the best choice to prepare V1/V2 or V3 vesicles, respectively. The problem with this procedure is that ethanol, which is present in the final vesicle system at a concentration of 3% (*v*/*v*), inevitably reduces the yield of protein production (Figure S1 in the Supporting Information). The intensity-weighted particle size distributions of V1/V2/V3 vesicles are shown in Figure 1B, in which dynamic light scattering (DLS) profiles are shown. Vesicles V3, which were obtained spontaneously by the ethanol injection method, were indistinguishable from the V2 vesicles, which were obtained by the

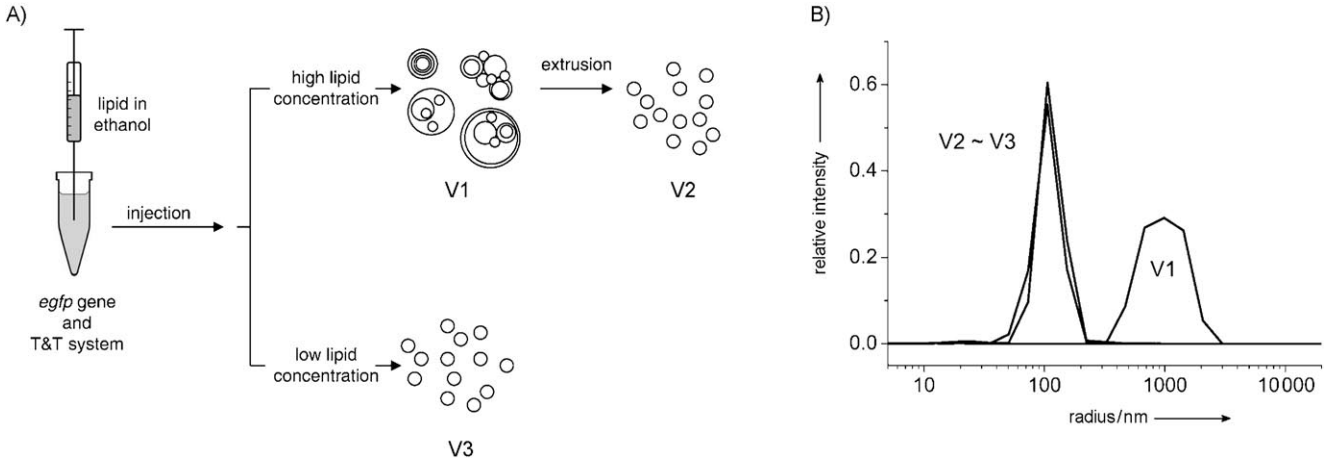

Figure 1. Experimental approach to the preparation of small vesicles (100 nm in radius). A) A small amount (3%, *v/v*) of a POPC/ethanol solution was injected into a vial containing the *egfp* gene and the transcription–translation (T&T) system. Depending on the POPC stock concentration, either a population of large (V1, multilamellar, heterogeneous, clustered) or small (V3, generally unilamellar) vesicles were obtained. Small V2 vesicles were be obtained from the V1 population by extrusion. After vesicle formation and processing, an external inhibitor was added, so that EGFP could be produced only inside the vesicles. Vesicles are not drawn to scale. B) DLS analysis of vesicles prepared by different methods. Under conditions used in this work, vesicles V2 and V3 had the same intensity-weighted size distribution.

classical extrusion method. Both consisted of a narrowly distributed population that was around 100 nm in radius (from these intensity-weighted profiles, it is possible to compute the corresponding number-weighted size distributions, which are more realistic measures of vesicles size; see the Supporting Information). Large vesicles (V1), the typical radius of which was around 500–2000 nm, were also visible by light microscopy (Figure S2). Due to the heterogeneous size and morphology of such V1 vesicles, it is difficult to calculate their entrapped volume, and interstitial entrapment cannot be ruled out. For these reasons, the quantification of synthesized EGFP "inside" such irregular compartments should be taken with care, since it does not mirror the entrapment capacity of giant unilamellar vesicles of similar size, as reported by others.[21,22] In other words, in this work V1 vesicles have been prepared only as precursors of V2 (extruded) vesicles.

A small aliquot of POPC (500 mM) in ethanol was added to the *egfp* gene/transcription–translation mixture, which was kept on ice in order to prevent the start of the reaction, while it was being stirred. In order to prevent EGFP synthesis in the solution outside the vesicles, inhibitors such as EDTA, RNase or proteinase K were immediately added after the formation of vesicles (these substances are unable to cross the lipid bilayer and therefore do not inhibit the reaction inside the liposomes). As a direct evidence of the occurrence of compartmentalized protein synthesis, we recorded the green fluorescence observed in the large V1 vesicles (Figure S2). The observed EGFP biosynthesis inside V1 vesicles demonstrates that externally added inhibitors, such as EDTA, proteinase K or RNase A, do not affect internal synthesis, but effectively act against synthesis in the external medium. The yield of EGFP in V1 vesicles was not expected to be high, because of the fact that such vesicles have low entrapped volume due to their multilamellar morphology (see below). We then tested the same reaction in smaller vesicles prepared by the extrusion procedure (Figure 1A). This assay was carried out by using purified components, that is, the PURE system. Similar results were obtained by using *E. coli* extracts (see the Supporting Information). As mentioned above, there are no reports on protein expression in 100 nm (radius) vesicles, which cannot be visualized by standard light microscopy or by flow cytometry.[20,23,24] In this case, the occurrence of EGFP synthesis must be followed by batch fluorescence measurements. Figure 2A shows the time course for the detection of green fluorescence in 100 nm (radius) POPC extruded vesicles, in the absence (curve a) or presence (curves b and c) of externally added EDTA. In the first case, EGFP synthesis outside as well as inside liposomes was observed, whereas in the latter cases, the fluorescence increase corresponded to the synthesis of EGFP inside vesicles.

When EDTA was added before the formation of vesicles, no fluorescence was observed (curve d). When the reaction was complete, the emission spectrum of vesicle samples was recorded in the presence of sodium cholate to eliminate vesicle scattering (Figure 2B). The emission maximum of 511 nm and the peak shape further confirm the occurrence of EGFP expression. Finally, the size of the vesicles was measured by DLS analysis and the results revealed a sharp monomodal distribution with no particles larger than 200 nm in radius (Figure 2C). These data collectively show that EGFP was effectively produced inside small vesicles. It is possible to quantify the internal EGFP production in relative and absolute terms. The relative yield (yield%) was calculated as the ratio between fluorescence intensity in the presence and absence of the external inhibitors (internal EGFP/total EGFP). The absolute amount of EGFP was calculated instead by means of a calibration line (Figure S3) and normalized with respect to the lipid concentration. These two yield measurements are shown in Figure 2D, in which the production of EGFP is evaluated in large (V1) and small vesicles (V2) and the PURE system is compared with cell extracts. The relative EGFP yield of large V1 vesicles was

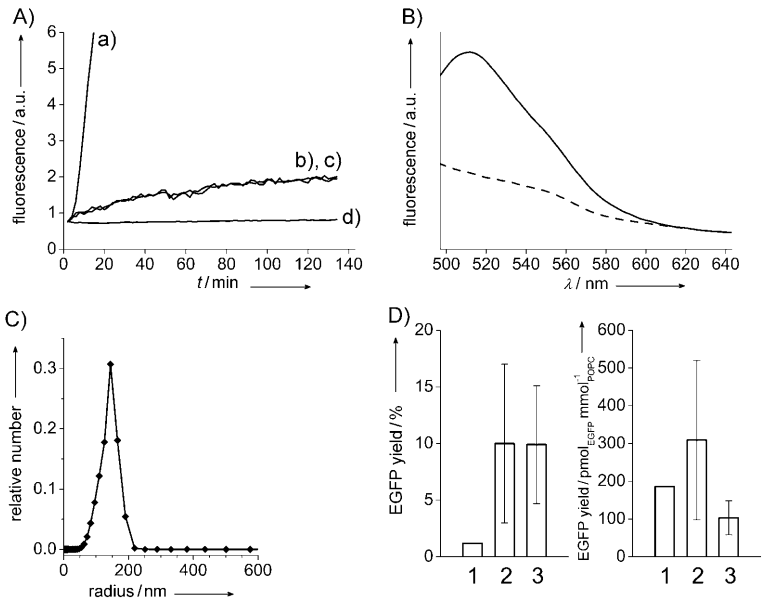

Figure 2. EGFP expression inside 200 nm (diameter) extruded V2 vesicles. A) Time profile of EGFP production; curve a: EGFP expression in the absence of externally added EDTA; curves b and c: EGFP expression inside vesicles, EDTA was added externally; curve d: negative control, EDTA was added before the formation of vesicles. B) Fluorescence emission spectra of EGFP-expressing vesicles after 3 h incubation: EGFP-expressing vesicles (continuous line); negative control (dashed line). Spectra were recorded in the presence of sodium cholate in order to eliminate scattering from the vesicles. C) Number-weighted DLS size distribution of extruded vesicles. DLS analysis was carried out at the end of the incubation period. D) Left: EGFP yield (%) was calculated as internal/(internal+external) protein production. Right: normalized EGFP yield (pmol EGFP per mmol POPC). Sample 1: POPC (10 mM) vesicles V1 (radius ~1000 nm), cell extracts (Promega); sample 2: POPC (15 mM) extruded vesicles (radius ~100 nm), cell extracts (Promega); sample 3: as in sample 2, but with the reconstituted kit (PURE system). Error bars refer to the standard deviation of two or three different experiments; for details see Table S2.

around 1.2%, which was calculated with respect to total EGFP yield in the aqueous phase. The multilamellar/multivesicular nature of these large irregular vesicles (or vesicle clusters) could account for the poor EGFP yield, especially when compared to other studies carried out with giant unilamellar vesicles.[21,22] The V2 vesicles, despite their small individual volume and irrespective of the transcription–translation system used, produced EGFP with relative yields of about 10%. The cumulative internal volume of all vesicles, which was calculated under the hypothesis of spherical shape and at the concentration of POPC (15 mM) used in these experiments, was also about 10% in both cases. Concerning the absolute yield, it was possible to estimate that inside small vesicles the PURE system and *E. coli* extracts produced 103 and 309 pmol EGFP per mmol POPC, respectively. These yields correspond to 5–10 EGFP molecules per 100 vesicles (considered unilamellar). The higher yield obtained with the *E. coli* extracts is in all likelihood due to the fact that it contains a much greater number of enzymes and other components compared to the minimal number of constituents in the PURE system. As evident from the large error bars of Figure 2D, experiments were often characterized by a quite variable output, probably due to the intrinsically stochastic nature of solute entrapment and confined reactivity.

The preparation method described above starts with the encapsulation of the transcription–translation components in large vesicles, which are then forced to divide by shearing forces (extrusion). Alternatively, small vesicles can be prepared directly in one step by the injection method to yield V3 vesicles (Figure 1A), which are indistinguishable from extruded V2 vesicles. Figure 3A shows the fluorescence time course for vesicles prepared by the multiple injection method in the presence of external inhibitor (curve a). When the inhibitor was added to the transcription–translation system before the formation of liposomes, curve b was obtained. The size of the vesicles was measured by DLS analysis, and the results indicate that their radius was always below 220 nm (Figure 3B). The amount of EGFP produced in this case was lower. In particular, the relative yield with *E. coli* cell extracts entrapped in vesicles (radius: 124 nm) was (0.3±0.1)%, which corresponds to about 27 pmol EGFP per mmol POPC. In addition, EGFP produced by the reconstituted PURE system could not be detected. Therefore, the maximal yield we could measure corresponds to one EGFP molecule per 100 vesicles. Very probably, there are few vesicles synthesizing EGFP, so that a realistic picture based on individual vesicles might substantially differ from average values. For example, results assumed to be one EGFP molecule expressed per 100 vesicles might actually be ten EGFP molecules synthesized inside one vesicle out of 1000. (A summary of yields for all systems tested in this study can be found in Table S2.)

In conclusion, it was possible to show that ribosomal protein synthesis can take place in compart-

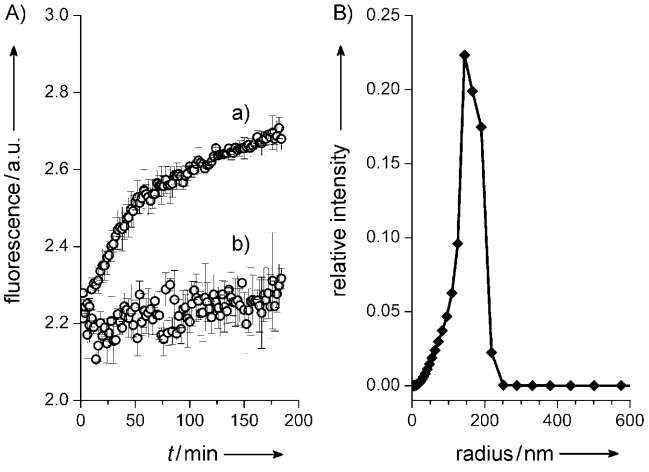

Figure 3. EGFP expression inside 200 nm (diameter) spontaneously formed V3 vesicles. A) Time profile of EGFP production; curve a: EGFP expression inside vesicles, EDTA was added externally; curve b: negative control, EDTA was added before the formation of vesicles. Error bars represent the standard deviation of three independent samples (curve a) and two negative controls (curve b). B) Number-weighted DLS size distribution of vesicles obtained by the injection method. DLS analysis was carried out at the end of the incubation period.

ments that have a radius as small as 100 nm; these were formed either by large vesicles that were then reduced to smaller ones, or by directly forming small vesicles by the injection method. Because these vesicles are capable of hosting protein biosynthesis, we suggest that this can be taken as the first experimental evidence that a dimension of about 200 nm can be compatible with full-fledged cells.

The "conundrum" of the multiple entrapment

Data presented in the previous section provide clear evidence of the ribosomal synthesis of protein inside liposomes with a radius of about 100 nm. Although this was clearly observed, the existence of this fact is not self evident from a mere theoretical point of view. We would now like to dwell upon this "entrapment conundrum", which indeed represents one of the most interesting aspects of the work with liposomes. The starting consideration is that, since POPC liposomes do not fuse with each other,[29] and nucleic acids, proteins, and small charged molecules cannot cross the liposome membrane,[30] all required components must be present inside every EGFP-producing vesicle from the moment of its formation. The PURE system—as the simplest transcription–translation kit capable of protein synthesis—is composed of 83 different components (enzymes, ribosomes and tRNAs, plus the *egfp* gene; Table S1), so that the inescapable conclusion from our work is that we have obtained vesicles that have entrapped more than eighty different macromolecular components. *E. coli* cell extracts certainly contain more components, but we can base our considerations on the minimal number of the PURE system.

The internal volume of a 100 nm (inner radius) vesicle is about (4.2×10^6) nm^3, so that there is enough space to accommodate several bulky ribosomes ($\sim$1000 nm^3) and all enzymes, such as the complete set of aminoacyl-tRNA synthetases, and translation factors, low-molecular-weight compounds etc., in several copies. However, the key point is not the lack of sufficient space, rather the adverse co-entrapment statistics of all components in the same lipid vesicle. We are dealing with the probability of entrapping 83 different macromolecular components (i.e., the PURE system) inside one single compartment, whereby under our set of experimental conditions only random statistics should govern the entrapment. Let us calculate such a probability by assuming that the entrapment events follow the Poisson distribution (justification and discussion are given in the Supporting Information). The probability, $\wp_c$, of finding a lipid vesicle that contains at least one molecule of each of the 83 species is given by Equation (1):

$$\wp_c(R) = \prod_{k=1}^{N} [1 - \exp(-C_k V(R))] \qquad (1)$$

where the product is extended over $N = 83$ species, and C_k are the numerical concentrations of the k^{th} species, and V is the vesicle volume. The dependence of the probability and of the volume on vesicle radius (R) has been shown explicitly. The probability function [Eq. (1)] is very useful for analyzing protein expression in small (V2 or V3) vesicles. Probability versus radius is illustrated in Figure 4, in which the three different solid curves show the probability of formation of a "viable" liposome as a function of its size, under the assumption that at least one (curve a), five (curve b) or ten (curve c) molecules of each molecular species are present inside a liposome of a given size. The calculation clearly shows what we have called the "entrapment conundrum". In fact, according to these curves, for vesicles with a radius below about 250 nm, the probability of simultaneous co-entrapment of the transcription–translation components should be vanishingly small. For example, in the case of 100 nm radius vesicles (our V2 and V3 systems), the probability of independent entrapment of at least one copy of the 83 components (each present at the concentrations used in this study) is 10^{-26}. In contrast, larger vesicles—for example, those with a radius of 1 μm—have a probability $\sim$1 in the three cases (note that expression of EGFP in extruded V2 vesicles also cannot be easily justified, despite the fact that they derive from large V1 vesicles; Figure S4).

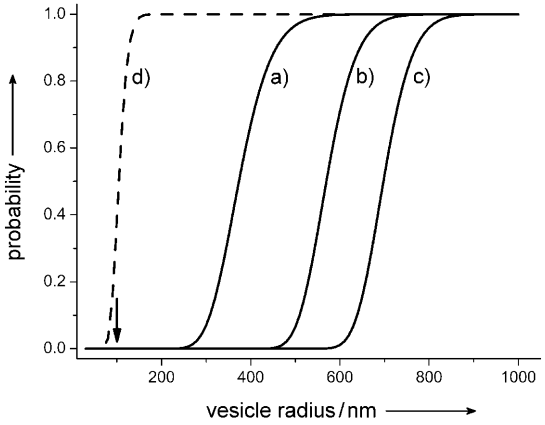

Figure 4. Probability of co-entrapment of all macromolecular components of the transcription–translation kit inside lipid vesicles of a given radius. The entrapment of each molecule was modelled as a Poissonian process, and the cumulative probability was calculated as product of probabilities of independent events. The concentrations of enzymes, tRNA, and other factors correspond to those of the PURE system. The arrow points to the vesicle size used in these experiments. The three solid curves indicate the probability of entrapping at least one (curve a), five (curve b) or ten (curve c) copies of each molecular species inside the same vesicle. The dashed curve d indicates the probability of entrapping at least one copy of each molecular species, under the assumption that their concentrations are all 50-times higher than the nominal (bulk) concentrations.

The argument of association between molecules does not hold in this case. In fact, we excluded the possibility of massive association of enzymes, tRNA and other components in solution, by measuring the size of the particles present in the reaction mixture. DLS measurement of the initial bulk PURE system in solution showed no aggregates larger than ribosomes (Figure S5). Despite this, we also calculated the probability curve under the assumption that the 83 components were associated in 50, 20 or 10 molecular clusters (Figure S6). In no case did the probability reach experimentally significant values (note

also that the enzymes present in the PURE system are water soluble). What can be then the explanation for the contradiction between experimental facts, and the curves in Figure 4? It is interesting to note that this question has not been raised before in the literature, except by us in a very tentative way.[31,32] The entrapment conundrum is accompanied by another interesting anomaly: the higher efficiency of protein expression inside liposomes compared to in bulk water. Previously data on the enhanced rate of EGFP production in compartments have also been reported.[21] In our case, the *average* concentration of EGFP produced inside V2/V3 vesicles was 28 nM (see the third and seventh columns of Table S2 in the Supporting Information; V3 vesicles were not considered), whereas the typical bulk EGFP production under similar conditions (in the presence of liposomes and 3% (*v/v*) ethanol) was about 4.6 nM (we carried out EGFP expression in bulk aqueous phase in an independent experiment). It follows that the expression of a protein occurs about six times more efficiently inside small liposomes than in an equal volume of noncompartmentalized bulk water. This calculation is based on the assumption that all liposomes contribute equally to EGFP expression. Most likely, only a fraction of the liposomes will be fully viable (containing all 83 macromolecular components plus small molecules), and accordingly, the efficiency will have referred only to this fraction and will be higher than the factor six mentioned above (we are not capable at the moment to determine which percentage of the liposomes are active). For these reasons, although V3 vesicles were not considered in the average, it can be stated that the EGFP yield for an individual V2/V3 vesicle reaches (and exceeds) the average value stated above (28 nM). How can we explain all this, namely the abnormal entrapment efficiency and the higher activity? The most interesting possibility is that entrapment of the components is accompanied by concentration enrichment, so that the concentration of the components inside the vesicles is larger than the bulk. This would mean that the C_k values in Equation (1) are larger than we assumed them to be. How much larger should they be in order for the probability of total entrapment to reach finite probability values? For example, Equation (1) gives probability values equal to 1% only if we assume local enzyme concentrations 20-times higher than nominal bulk concentrations. Higher probability values correspond to higher enhancement factors, up to 50× (~40%) or 100× (~90%; Figure 4, dashed curve d, and Figure S7). This very high local concentration would also provide the simplest rationale for the super-activity. The mechanism of concentration enrichment inside vesicles cannot be explained with the present data. Our working hypotheses, which will guide future work, focus on the possible cooperative expulsion of water during solute entrapment, so that there might be an increase in local concentration [Eq. (1)]. This is also related to possible depletion effects[33] and consequent internal crowding in view of the fact that the 100 nm (inner radius) vesicles have a confinement volume of 4.2 aL (10^{-18} L), or a simultaneous enhancement of reactivity in confined systems. Further study will be devoted to provide a physical explanation of these not yet clarified aspects of vesicle systems.

More generally, however, all these considerations open new perspectives to the involvement of a closed membrane in the reactivity of cell models and most probably, by inference, of biological cells, and might therefore shed a new light on the importance of (micro)compartmentation effects in the origin-of-life scenarios—a notion which, on the basis of self-reproducing micelles and vesicles, has long been emphasized.[34,35] Clearly, an in-depth investigation is needed at this point to validate all these effects of compartmentation and to study their mechanisms, for example, as a function of liposome size and/or type and amount of solute molecules. Similarly, membrane-association effects could also play a role and deserve special investigation. This is now in progress in our group with different types of solutes (with or without macromolecules) in differently sized vesicles. Furthermore, studies on entrapped simpler metabolic routes will allow more detailed work, such as the possible rate enhancement of compartmentalized reactions.

Experimental Section

Materials: 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC) was from Avanti (Alabaster, AL, USA). The plasmid pWM-T7-EGFP (3026 bp) was from Biotecton (Zürich, Switzerland). Recombinant EGFP was from BD Bioscience (Basel, Switzerland). The cell-free expression kit (code: L1130: *E. coli* T7S30 extract system for circular DNA) and the amino acid mixture (code: L4461) were from Promega. The transcription–translation kit composed of purified components (PURESYSTEM® Classic) was from the Post-Genome Institute Co., Ltd. (Tokyo, Japan) and from the laboratory of Prof. T. Ueda (University of Tokyo). Acetic acid, Tris base, KOH, sodium cholate, EDTA and ethanol were from Sigma–Aldrich. RNase A and proteinase K were from Clontech. Bio-Gel A50m gel was from BioRad (#151-1340). Trp-Trp-Trp was from Bachem (Bubendorf, Switzerland). All solutions and dilutions were done by using sterile ultrapure water from Pharminvest S.p.A. (Milan, Italy). Multicore-like buffer (MC buffer) was prepared by KOH titration (pH 7.5) of a mixture of acetic acid/Tris base (125 mM/25 mM).

Method summary: Mixing of transcription–translation elements, preparation of vesicles and vesicle manipulations were all done at 4 °C and samples were kept constantly on ice in order to inhibit the premature start of transcription–translation reactions. All equipment used to manipulate or process vesicles were placed at 4 °C at least 30 min before experimentation.

The components of the cell-free protein expression kit (Promega or PURE system) were mixed in an Eppendorf tube placed on ice, and the liposomes were then formed in situ by using the ethanol injection method. Following the indications of the manufacturer, we mixed for the Promega kit: S30 extracts (20 μL), T7S30 extracts (15 μL), amino acid complete mixture (10 μL) and pWM-T7-EGFP plasmid (2.0 μg); and for the PURE system: solution A (25 μL), solution B (10 μL), and pWM-T7-EGFP plasmid (1 μg). In both cases, the final volume was adjusted with distilled water to 48.5 μL. In all experiments, the stock concentration of pWM-T7-EGFP plasmid in pure water was adjusted to 1 μg μL^{-1}, so that 1 or 2 μL were added to the reaction mixture.

The expression of EGFP in the water phase was carried out first, also in the presence of ethanol or methanol (Figure S1). In order to compare the internalized yield and the bulk-reaction yield, we determined the yield of EGFP production in the bulk phase (cor-

responding to 4.6 nM) in the presence of ethanol (3%, *v/v*) and preformed "empty" POPC (100 nm radius) extruded liposomes.

Vesicles were formed by injecting a solution of POPC in the transcription–translation mixture, and processed to form 100 nm radius vesicles. In order to inhibit protein expression, we added three different inhibitors after liposome formation: 1) EDTA (45 mM), or 2) RNase A (0.18 mg mL^{-1}), or 3) proteinase K (0.18 mg mL^{-1}). As shown in Figure S8, in negative control samples EDTA was added before the formation of liposomes; in positive control samples no inhibitor was added after the formation of liposomes (protein expression occurred inside and outside liposomes). When external protein synthesis had to be blocked, we added to the reaction mixture, on ice: 1) EDTA (5 μL; 500 mM pH 8.0), or 2) RNase A (5 μL; 2 mg mL^{-1} in MC buffer), or 3) proteinase K (5 μL; 2 mg mL^{-1} in MC buffer). The resulting EGFP expression was measured fluorometrically, and the DLS analysis was carried out at the end of the incubation time.

Method details

Preparation of vesicles by ethanol injection (general): The reaction mixtures were put in a plastic Eppendorf tube (1.5 mL) together with two Teflon-covered followers (length 5 mm, diameter 2 mm). The tube was kept in ice and put over a magnetic stirrer just before the injection. Then, a solution of POPC in ethanol (typically in the range 150–500 mM) was quickly injected by means of a 10 μL Hamilton microsyringe under vigorous stirring (about 500 rpm). The formation of liposomes was immediate. The tube was kept over the magnetic stirrer for 10 s, and then put back on ice. Prechilled inhibitor was added immediately after injection when required. The final ethanol concentration in the reacting mixture was always 3% (*v/v*), or 6% in the case of the "multiple injection" protocol (see below).

Preparation of V1 vesicles: Vesicles were prepared by using the injection method. In particular, POPC (1.5 μL, 500 mM) in ethanol was injected into the reaction mixture (48.5 μL), which was kept at 4 °C, so that the final POPC concentration was 15 mM and the ethanol fraction was 3% (*v/v*).

Preparation of V2 vesicles: V1 vesicles were extruded by being passed through two stacked polycarbonate membranes (Nuclepore Track-Etch Membrane, Whatman) with 200 and 100 nm pore size (diameter). Easier extrusion was achieved by sandwiching the two membranes with three drain discs. All operations were done at 4 °C. However, in order to further reduce the extrusion time, we used two hand extruders (Liposofast, Avestin, Mannheim, Germany). The first was equipped with two 200 nm membranes, the second with two 100 nm membranes. After extrusion through the 200 nm membranes (five times), liposomes were moved to the second extruder by means of a Hamilton syringe, and then extruded through the 100 nm membranes (three times).

Preparation of V3 vesicles: Vesicles were prepared by using the (multiple) injection method.[36] The procedure was the same as in the general description (see above). In particular, 2×1.5 μL aliquots of POPC (150 mM) in ethanol were sequentially injected into the reaction mixture (48.5 μL), which was kept at 4 °C, so that the final POPC concentration was 8.5 mM and the ethanol fraction was 6% (*v/v*). In order to reduce the ethanol content in the preparation, because it inhibits protein expression, vesicles (51.5 μL) were applied to a home-made, size-exclusion minicolumn, which was prepared by filling 1 mL disposable syringes with Bio-Gel A50m. Elution was carried out at 4 °C by adding cold MC-type buffer to the top of the column. Vesicles were collected typically as 150 μL fractions, that is, three-fold dilution occurred inside the column; the final POPC concentration was 2.8 mM. To remove small vesicle aggregates, which were detected by using DLS in preliminary experiments, prechilled H-Trp-Trp-Trp-OH (3.9 mM dissolved in MC-type buffer) was added to eluted vesicle fractions (5 μL were added to 50 μL aliquots of eluted vesicles).[37] When required, inhibitors (EDTA, RNase A or proteinase K) were added to the eluted vesicles (i.e., 5 μL of inhibitor was further added).

Note: in principle, small vesicles can also be prepared by lipid-film hydration and sonication. In this study we avoided this procedure in order to minimize potential loss of activity of the transcription–translation machinery.

Measurements: Fluorescence emission and excitation spectra were recorded with a Jasco FP-6200 spectrofluorometer, by using a 600 μL quartz cuvette (Hellma, code: 101-016QS, 5×5 mm). The following parameters were used to record emission spectra; λ_{ex}: 470 or 483 nm, λ_{em}: 490–600 nm; and for excitation spectra λ_{ex}: 400–505 nm, λ_{em}: 520 nm. Other settings were excitation and emission slits: 5 nm; response time: medium; sensitivity: high; data pitch: 1 nm; scan speed: 250 nm min^{-1}.

The fluorescence intensity of expressed EGFP was measured by using a Victor 3V plate reader, with a standard 96-well plate. Samples were distributed in the plate so that at least three empty wells were present between different samples. This instrument was more reliable than the spectrofluorometer for quantitation studies. Before fluorescence measurements, vesicles (30 μL) were transformed in micelles by the addition of sodium cholate (300 mM) solution (70 μL), incubated for 10 min, and then measured. Instrument settings: CW-lamp energy: 20000 a.u. (stabilized); excitation filter F485; excitation aperture: normal; emission filter F535; emission aperture: large; position: bottom; counting time: 0.5 s.

The kinetics of EGFP production was followed by using a RT-PCR Corbett Rotor-Gene 6000, which was used as sensitive fluorometer, and was run at constant temperature. The use of such an instrument improves reproducibility, enhances sensitivity, allows the use of small sample volumes (25 or 50 μL) and provides homogeneous heating. Vesicle samples were placed in PCR-type 200 μL Eppendorf tubes and kept on ice before the start of kinetic measurements. They were then quickly placed in the instrument rotor and the variation of fluorescence was observed over time. Instrumental settings: fluorescence channel: green (excitation 470 nm, emission 510 nm); gain 5, 7 and 10; cycle setting: 120 s at (37.0±0.5) °C; 400 rpm.

Fluorescence microscopy was performed with a Leica TCS SP5 confocal microscope; vesicles were placed on a glass stage and gently covered by a 0.17 mm coverslip which was then sealed with nail varnish.

Dynamic light scattering (DLS): Measurements were carried out with an ALV home-assembled light scattering photometer made of a 25 mW He-Ne laser (Model 127, Spectra-Physics Lasers, Mountain View, Canada), an ALV DLS/SLS-5000 Compact Goniometer System (ALV, Langen, Germany), two SPCM-AQR avalanche photodiodes (PerkinElmer Optoelectronics, Vaudreuil, Canada) and an ALV-5000 Multiple-tau Digital Correlator (ALV, Langen, Germany). The cylindrical scattering cells were immersed in a fuzzy-thermostated decaline bath (ALV), which was kept at 25.0 °C. All experiments were performed at the scattering angle of 90°; other settings were: solvent viscosity 0.899 MPa s, solvent refractive index 1.330. Great care was taken at every step to avoid the presence of dust in the liposome preparations. Liposomes (after the incubation period) were

diluted (tenfold) in isotonic buffer and measured without any pretreatment. Particle-size distribution was computed by using two different algorithms (ILT and CONTIN), which gave similar results. Number-weighted size distributions were calculated within the Rayleigh–Gans–Debye approximation, as shown in the Supporting Information.

Calibration lines where constructed to correlate fluorescence intensities recorded by the plate reader and by the RT-PCR machine to EGFP concentration, in the proper matrix; A) plate reader: EGFP, transcription–translation matrix, POPC (8.5 or 15 mM) extruded vesicles (100 nm radius), sodium cholate (150 mM); B) RT-PCR machine: EGFP, transcription–translation system, POPC (8.5 or 15 mM) extruded vesicles (100 nm radius). The presence of sodium cholate was necessary in order to reduce the scattering of the sample. Sodium cholate was not added to the RT-PCR calibration line since the RT-PCR reading was used to monitor EGFP expression in real time, in the presence of vesicles. Preliminary experiments have shown that sodium cholate does not modify EGFP fluorescence under the experimental conditions used. Fluorescence values were dependent on sample volumes. The amount of EGFP was calculated by means of the following calibration lines:

A) plate reader: $F_{net} = 3.75 \times 10^4\ C_{EGFP}$ (100 μL)

B) RT-PCR machine: $F_{net} = 8.46\ C_{EGFP}$ (50 μL; gain = 5)

B′) RT-PCR machine: $F_{net} = 127\ C_{EGFP}$ (50 μL; gain = 10)

where F_{net} is the difference between the fluorescence of EGFP-expressing liposomes and the negative control, and C_{EGFP} is the EGFP concentration ($\mu g\,mL^{-1}$).

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