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The Use of Liposomes for Constructing Cell Models

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Abstract. We illustrate here in a form of a short review some of the work developed in our and other groups aiming at performing inside liposomes enzymatic reactions relevant for the origin of life. The work on giant vesicles will not be considered here. The long-range goal of our work with SUVs or LUVs (small unilamellar vesicles or large unilamellar vesicles) is the construction of a model minimal cell. By this we mean a cell-like system containing the minimal and sufficient number of macromolecular components for expressing some of the basic functions of a living cell- such as protein biosynthesis, growth and self-reproduction, homeostasis based on a primitive metabolism. We begin describing a POPC liposomal system containing some of the enzymes of the salvage cycle for the synthesis of lecithin; then vesicles containing the nucleotide phosphorylase enzyme for the polymerisation of ADP into poly(A); an oleate self-reproducing vesicular system which hosts Qβ replicase for the replication of a RNA template; a POPC systems (POPC = 1-palmitoyl-2-oleoyl-*sn*-phosphatidylcholine) hosting the elements for a polymerase chain reaction; and finally the attempts to organize inside liposomes the ribosomal system capable of the synthesis of poly(phenylalanine). This analysis of published work will be followed by the description of novel work aimed at expressing a protein (green fluorescent protein) inside liposomes. The possible development of this work and its limits will be discussed.

Key words: cell models, early cells, liposomes, minimal cells, origin of life

1. Introduction

As is well known, vesicles are spherically closed structures constituted in water by a double layer of surfactant molecules. When this surfactant is a phospholipid, vesicles are commonly referred to as liposomes, although often in the literature the two terms are used equivalently. The dimensions of conventional liposomes are typically in the range 50 to 500 nm, and a particular terminology is used for distinguishing the various classes, for example one talks about SUVs or LUVs, small unilamellar and large unilamellar vesicles (liposomes) respectively [1, 2]. Liposomes are considered to have been of importance in prebiotic chemistry, in particular as precursors of protocells [3, 4]. However, liposomes per se are only lipid shells, and in order to transform them in closer models to cells, they have to be filled with biochemicals and in particular with macromolecules that eventually lead to primitive forms of metabolisms.

In the scenario of the origin of life, one should look for ways by which the specific sequences of the first nucleic acids and proteins that were formed inside the liposomes starting from monomers or other simple low molecular weight precursors. This is the so-called 'bottom-up approach' [5] for the origin of life, that has been proved till now to be difficult to be implemented as we do not have until now methods to device, either experimentally or conceptually, how some specific macromolecular sequences may have originated. The alternative procedure to move towards primitive cells is the so-called 'top-down approach' [5], according to which extant DNA, RNA or enzyme are utilised and inserted directly into liposomes. This approach may lead to the construction of minimal cells, i.e., to liposomal systems characterised by the minimal and sufficient conditions for being defined as living [6–8].

In this paper we wish to review the work done in our and other groups for incorporating molecular biology reactions inside liposomes, work aimed finally to implement a minimal cell. Work with giant vesicles, another direction of the work in our group, will be not reviewed here. Also, the methodology for incorporating macromolecules inside the liposomes will not be discussed here, as it is described in detail in our previous papers [8–10].

2. Enzymatic Reactions Inside Liposomes

2.1. SYNTHESIS OF LECITHIN

The first attempts in our group to realise cell models utilising POPC liposomes (POPC stands for 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) have been performed already ten years ago [11]. The idea was to host inside the liposomes the enzymatic chain of reactions (the so-called salvage pathway) that, starting from glycerol-3-phosphate, lead to lecithin. The system would then be seen as a lecithin microreactor that produces lecithin, leading to a process of growth and eventually to a splicing of the original liposomes. In fact, the final dimensions of the liposomes were smaller than the initial ones.

Only preliminary results were carried out in this work, and although they were encouraging, it was not pursued further at that time. One of the problems was the poor availability of the enzymes of the salvage cycle, which are not commercially available and had to be isolated from animal tissues. Presently, there are studies in our group going on in which recombinant *E. coli* enzymes are used instead.

2.2. SYNTHESIS OF POLY(A)

In the following years, a series of experiments have been carried out utilising oleic acid/oleate vesicles. There are two good reasons for this choice: due to the relatively simple chemical structure, they can be considered as possible prebiotic vesicle-forming surfactants; and furthermore we have found in our lab conditions

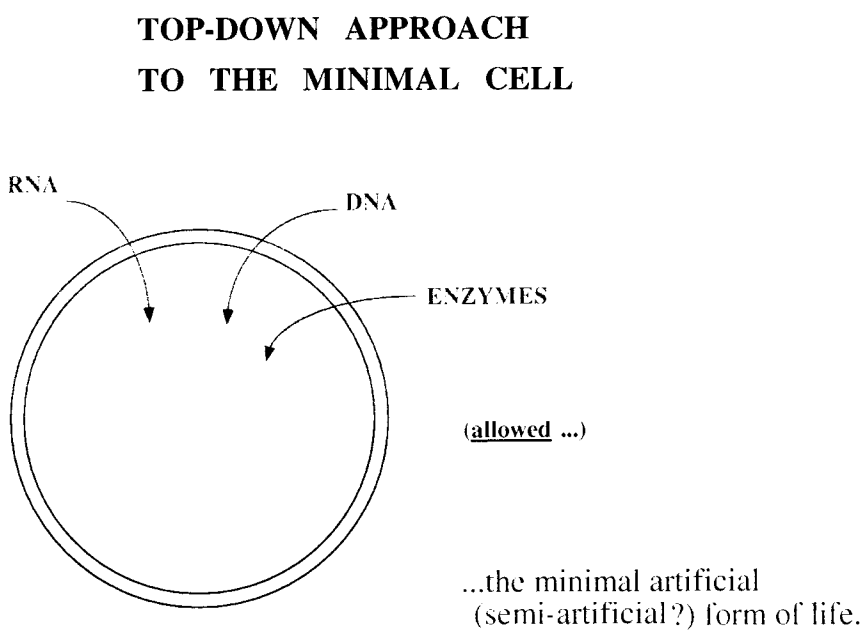

Figure 1. Schematic presentation of the 'top-down' approach.

under which these vesicles can auto-catalytically self-reproduce, i.e., they can catalyse their own formation owing to hydrolysis reactions of water-insoluble precursors (e.g. oleic anhydride or ethyl oleate) that bind on the vesicle membrane [12, 13]. One typical reaction that we considered with this system is the polymerisation of ADP by polynucleotide phosphorylase to yield poly(A). It is interesting to recall that this reaction has been originally proposed by A.I. Oparin using so-called coacervates [14, 15].

In our lab, conditions were chosen under which the polymerisation of ADP proceeded simultaneously with the self-reproduction of oleate vesicles [6]. The substrate ADP, added externally, is partly capable of permeating across the vesicle membrane. In this way, we have realised a 'shell and core reproduction' system, whereby the vesicular system undergoes reproduction while producing a RNA-analog in its interlor. Independently, the group of Deamer and Joyce utilised the same ADP polymerising enzyme inside dimyristoyl-PC liposomes, however, without self-reproduction of liposomes [16]. In this paper an interesting technique has been proposed for increasing the permeation of ADP across the liposome membrane.

More recently, we have studied again this reaction utilising POPC liposomes. In this case, we attempted to increase the permeability of ADP by using sodium cholate, a method suggested for other systems by Schubert et al. [17]. In fact, in the presence of cholate added externally to liposomes containing entrapped en-

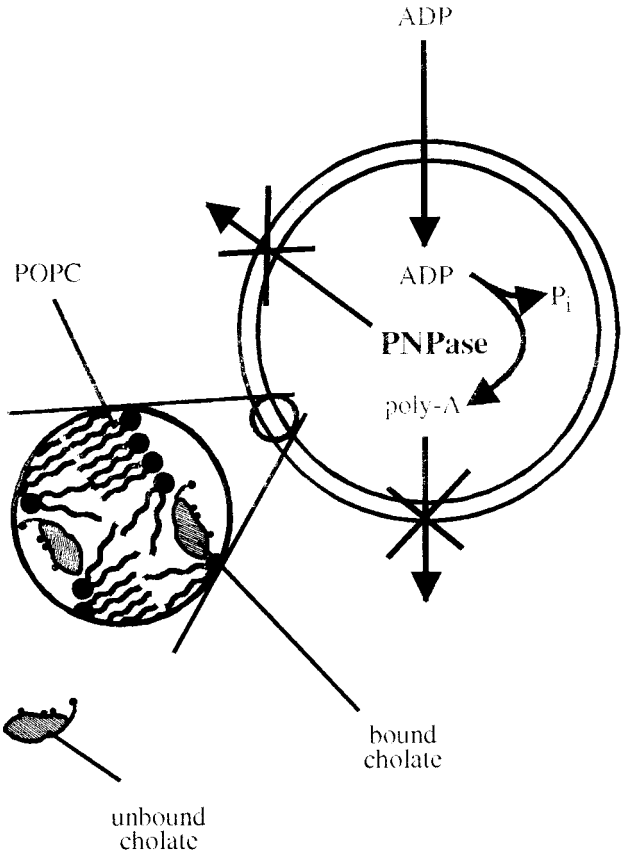

Figure 2. Illustration presenting the poly(A) synthesis by polynucleotide phosphorylase (PNPase). The vesicle membrane is semipermeable and allows the uptake of ADP, whereas the macromolecular components cannot leak out.

zyme, the enzymatic reaction inside the liposomes can be detected and measured, whereas in the control experiment (no cholate added), no reaction is observed. This is illustrated in Figure 2, described in more detail by Treyer et al. [18].

2.3. SYNTHESIS OF GLYCOGEN

Using the same methodology – entrapment of macromolecules within POPC liposomes, usage of sodium cholate to make them permeable to the substrate molecules – it was demonstrated in a further attempt that glycogen can be synthesized by glycogen phosphorylase with the monomers being added from the outside [19]. In this study, two macromolecules had to be entrapped in the same liposome: the enzyme glycogen phosphorylase and glycogen as an initiator. To be sure that no glycogen synthesis occurred outside the liposomes, aberrant amyloglucosidase –

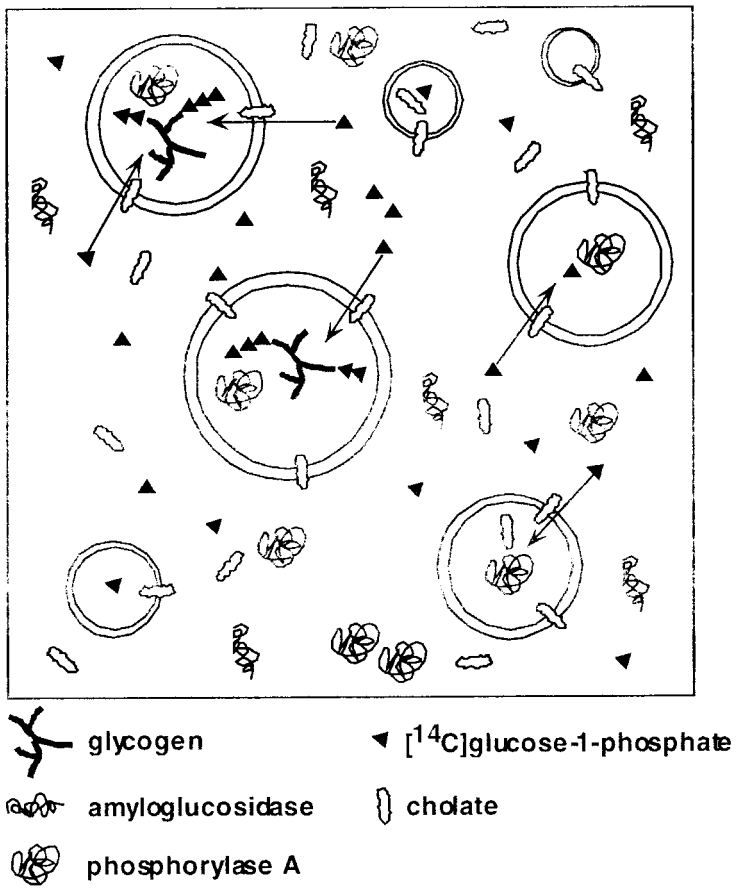

Figure 3. Illustration presenting the glycogen synthesis by phosphorylase A inside POPC liposomes. The liposomes are semipermeable and allow the uptake of the substrate molecules glucose-1-phosphate. In the vesicle, glucose-1-phosphate is linked to the glycogen primer macromolecules. Outside the vesicles, all the glycogen primer molecules have been digested by amyloglucosidase and, therefore, the incorporation of glucose-1-phosphate is not possible.

an enzyme that degrades glycogen – was added outside the liposomes (see Figure 3). One difficulty when working with phospholipid-cholate systems is that an overconcentration of cholate could lead to the solubilization of liposomes and, consequently, leads to a release of once entrapped enzyme. Because the experiment was designed in such a way that a 'short-term' overconcentration could not be totally excluded, in control experiments some glycogen was added together with the substrate molecules to the external medium. Even under these conditions, no relevant amount of glycogen was synthesized outside the liposomes; proof that an eventual release of encapsulated glycogen phosphorylase/glycogen due to an overconcentration of cholate had no effect.

2.4. REPLICATION OF RNA BY Qβ REPLICASE IN SELF-REPRODUCING OLEIC ACID/OLEATE VESICLES

A more complicated case of a self-reproducing vesicular system that simultaneously produces RNA internally has made use of the enzyme Qβ replicase. As is well known [20], this enzyme is able to synthesise RNA based on a RNA template. As substrate molecules, one needs the four nucleotides ATP, CTP, GTP, and UTP as well as Mg^{2+} ions. All ingredients required for Qβ replicase in oleic acid/oleate vesicles were added to a lipid film and 100-nm extruded vesicles were prepared. Then, EDTA was added to the external medium to inhibit the enzyme reaction outside the vesicles and the incorporation of [^{35}S]ATP was followed with time [7].

This reaction already acquires a considerable complexity level. In fact, we have inside the vesicles both an enzyme and a RNA template together with the substrate molecules, and novel RNA is being produced while the vesicles grow and eventually split. Of course, in this and in the previous experiments, the enzymatic reaction inside the vesicles and the self-reproduction of vesicles are not coupled with each other. The search for a good chemical or biochemical coupling is still in progress.

2.5. THE CASE STUDY OF THE POLYMERASE CHAIN REACTION INSIDE CONVENTIONAL POPC LIPOSOMES [21]

Carrying out a complex DNA polymerase reaction such as the polymerase chain reaction by thermostable DNA polymerases in liposomes was a particular challenge: The liposomes had to exhibit a high stability even at temperatures between 90 and 95 °C. In addition, there had to be at least one enzyme molecule, one template DNA molecule as well as several shorter oligonucleotide molecules present in a single compartment. Because no polymerase activity can be measured before melting the double-stranded DNA, the liposome preparation and entrapment techniques can be performed simultaneously without being afraid of producing product molecules outside the vesicles during this preparation period.

In fact, all ingredients were added to a lipidic film and the resulting dispersion was treated by freezing/thawing. Afterwards, the dispersion was extruded through filters with 400-nm pores. The extruded dispersion was then treated with high amounts of DNase I for several hours, before the nonentrapped material was removed by spin column gel permeation chromatography. The fractions containing turbid material were then combined and the liposomes were subjected to 25 temperature cycles. For a determination of the newly synthesized DNA, the liposomes were destroyed by adding sodium cholate and the DNA was isolated and analysed by polyacrylamide gel electrophoresis (see Figure 5).

This case study permitted us to show that even relatively complex biochemical reactions can be performed inside liposomes, even if they have to be carried out at temperatures between 60 and 95°C. On the other hand, these experiments also demonstrated the main difficulty when working with conventional liposomes: The likelihood that an individual liposome can host all ingredients is relatively small. At

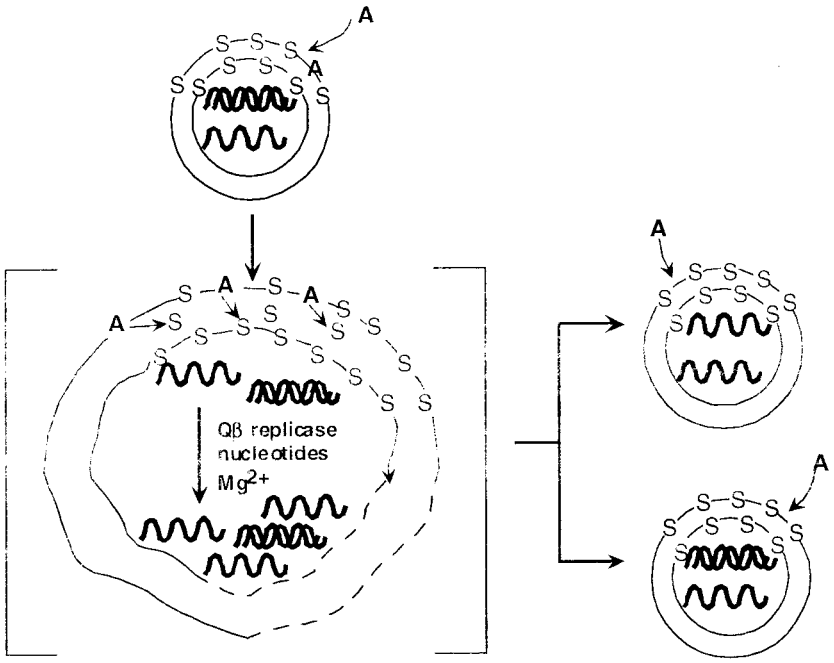

Figure 4. Schematic presentation of the MDV replication by Qβ replicase while the vesicles undergo growth and (eventually) division.

the concentrations of plasmid DNA and DNA polymerase applied for the presented PCR experiments in liposomes, the probability the an average sized liposome – having a diameter of 180 nm – as evaluated by freeze-fracture electron microscopy – may contain both, enzyme and DNA template was calculated to be $< 0.5\%$. In addition, inside such a model average-sized liposome there were enough nucleotides present so that 3–4 newly synthesized DNA molecules could be produced. These calculations also confirmed why the yield of obtained product was relatively modest. And they demonstrated that for the design of a better microreactor model, it was inalienable that substrate molecules could be added externally.

2.6. POLY(PHE) PRODUCTION IN RIBOSOME-CONTAINING POPC LIPOSOMES

The expression of proteins inside liposomes with an internalised translation machinery (containing the ribosomal subunits, the mRNAs/tRNAs, the aminoacyl synthetases, all protein factors needed for protein synthesis, together with all sort of smaller molecules) is one of the main challenges within the project of using liposomes for constructing cell models – see Figure 6. This is a problem of considerable complexity also from the technical point of view because one liposome must

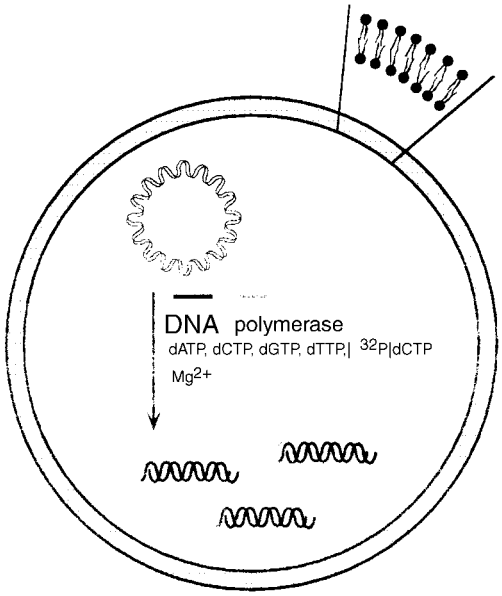

Figure 5. Polymerase chain reaction inside POPC or POPC/PS (90:10) liposomes. All ingredients required for PCR were entrapped in the liposomes and the liposomes purified by gel filtration chromatography. Then the liposomes were subjected to temperatures between 60 and 95 °C and the reaction products (the 369-bp DNA fragments) isolated and analyzed by gel electrophoresis (for details see Ref. 21).

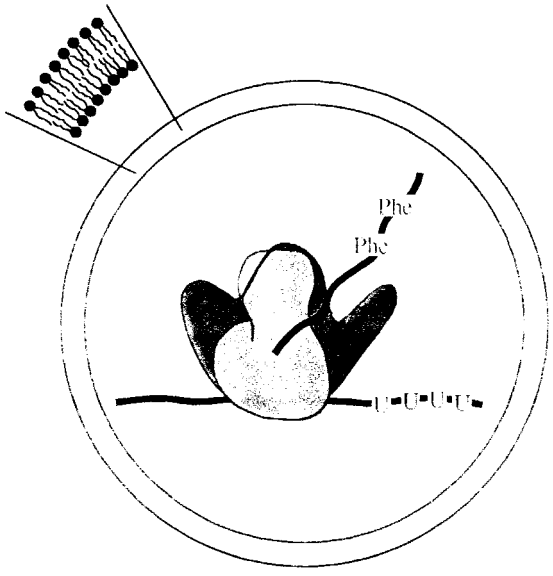

Figure 6. Poly(Phe) synthesis in POPC liposomes. The illustration shows the two ribosomal subunits, and the nucleic acid poly(U). tRNAs and other protein components required for translation are not shown (for the detailed experiments, see Ref. 19).

host all the above mentioned macromolecules. In view of this complexity, we have tackled this problem by studying first the expression of a simple polypeptide such as poly(Phe) [8]. In this case several things are simplified: For instance, we need a very simple, commercially available mRNA, namely poly(U). In addition, only one tRNA, namely $tRNA^{Phe}$ is necessary for the expression of poly(Phe). The fact that poly(U) instead of a naturally occurring mRNA was used made the experiments easier in the way that the process of transcription could be omitted.

Clearly, expressing a peptide such as poly(Phe) is far removed from expressing a protein. However, this experiment has shown that it is possible to incorporate ribosomes inside liposomes in a still active form. The major problem when scaling up to proteins, as already mentioned, is the necessity to incorporate into a single liposome many macromolecular components at a time, which are usually not commercially available. Eventually, all the single macromolecular components have to be isolated and than inserted, one after the other, inside the liposomes, a procedure which is theoretically possible, even if not in our group [22]. While this is the correct way to proceed, for the moment we have tackled the problem utilising a commercially available kit for protein biosynthesis, the so-called T7 S30 extract for circular DNAs, supplied by Promega. This translation kit contains already all macromolecular components, including the ribosomes, needed for protein synthesis, and what is necessary for the synthesis of a given protein is only the appropriate gene behind a T7 promoter on a plasmid cloning vector.

2.7. SYNTHESIS OF THE ENHANCED GREEN FLUORESCENT PROTEIN (EGFP)

We will describe now an experiment aimed at performing the biosynthesis of EGFP inside POPC liposomes. For this purpose, we have used the above mentioned plasmid cloning vector together with the T7 S30 Promega protein expression kit. The basic idea was quite simple and similar to the one published for the synthesis of poly(Phe). All ingredients were added and mixed and the protein expression was allowed to start for a short period (preincubation time 1–5 minutes). Then a methanol solution containing POPC (33 mM) was added very quickly by a injection method and the resulting suspension was mixed for 5–10 sec, before EDTA was added to the external medium (final concentration was 30–33 mM) at a concentration which should inhibit protein expression. Even if the liposomal dispersion was turbid, it was possible to follow spectroscopically the fluorescence intensity above 500 nm, starting at time zero (corresponding to the situation after the preincubation) and performing the incubation for 30–60 min.

One problem with these kind of measurements is that the suspension is not really homogeneous; therefore, one measurement results in an apparently higher fluorescence intensity, while in the next measurement, much lower intensities were recorded. However, when all these values were normalized to a certain ‘turbidity value’, then a clear increase in EGFP fluorescence was obtained (Figure 7, Ref. 23).

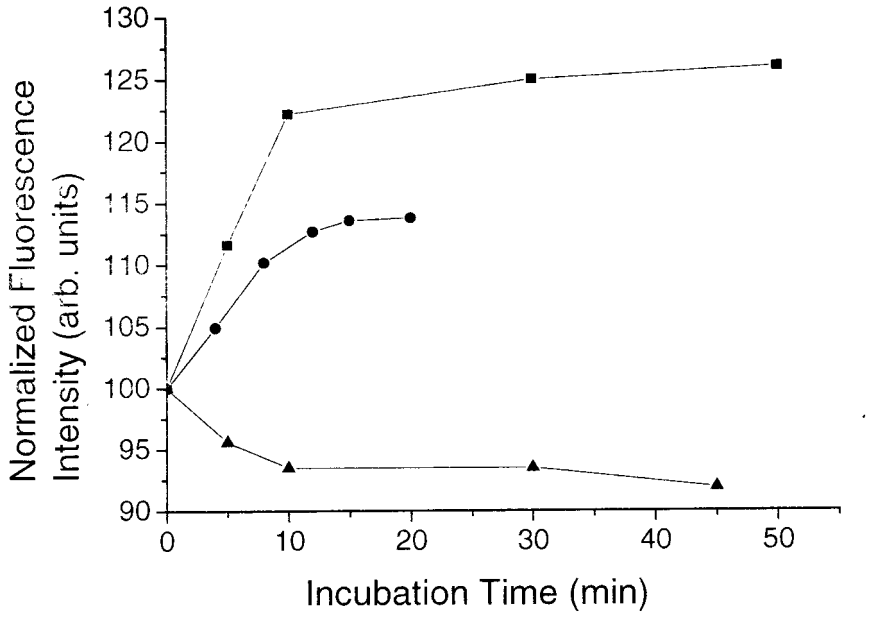

Figure 7. EGFP synthesis in POPC liposomes prepared by MeOH injection. All ingredients necessary for the synthesis of EGFP were mixed (S30 extract, premix, DNA containing the EGFP gene, amino acids) and incubated at 37 °C for 2.5 min (squares) or 5 min (circles). Then a methanolic solution containing POPC was injected (final POPC concentration = 1.5 mM, final MeOH concentration 4.3%), the resulting dispersion was mixed and EDTA was added to the external medium (final EDTA concentration = 33 mM). The fluorescence intensity was measured (the time zero point corresponds to the time point 1–2 min after the addition of EDTA) and the reaction was followed fluorometrically. Each point reflects the difference in the fluorescence intensity at 511 nm and 500 nm at the given time. To compare various experiments, the obtained difference values were normalized to 100. To check whether EDTA did inhibit any protein expression outside the liposomes, EDTA was added immediately before the addition of MeOH/POPC (triangles).

3. Concluding Remarks and Outlook

What have we learned from the experiments described above? One solid piece of information is that a series of molecular biology reactions, also those of noteworthy complexity, can be hosted in liposomes and almost in all cases, the enzymatic systems appeared to work well in the restricted closed environment of this special microreactor. The fact that even the PCR reaction, that proceeds under extreme temperature conditions, can be carried out in liposomes; and the fact that the large ribosome system can be incorporated inside the liposomes; all this is further evidence to the versatility and robustness of liposomes as biological microreactors. In addition, it is of considerable importance that a reaction inside vesicles can run simultaneously with an auto catalytic self-reproduction of the vesicles – as

it appears then that conditions for building up closer models to cell are indeed reachable.

How long is the path from here to a real 'semisynthetic' living cell? The answer depends of course on the definition of cellular life, a question which has been debated at length, see for example [24]. If one wishes a liposomal system that is capable of performing all basic functions of living cells, namely self-maintenance, self-reproduction and mutation/evolution, then it would be difficult at this stage to give a clear answer to the question. However, it appears safe to say that the proposition of a DNA minimal cell is not impossible, actually it lies within the possibility domain of chemists and molecular biologists.

At this point, however, is more reasonable to foresee intermediate stages of cell models, before reaching the complexity of full-fledged cellular life. One of these intermediate stages is the construction of a liposome cell-like system that is able to express proteins in a satisfactory way. Here the main difficulty is again of technical nature, i.e., one should be able to incorporate several dozen of different macromolecules inside one liposome at a time. For this reason we are now so interested in the approach described in the last experiment illustrated in this paper, the one leading to the synthesis of GFP. Although the data shown here are preliminary, it appears that the technique of forming liposomes by the injection method directly on the concentrated mixture of all biomolecular components is feasible. It is now necessary to study this type of experiments in more detail, considering also a more stringent elaboration of the fluorescent data. This is now in progress in our group.

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