



# A Remarkable Self-Organization Process as the Origin of Primitive Functional Cells\*\*

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Research into the origin of life has often focused on the chemistry of self-replication,<sup>[1–3]</sup> whereas less attention has been devoted to experiments that might reveal the emergence of cells as compartmentalized and dynamic chemical systems. Modern views consider lipid vesicles (liposomes) as the most plausible model of primitive cellular systems;<sup>[4–6]</sup> however, experimental investigations into liposome formation and solute entrapment, a key event for the origin of cells, has not lead to an explanation for the assembly of a functional genetic–metabolic network inside liposomes to date.

One way to tackle the question about the origin of cells is to carry out experimental research on simplified chemical systems. Such models should be complex enough so as to display a simple-cell-like behavior; however, they should be well-characterized in terms of composition and function.

As a viable model for investigating the onset of “minimal” metabolism inside liposomes, we investigated protein synthesis by cell-free transcription and translation (TX-TL) systems. These multimolecular systems contain a total of about 80 different macromolecular components, including RNA polymerase, ribosomes, tRNAs, aminoacyl-tRNA synthetases, translation factors, and bioenergy-related enzymes (as well as low-molecular-weight compounds such as amino acids and nucleotides), and are capable of synthesizing a protein starting from the corresponding DNA sequence.

In a TX-TL system, such as a commercially available *E. coli* cellular extract or the well-characterized PURE system<sup>[7]</sup> (a reconstituted kit that contains the minimal number of molecules necessary for TX-TL activity), a functional protein is produced as soon as the corresponding DNA sequence is supplied to the mixture. This happens because the commercial TX-TL mixture is concentrated, as it comes directly from a cell extract. The real question then is, how such a critical concentration of the components might have been reached under prebiotic conditions. In fact, when we consider a prebiotic solution and assume that the macromolecular components have somehow developed by themselves, the solution in a primitive marine or lagoon environment can only be highly diluted and therefore unreactive.

We reasoned that such an accumulation of components could occur within liposomes, which might thus provide an active role in concentrating the TX-TL molecules, as we reported for a simple case of single-species entrapment (ferritin<sup>[8]</sup> or ribosomes<sup>[9]</sup> were used as model compounds). In other words, we propose a scenario where the formation and closure of lipid membranes to form liposomes could have been the driving force for a high local concentration (overcrowding) of diluted solutes, an event that, in principle, may have led to the onset of metabolism in primitive cells.

Herein, we therefore focus on the investigation of the synthesis of proteins inside liposomes, which are formed in situ in the presence of a diluted TX-TL mixture. Such a dilute, non-reactive solution simulates a possible origin-of-life scenario in a marine or fresh-water lagoon, where the components of life (DNA, RNA, and proteins) have formed independently, but cannot react because of the extremely high dilution. In particular, we address the question whether the spontaneous formation of liposomes might bring about a spontaneous concentration of all components of the mixture inside vesicles, so that protein synthesis proceeds efficiently within these cell-like compartments, whereas the same reaction does not proceed in free solution (Figure 1).

The original TX-TL mixture already becomes unreactive when it is diluted with a buffer in a 1:1 ratio (for details, see the Supporting Information). A small aliquot of a phosphatidylcholine–ethanol solution is now injected into this dilute and unreactive TX-TL mixture to form liposomes of various size and morphology by spontaneous self-assembly. In particular, we used 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC), a well-characterized phospholipid that has been extensively used in similar protein-synthesis experiments, because of its stability and minimal interference with TX-TL reaction, as well as its low melting temperature (ca. –4 °C). POPC vesicles entrap the components of the TX-TL mixture during their formation, whereby the macro-

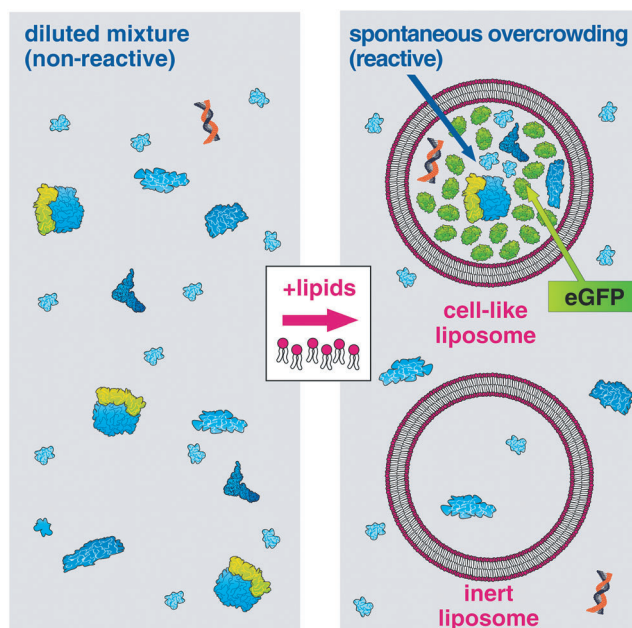
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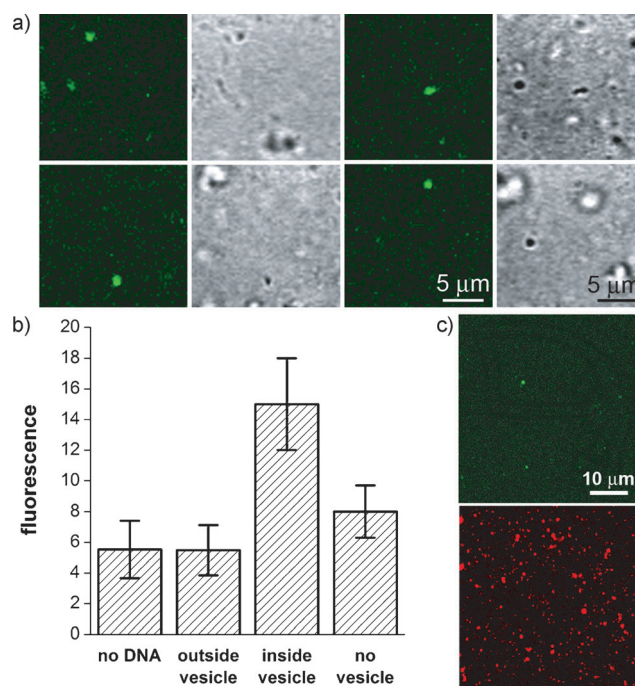
Supporting information for this article is available on the WWW under <http://dx.doi.org/10.1002/anie.201306613>.



**Figure 1.** An experimental model for the origin of cellular metabolism. Lipid vesicles (liposomes) are formed in a diluted, sluggishly reacting (or unreactive) functional macromolecular mixture that simulates diluted primitive aqueous solutions. Because of lipid self-assembly, liposomes form spontaneously in this solution and entrap, also spontaneously, the available solutes, which gives rise to “protocells”. Under these diluted conditions, the formation of inactive compartments is expected. A small but measurable number of solute-filled liposomes form spontaneously against the statistical expectation, which gives rise to cell-like systems where the high local (intraliposome) solute concentration triggers the onset of protocell metabolism. The transcription–translation (TX–TL) biomolecular machinery was taken as a model of complex cellular function.

molecular species cannot escape through the POPC bilayer because of their large size. Similarly, non-entrapped macromolecules cannot enter the vesicle. We incubated the sample for 2–4 h at 37°C to allow the synthesis of enhanced green fluorescent proteins (eGFPs) inside and outside vesicles. Aside from small vesicles, the sample contained large vesicles, the size of which generally ranges from approximately 0.7–2  $\mu\text{m}$ , which could be well visualized by confocal microscopy.

Typical images of the vesicles in the sample are shown in Figure 2a. Green fluorescent vesicles are clearly visible against a dark background, which indicates that eGFP is effectively produced inside the vesicles, whereas the same reaction does not proceed at a measurable rate in the free solution. In control experiments, we confirmed the fluorescence versus concentration profiles to be linear. Deviations of the inner filter effect are negligible because of the short path length of the specimen ( $< 50 \mu\text{m}$ ). A quantitative comparison of the external fluorescence with a negative control sample (a similarly diluted TX–TL mixture prepared in the absence of DNA) confirmed that eGFP is not produced outside the vesicles, or, strictly speaking, that its concentration remains below the detection limit (Supporting Information, Figure S1). The resulting structure, a liposome compartment containing the biochemical machinery most important for life, can be seen as good model for primitive cells that form



**Figure 2.** Liposomes that synthesize eGFP are prepared by the ethanol injection method. a) Confocal microscopy images of POPC liposomes (3.3 mM) prepared by the ethanol injection method in the presence of a diluted PURE system solution ( $\times 0.65$ ). Bright vesicles are visible against a dark background, which indicates intraliposomal eGFP synthesis. Images have been modified for higher contrast (see the Supporting Information for details). Additional images of fluorescent liposomes that were prepared by other methods, and in the presence of cell extracts and different dilution factors are presented in Figure S6. b) Quantitation of eGFP fluorescence in negative-control samples (no DNA), in samples of the bulk solutions (extra-liposome), in liposomes, and in the absence of liposomes. c) Nile-Red staining (bottom panel) of eGFP producing liposomes (top panel) reveals that the large majority of liposomes do not synthesize the protein.

spontaneously by a self-organization process. In other words, the mechanism of vesicle self-assembly might promote the spontaneous concentration of solutes within these microcompartments. This allows and triggers biochemical reactions that would not occur in diluted solutions. The on/off behavior that follows from the remarkable enhancement in the yield of protein synthesis that is induced by the high concentration of solutes inside liposomes is clearly shown in Figure 2b. The presence of POPC liposomes hardly affects the eGFP synthesis.

The number of green fluorescent vesicles (eGFP synthesizing) within the sample was quite low. To estimate the fraction of green fluorescent vesicles within the vesicle population, we stained the vesicles with a red-fluorescent dye (Nile Red), as shown in Figure 2c. Image analysis reveals that less than approximately 0.5% of the vesicles display an overcrowding of the components of the TX–TL kit. This percentage, even if low, sharply contrasts with the statistical expectation value, which is essentially zero (Supporting Information, Table S1). The probability of co-entrapping 83 different macromolecules (the TX–TL components of the PURE system), considered as a product of independent

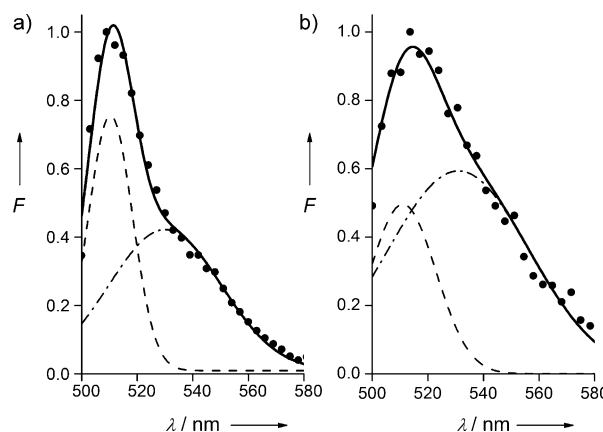
events, is our null-hypothesis against which we contrasted our experimental data. As it is expected that the TX-TL components interact dynamically with each other, and perhaps form hyperstructures,<sup>[10,11]</sup> we have also calculated the co-entrapment probability for 40 or 20 different macromolecular complexes that originate from macromolecular associations within the TX-TL kit. The expected values would not explain the observations under any circumstances. Expected probabilities become similar to experimentally observed frequencies only if few and very large complexes between the TX-TL components are considered (such as five or ten large complexes).

Alternatively, in a scenario where protein synthesis would depend on the concentration of only a few key components, spontaneous two-fold concentration of these components could also explain the data. Poisson statistics suggest that components that are present at the lowest concentration could play such role (for example, DNA), or components, whose activity is critical for the success of protein synthesis (for example, ribosomes).

On the other hand, the amount of eGFP-producing vesicles (0.5 %) found is similar to the amount of super-filled vesicles found in the case of ferritin and ribosomes entrapment,<sup>[8,9]</sup> which suggests that the encapsulation of the multicomponent TX-TL kit could also follow a power-law distribution (as for ferritin and ribosomes), rather than a Poisson distribution. It is clear that further evidence is required to establish what is the best suitable statistical model for a comparison with the experimental data.

In comparative experiments, POPC vesicles were formed by the hydration of thin films deposited on glass beads (2 mm), with or without an extrusion step (800 nm). Similar results were observed in all cases, both for *E. coli* cell extracts and the PURE system. The full set of experimental conditions and typical results can be found in the Supporting Information. Aside from POPC, vesicles were also prepared from a sodium oleate/POPC mixture in a 1:4 ratio by the injection method. A discussion on the effect of experimental variables and on the relation between vesicle size and internal fluorescence is given in the Supporting Information (Table S2 and Figure S2).

We also checked the spectral features of eGFP fluorescence inside vesicles to further strengthen our conclusions about its intravesicle production. The emission spectrum obtained by microspectrofluorimetry of eGFP produced in bulk solution is shown in Figure S3 a, and it is compared with the spectrum recorded by a fluorimeter; these show perfect similarity. The eGFP emission spectrum can be deconvoluted as a sum of two Gaussian emission bands, centered at 511 and 531 nm, respectively.<sup>[12]</sup> The ratio between the two band intensities for bulk eGFP is  $1.8 \pm 0.1$  (Figure 3 a). Whereas some green fluorescent vesicles actually show an emission spectrum that fits well with that one of eGFP in bulk solution, others display a significantly different spectrum (Figure S3 b), which is characterized by a different ratio of the two band intensities. In particular, the intensity of the 511 nm band decreases, whereas the one of the 531 nm band increases, and their ratio dramatically drops to lower values, in the range of  $0.9\text{--}0.5 \pm 0.1$ . A typical case is shown in Figure 3 b. Previous



**Figure 3.** eGFP emission spectra recorded by microspectrofluorimetry. a) Bulk eGFP spectrum (●), b) intra-liposome eGFP spectrum (●). Each emission spectrum was fitted as a sum (—) of two Gaussian bands centered at 511 nm (---) and 531 nm (---), respectively. The different spectral shape in (b) is due to the varied ratio between the two peaks, owing to locally concentrated eGFP.

studies have shown that closely packed eGFP molecules that were immobilized in a gel display a similar change in the emission spectrum,<sup>[13]</sup> which has been tentatively explained as energy transfer between molecules. In our case, the observation of such anomalous eGFP emission spectra inside liposomes suggests that in those vesicles that were able to over-concentrate the TX-TL solutes, the internal eGFP synthesis proceeded so well that its local concentration becomes comparable to densely packed eGFP arrays. This evidence further supports our interpretation of the data in terms of few super-active vesicles formed thanks to exceptionally favorable entrapment events. In turn, the locally concentrated TX-TL molecular machinery efficiently produces functional eGFP molecules that accumulate inside the vesicles.

We believe that the low amount of such overcrowded vesicles is a signature of the underlying specific, local, and stochastic conditions that favor their formation. The driving force could be related to the release of free water (at the expense of macromolecule surface-bound water, which also extends down to multiple layers), so that the observations could be tentatively explained as a kind of phase transition. We are currently investigating possible generative mechanisms by means of stochastic simulations.

From the viewpoint of experimental investigations, the mechanism leading to solutes of “super-concentration” inside vesicles is a conundrum, and several questions are still unanswered. A more detailed and quantitative analysis of protein encapsulation can be obtained by fluorescence-tagged proteins, possibly using several proteins to assess the statistics of co-encapsulation by confocal microscopy or a flow cytometer. Averaging techniques based on the quantitation of the overall solute encapsulation (or the overall reaction products) are not suitable in this context. A detailed entrapment analysis would be informative for moderately complex mixtures, and help to formulate a quantitative model for this intriguing phenomenon. However, a functional assay on the success of a reaction in terms of end product detection as

in the case of eGFP synthesis remains a very stringent test for real-case mixtures. The exploration of over-concentration effects in vesicles prepared by other methods, such as detergent depletion, reverse phase evaporation, and water-in-oil droplet transfer, would further expand the current available observations<sup>[8,9,14]</sup> (when the method itself does not impair the activity of the solutes of interest).

In conclusion, we have shown that the formation of functionally active primitive cell models is due to a remarkable self-organization step that involves lipids and water-soluble molecules. Because of the spontaneous concentration of the solutes inside micrometer-sized liposomes, it was possible to observe the occurrence of a cell-like complex biochemical pathway, whereas no reaction occurred in free solution. Aside from obvious routes for concentrating solutes, including the evaporation of water in lagoons, our data emphasize the role of micro-compartmentalization during the origin of life, and provide a physically realistic model for understanding the emergence of early cells. Moreover, understanding the generative mechanism for the formation of solutes of super-concentration inside vesicles could allow the development of preparative methods that facilitate their formation. This, in turn, would be of great relevance for practical applications, especially in the nano-biotechnological field.

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- [1] L. E. Orgel, *Nature* **1992**, 358, 203–209.
- [2] J. W. Szostak, D. P. Bartel, P. L. Luisi, *Nature* **2001**, 409, 387–390.
- [3] T. A. Lincoln, G. F. Joyce, *Science* **2009**, 323, 1229–1232.
- [4] D. W. Deamer, *Nature* **1985**, 317, 792–794.
- [5] P. Walde, A. Goto, P. A. Monnard, M. Wessicken, P. L. Luisi, *J. Am. Chem. Soc.* **1994**, 116, 7541–7547.
- [6] S. S. Mansy, J. P. Schrum, M. Krishnamurthy, S. Tobé, D. A. Treco, J. W. Szostak, *Nature* **2008**, 454, 122–125.
- [7] Y. Shimizu, A. Inoue, Y. Tomari, T. Suzuki, T. Yokogawa, K. Nishikawa, T. Ueda, *Nat. Biotechnol.* **2001**, 19, 751–755.
- [8] P. L. Luisi, M. Allegretti, T. Souza, F. Steiniger, A. Fahr, P. Stano, *ChemBioChem* **2010**, 11, 1989–1992.
- [9] T. Pereira de Souza, F. Steiniger, P. Stano, A. Fahr, P. L. Luisi, *ChemBioChem* **2011**, 12, 2325–2330.
- [10] M. Hoppert, F. Mayer, *Cell Biochem. Biophys.* **1999**, 31, 247–284.
- [11] V. Norris, T. den Blaauwen, A. Cabin-Flaman, R. H. Doi, R. Harshey, L. Janniere, A. Jimenez-Sanchez, D. J. Jin, P. A. Levin, E. Mileyskova, A. Minsky, M. Saier, Jr., K. Skarstad, *Microbiol. Mol. Biol. Rev.* **2007**, 71, 230–253.
- [12] M. Chattoraj, B. A. King, G. U. Bublitz, S. G. Boxer, *Proc. Natl. Acad. Sci. USA* **1996**, 93, 8362–8367.
- [13] R. A. G. Cinelli, A. Ferrari, V. Pellegrini, M. Tyagi, M. Giacca, F. Beltram, *Photochem. Photobiol.* **2000**, 71, 771–776.
- [14] T. Souza, P. Stano, F. Steiniger, E. D'Aguzzo, E. Altamura, A. Fahr, P. L. Luisi, *Origins Life Evol. Biospheres* **2012**, 42, 421–428.