Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications xxx (2007) xxx–xxx
BBRC
www.elsevier.com/locate/ybbrc

Protein synthesis in liposomes with a minimal set of enzymes

Giovanni Murtas [a,b,d,*], Yutetsu Kuruma [a,b], Paolo Bianchini [c], Alberto Diaspro [c], Pier Luigi Luisi [b,*]

[a] *Centro Studi e Ricerche "E. Fermi", Compendio Viminale, 00184 Rome, Italy*
[b] *Dipartimento di Biologia, Università degli Studi di Roma Tre, Viale G. Marconi 446, 00146 Rome, Italy*
[c] *INFM and Dipartimento di Fisica, Università degli Studi di Genova, Via Dodecaneso 33, 16146 Genova, Italy*
[d] *KITP, Kohn Hall, University of California, Santa Barbara, Santa Barbara, CA 93106, USA*

Received 23 July 2007

Abstract

In a significant step towards the construction of the semi-synthetic minimal cell, a protein expression system with a minimal set of pure and specific enzymes is required. A novel cell-free transcription and translation system named PURESYSTEM (PS), consisting of a specified set of 36 enzymes and ribosomes, has been entrapped in POPC liposomes for protein synthesis. The PS has been used to transcribe and translate an Enhanced Green Fluorescent Protein (EGFP) gene from plasmid DNA. The synthesis is confirmed by the EGFP fluorescence emitting liposomes on fluorometric analysis and on confocal microscopy analysis. Furthermore the PS encapsulated into POPC liposomes can drive the expression of the *plsB* and *plsC* genes encoding for the *sn*-glycerol-3-phosphate acyltransferase (GPAT) and 1-acyl-*sn*-glycerol-3-phosphate acyltransferase (LPAAT) involved in the first step of the "salvage pathway" for synthesis of POPC. The expression of GPAT and LPAAT in liposomes would in principle allow the production of the cell boundary from within.

Keywords: Synthetic biology; Minimal cell; Liposomes; Origin of life; Minimal genome; Cell-free transcription and translation; Protein synthesis

In the last few years the attention of several groups has focused on the laboratory construction of the minimal cell, one namely characterized by the minimal and sufficient number of components to be defined as alive. Of course the term "minimal cell" represents a vast family of possibilities, and in fact in the literature different proposals have been formulated. This is apparent from a few published reviews [1–3] and on the complementary studies on the minimal genome [4,5]. It is also important to mention the work by Venter and collaborators that following what is known as a top down approach, he has used transposon mutagenesis to determine the name and number of the essential genes of an extant minimal bacterium[6]; this is different from the work of Luisi's team that focus more on a synthetic biology approach to build a non extant semi-synthetic minimal cell using extant molecules and synthetic cell membranes (liposomes) creating an experimental minimal cell model for early living cells of early evolution [7].

Generally, the method of choice is to encapsulate in vesicles the components for complex biochemical processes [8,9] or protein synthesis [10,11]. It has also been possible to show that vesicles formed by fatty acids are capable of self-reproduction [12,13]. Concerning protein synthesis, several successful experiments have been carried out using the Green Fluorescent Protein (GFP) for obvious reasons of facile detection [14].

In all these experiments, GFP has been synthesized by using a commercially available *E. coli* extract for cell-free protein synthesis. In all these cases, one is dealing with a kind of "black box", in the sense that the composition and the relative concentration of the very many components are not made available by the commercial companies. Obviously, this represents a considerable handicap in the

* Corresponding authors. Fax: +39 (0)6 55176321.
E-mail addresses: murtas@uniroma3.it (G. Murtas), luisi@mat.ethz.ch (P.L. Luisi).

0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.07.201

work, as ideally one would like to investigate the activity just as a function of the relative composition and concentration of the components.

Removing this handicap is the main reason of the present work, and with this aim we managed to replace this "black box" with a cell-free transcription and translation system "reconstituted" from translation factors expressed and purified from *E. coli*, named PURESYSTEM (PS) [15]. This is a kit composed of highly purified 36 enzymes, 70S ribosomes, tRNA and low molecular weight cofactors and substrates, which are all known in number and concentration. Stock solutions are mixed to obtain the reaction mixture for a minimal protein expression system.

In this work we succeeded in entrapping the PS and a plasmid encoding for the enhanced GFP (EGFP) in liposomes, and—using fluorometric analysis and confocal microscopy to show that EGFP is produced inside the liposome.

Whereas EGFP is interesting as a tracer molecule for protein synthesis, the internal synthesis of more functional enzymes is needed in order to bring about more complex and specific processes, including eventually self-replication. We therefore investigated here, in a preliminary form, the expression of two of the glycerol acyltransferase that catalyze the reactions of the "salvage pathway" to synthesize phospholipids within the vesicle membrane [16].

Results and discussion

In order to express a functional protein inside liposome, the PS was encapsulated in liposomes together with the pWM/T7/EGFP plasmid, which encodes the respective gene of the EGFP. The PS is a protein-synthesizing cell-free system composed of 36 recombinant His-tagged protein factors purified from *E. coli* [15]. In addition to these, the PS also contains highly purified 70S ribosomes and low molecular weight components such as amino acids, tRNAs, and NTPs. The system is able to produce the protein at a rate of several hundred μg/ml without any supplementary apparatus [15].

To measure the EGFP fluorescence produced within 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC) liposomes, we used fluorometric analysis [17] testing aliquots of the liposome-EGFP expression reaction at one hour interval for three hours. Here, to prevent the EGFP synthesis at the outside of liposomes, the RNase A was added to the reaction mixture soon after liposome formation (see Materials and methods). Fig. 1 shows the EGFP fluorescence spectra peaking at 508 nm, as expected for the green fluorescence of this molecule. It was necessary to subtract the scattering typical in the fluorescence spectra, and to this aim we used Dihydrofolate reductase (DHFR) as the control protein to obtain a value for the background fluorescence so as to subtract this from the values obtained from EGFP (see Materials and methods).

The collected data were normalized in both cases and the DHFR fluorescence values were subtracted from the EGFP spectra values at each time point. The result is shown in Fig. 1, which clearly shows that specific fluorescence of the EGFP was observed within liposomes.

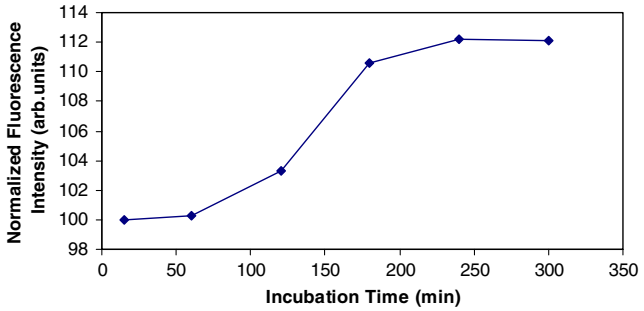

Fig. 1. Fluorometric analysis of EGFP produced within liposome. Time course of EGFP fluorescence in liposome testing aliquots of the PS-EGFP in liposome reaction. The EGFP fluorescence values were subtracted with the negative control DHFR values at each time point, and the resulting values normalized to 100 at the first 15 min time point. Therefore in this graph we report the percentage increase in EGFP fluorescence relative to DHFR control. Each fluorescence value for EGFP and DHFR was collected with emission picking at 508 nm as expected for the EGFP green fluorescence.

To verify the efficiency of the RNase A action in the system, the EGFP synthesis was performed without liposomes in the presence or absence of RNase A. With RNase A no EGFP fluorescence was observed. This result shows that RNase A completely inhibits the transcription and translation in the system, so that detected EGFP fluorescence can clearly be ascribed to the protein synthesizing within liposomes (data not shown).

The total amount of synthesized EGFP inside liposomes was estimated as almost 10% of the yield in balk. It is however difficult to provide a clear estimate of the synthesis efficiency (mg/ml/h). In fact, only those liposomes which entrap all components are capable of protein synthesis. The determination of this fraction is very difficult with the presently available techniques. This is one issue that we should solve in the near future.

Confocal microscopy was used in order to obtain further evidence about the localization of protein synthesis inside the liposome. The samples were prepared under the same conditions mentioned above, and were observed after 3 h of incubation at 37 °C.

Various concentrations of the enzyme solution of PS and the template DNA of EGFP were tested in order to vary, and possibly optimize, the fraction of vesicles entrapping all components for protein synthesis.

As shown in Fig. 2a, the sample monitored by confocal microscope showed vesicles with size raging from 600 nm to 2 μm and possessing the expected green fluorescence spectrum from EGFP. On the other hand, multiple tests to verify the fluorescence spectra on the outside of these vesicles failed to shown EGFP fluorescence, demonstrating again that the fluorescence is confined to the vesicle compartments (see details in Fig. 2b).

Please cite this article in press as: G. Murtas et al., Protein synthesis in liposomes with a minimal set of enzymes, Biochem. Biophys. Res. Commun. (2007), doi:10.1016/j.bbrc.2007.07.201

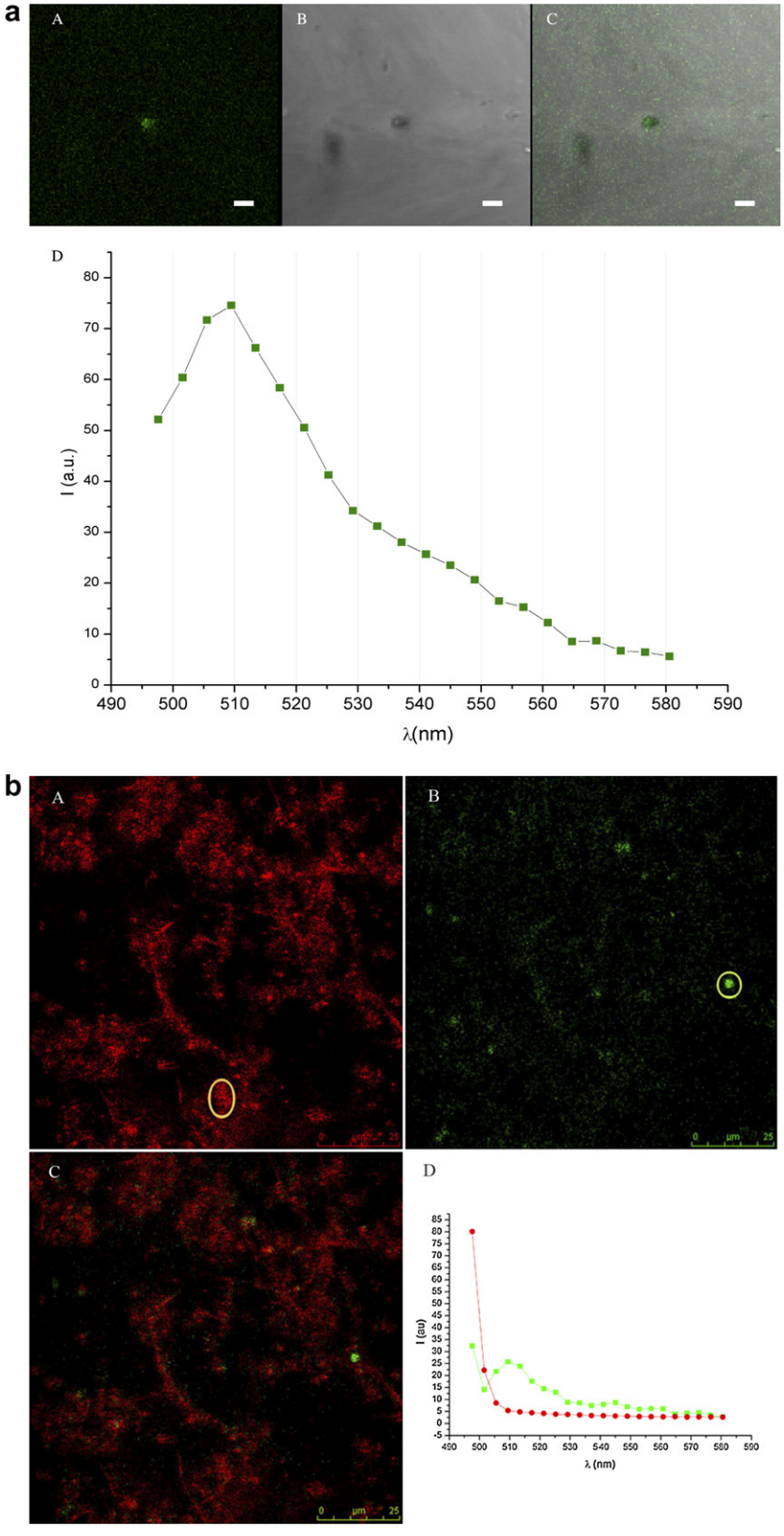

Fig. 2. Confocal images of EGFP fluorescent liposomes. (a) Confocal Images of a liposome excited at 488 nm. (A) Fluorescence acquired in the range 500–560 nm (green). (B) Transmission image (grey). (C) Overlay of A and B. The unit bar is 1 μm. (D) Graph extracted from the spectral series done on the same liposome (the spectral series is not shown). (b) (A) image extracted from the spectra of the reflection (red). (B) Image extracted from the fluorescence spectra (green). (C) Overlay of A and B. (D) Graph extracted from the spectral series calculated within the circles: EGFP fluorescence in liposomes (green dots) and the reflection light in the background (red dots) (the spectral series is not shown). In the Graphs 2a and 2b I (a.u) corresponds to fluorescence intensity in arbitrary units. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

Please cite this article in press as: G. Murtas et al., Protein synthesis in liposomes with a minimal set of enzymes, Biochem. Biophys. Res. Commun. (2007), doi:10.1016/j.bbrc.2007.07.201

We proceeded then to study the synthesis of two enzymes involved in the biosynthesis of phosphatidic acid in *E. coli*: the *sn*-glycerol-3-phosphate acyltransferase (GPAT) and the 1-acyl-*sn*-glycerol-3-phosphate acyltransferase (LPAAT). Both proteins are localized in the cytoplasmic membrane. The molecular size of GPAT is estimated a 83 kDa [18] and the LPAAT is a 27.5 kDa [19]. The aim of synthesizing these proteins in liposome is to produce endogenously phospholipids by means of internalized enzymes. Although the enzymatic synthesis of phospholipids inside liposomes is not a novel idea [20], a protein synthesis inside liposome with a limiting number of molecules and following biosynthesis of phospholipids which resulted from an enzymatic activity of the synthesized protein have yet to be achieved.

The synthesis of these proteins was demonstrated by radiolabeling (using radio-labeled [^{35}S] methionine in the system). The synthesis of GPAT and LPAAT is successfully observed at expected position on the SDS–PAGE gel. When this reaction mix was not encapsulated within POPC liposomes, protein synthesis was completely inhibited by RNase A. On the contrary, when the same reaction was encapsulated in liposomes, protein synthesis was observed even in the presence of external RNase A. The ratio of the inside synthesis versus the whole synthesis can be evaluated by comparing the band intensities of the products on the SDS–PAGE gel. The result is that about 3.5–9% of the protein was synthesized inside liposomes.

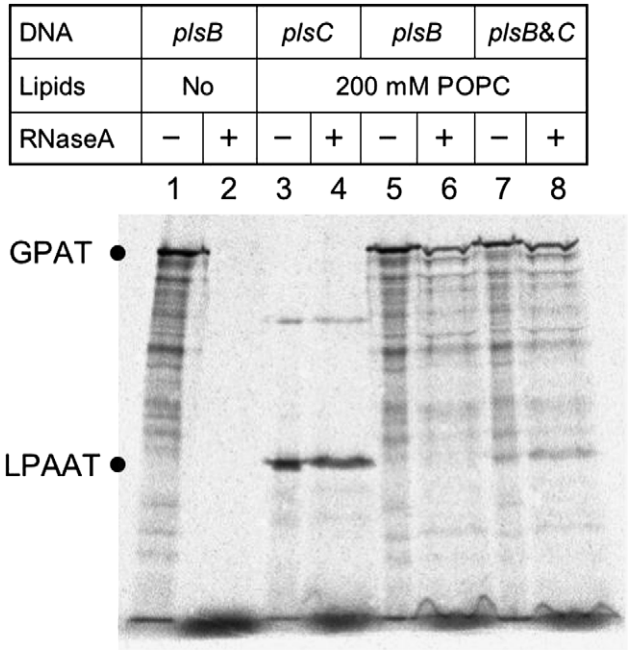

Fig. 3. Membrane proteins syntheses inside liposomes. The membrane proteins GPAT and LPAAT were synthesized in the PS cell-free transcription/translation system, starting from the corresponding genes *plsB* and *plsC*, respectively. The synthesis reactions were carried out at 37 °C for 2 h in the presence of [^{35}S] methionine. The cell-free reaction mixtures were mixed with a lyophilized POPC film in order to entrap them in 200 mM (final concentration) POPC liposomes (lanes 3–8). The lanes 1 and 2 shows the same reaction mixtures but without POPC liposomes. After liposomes formation, RNase A was added (even lanes) or omitted (odd lanes). Synthesized proteins were precipitated by acetone and subsequently washed by diethyl ether before analyze by 12% of SDS–PAGE. The gel is visualized with a phosphorimager and MultiAnalyst software (Bio-Rad). A 10% of the samples without RNase A treatment were loaded on the gel, instead 100% of the samples with RNase A were subjected to SDS–PAGE analysis. The positions of synthesized GPAT and LPAAT are indicated in figure, respectively.

As shown in Fig. 3, simultaneous synthesis of GPAT and LPAAT was also performed under the same conditions by adding the two corresponding plasmid DNAs (see Materials and methods). The next target will be to demonstrate the synthesis of both enzymes within the same vesicle compartment.

Concluding remarks

We believe that this work represents a significant step in the construction of the minimal cell. In fact, in all previous work in the literature protein synthesis in liposomes has been carried out with commercial extract [10,11], a mix of enzymes in which the composition and the concentration were not defined. We are now able to reconstruct the protein synthesis inside liposomes with a system in which all components are known and have a definite concentration. This should enable one to fully regulate the response of the system as a function of the composition/concentration of the reagents. The detailed knowledge of all components should enable us to accomplish the next step in this project of the minimal cell—a further reduction in the number of genes involved in protein synthesis process, testing in particular which of the 36 enzymes of the PS and ribosomal proteins is really essential.

While EGFP is extremely useful as a reporter protein, expression of other enzymes inside the vesicles is necessary for achieving more elaborated cellular functions. In this work, we have also shown the possibility of synthesizing membrane proteins, by means of GPAT and LPAAT, involved in the ''salvage pathway'' of phospholipids synthesis [16]. Although this research is still in progress, we believe that the preliminary observation made in this work represents a key step for the synthesis of phospholipids, which in turn may be opening the door to the biosynthesis of membrane components from within. To initiate the phospholipids biosynthesis inside the vesicles, it is necessary that first both membrane proteins are associated with the membrane vesicle to perform in an active conformation. In the modern cells the membrane localization of proteins is tightly controlled by several cytoplasmic and membrane-integrated factors (e.g. signal recognition particle, SRP, and SRP receptor plus Sec translocon). Although we have mentioned only the protein synthesis step in this study, the membrane protein localization and activation inside vesicles is highly expected by technical association with these modern machineries. Another avenue of further work lies in the extension of the present system into one which is capable of continuous synthesis. In this sense,

Please cite this article in press as: G. Murtas et al., Protein synthesis in liposomes with a minimal set of enzymes, Biochem. Biophys. Res. Commun. (2007), doi:10.1016/j.bbrc.2007.07.201

preliminary work for constructing a continuous bioreactor has been already described by our group [21].

Materials and methods

Materials. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC) was purchased from Avanti Polar Lipids (Canada). The PURESYSTEM Classic II was purchased from Post Genome Institute Co., Ltd. (Tokyo, Japan) or kindly supplied by Prof. T. Ueda (The University of Tokyo). The pWM/T7/EGFP plasmid encodes a T7 promoter, a Shine-Dalgarno sequence followed by the EGFP open reading frame, this plasmid was kindly provided by Bio Tecton (Zurich, Switzerland). The DHFR DNA template, provided by Post Genome Institute Co., Ltd., is a plasmid DNA containing the Dihydrofolate reductase (DHFR) gene and the corresponding protein does not fluoresce. The plasmids pBT302_plsB and pBT302_plsC, containing the genes *plsB* and *plsC* and encoding respectively for the enzymes GPAT and LPAAT were supplied from BioTecton (Zurich). These genes are located under T7 promoter. The *Escherichia coli* strain used to grow plasmids was JM109 [22].

Plasmid purification. It was carried out using a QIAGEN plasmid midi kit (QIAGEN, Hilden, Germany) and following the QIAGEN manufacturer's recommendations.

Liposome preparation. Liposomes were prepared by the dehydration rehydration method as described by Yomo and coworkers [9]. Briefly, POPC liposomes were prepared by hydrating a thin POPC film with ultrapure water (Milli-Q, Millipore), followed by extrusion (10 passages) through two stacked 400 nm polycarbonate membranes (Nucleopore Track-Etch Membrane, Whatman) and freeze-drying. The resultant freeze-dried liposomes were therefore hydrated with 50 μl of the reaction-solution containing the plasmid of choice and the PS system for protein synthesis. The final POPC concentration was 50 mM in the samples used for fluorometric analysis, 100 mM in the reaction samples observed under confocal microscope and 200 mM used in the GPAT and LPAAT enzymes synthesis. In all cases, RNase A was added into the prepared reaction mixture to monitor the only liposome inside reaction.

Transcription and translation reaction. In vitro transcription and translation was carried out using the cell-free expression system PURESYSTEM classic II. Aliquots of sol. A (a concentrated buffer) and sol. B (a mixture of enzymes) of the PURESYSTEM kit and plasmid DNA were mixed in a 50 μl, then added to the lyophilized liposomes at 4 °C and vortexed briefly. Subsequently 3 μl of RNase A (100 μg/ml) was added to prevent protein synthesis occurring outside of the liposomes and the mixtures were vortexed briefly. The liposome reaction mix was incubated at 37 °C for 3 h. The reaction mix used in confocal microscopy was set up as follow: 0.3 μg of pWM/T7/EGFP plasmid DNA (5 nM) + 20 μl sol. A and 8 μl sol. B into a final 30 μl of reaction mixture. The negative control was set up using the same conditions but introducing the DHFR template instead of EGFP. The transcription and translation of the *plsB* and *plsC* genes in the PURESYSTEM (12.5 μl sol. A and 5 μl sol. B) was modified by adding of 1 μl of [^{35}S] methionine (0.5 MBq/ μl) and 0.5 μg of plasmid DNA (6.4 nM for *plsB* and 10 nM for *plsC*: final concentration) into a 25 μl of reaction mixture. When double protein expression occurs, the plasmids pBT302_plsB and pBT302_plsC were mixed as 0.25 μg each to reach a final 0.5 μg of plasmid DNA (3.2 nM for *plsB* and 5 nM for *plsC* final concentration).

SDS–PAGE analysis. After 37 °C incubation, the reaction mixtures were transferred on ice and subsequently subjected to acetone precipitation twice. The resulting precipitates were washed with diethyl ether and precipitates were employed for SDS–PAGE analysis. Proteins labeled by [^{35}S] methionine were resolved on a 12% SDS–PAGE gel, and visualized using the phosphorimager Bio-Rad GS-525 Molecular Imager and MultiAnalyst software (Bio-Rad).

Fluorometric analysis of protein synthesis in liposomes. Fluorescence spectra of EGFP from within liposomes were recorded by an LS50B Luminescence Spectrometer (Perkin-Elmer Ltd., UK). Excitation wavelength was set up at 480 nm (slit 5 nm) and the emission spectrum was recorded from 490 to 560 nm (slit 5 nm), using the cut-off filter open. A micro quartz cell was used in all the experiments (400 μl, Hellma).

Aliquots of 10 μl from the liposome reaction mix incubated at 37 °C, as described in *transcription and translation reaction section*, were diluted in 350 μl of the micro quartz cell using 0.2 M bicine (pH 8.5) in order to provide the same osmotic pressure inside and outside the liposome.

Confocal microscopy. Fluorescence images of EGFP from within liposomes have been acquired with confocal microscope "Leica TCS SP5". Confocal Images are the result of liposomes excited at 488 nm and fluorescence acquired in the range of 500–560 nm, 10 μl of the EGFP translation in liposomes reaction have been loaded over a glass slide and scanned soon after with the laser beam of the confocal microscope.

Acknowledgments

This work was funded by Museo Storico della Fisica e Centro Studi e Ricerche "E. Fermi" (Rome) supporting our research and providing a Senior Research Grant to Dr. Giovanni Murtas, and a Junior Research Grant to Dr. Yutetsu Kuruma. Dr. Giovanni Murtas research work was supported in part by KITP/National Science Foundation under Grant No. PHY99-07949. Prof. Ueda kindly provided some of the PURESYSYEM kit used in this work. We gratefully acknowledge the assistance of Dr. Pasquale Stano (Luisi's group) for expert assistance with the fluorometric assay and liposome preparation and the help of Davide Mazza (Diaspro's group) with the confocal microscope.

References

[1] P.L. Luisi, Towards the engineering of minimal living cells, Anat. Rec. 268 (2002) 208–214.
[2] A. Pohorille, D. Deamer, Artificial cells: prospects for biotechnology, Trends Biotechnol. 20 (2002) 123–128.
[3] H.J. Morowitz, Beginnings of Cellular Life, Yale Univ Press, New Haven: CT, 1992.
[4] E.V. Koonin, How many genes can make a cell: the minimal-gene-set concept, Annu. Rev. Genomics Hum. Genet. 1 (2000) 99–116.
[5] R. Gil, F.J. Silva, J. Peretó, A. Moya, Determination of the core of a minimal bacteria gene set, Microbiol. Mol. Biol. Rev. 68 (2004) 518–537.
[6] J.I. Glass, N. Assad-Garcia, N. Alperovich, S. Yooseph, M.R. Lewis, M. Maruf, C.A. Hutchison, H.O. Smith, J.C. Venter, Essential genes of a minimal bacterium, Proc. Natl. Acad. Sci. USA 103 (2006) 425–430.
[7] P.L. Luisi, T. Oberholzer, A. Lazcano, The notion of a DNA minmal Cell a general discrourse and some guidelines for an experimental approach, Helvetica. Chimica. Acta 85 (2002) 1759–1777.
[8] T. Oberholzer, M. Albrizio, P.L. Luisi, Polymerase chain reaction in liposomes, Chem. Biol. 2 (1995) 677–682.
[9] T. Oberholzer, R. Wick, P.L. Luisi, C.K. Biebricher, Enzymatic RNA replication in self-reproducing vesicles: an approach to a minimal cell, Biochem. Biophys. Res. Commun. 207 (1995) 250–257.
[10] K. Ishikawa, K. Sato, Y. Shima, I. Urabe, T. Yomo, Expression of a cascading genetic network within liposomes, FEBS Lett. 576 (2004) 387–390.
[11] V. Noireaux, A. Libchaber, A vesicle bioreactor as a step towards an artificial cell assembly, PNAS 101 (2004) 17669–17674.
[12] P.L. Luisi, P. Stano, S. Rasi, F. Mavelli, A possible route to prebiotic vesicle reproduction, Artificial Life 10 (2004) 297–308.

[13] P. Walde, R. Wick, M. Fresta, A. Mangone, P.L. Luisi, Autopoietic self-reproduction of fatty acid vesicles, J. Am. Chem. Soc. 116 (1994) 11649–11654.
[14] W. Yu, K. Sato, M. Wakabayashi, T. Nakaishi, E.P. Ko-Mitamura, Y. Shima, I. Urabe, T. Yomo, Synthesis of functional protein in liposome, J. Biosci. Bioeng. 92 (2001) 590–593.
[15] Y. Shimizu, A. Inoue, Y. Tomari, T. Suzuki, T. Yokogawa, K. Nishikawa, T. Ueda, Cell-free translation reconstituted with purified components, Nat. Biotechnol. 19 (2001) 751–755.
[16] L. Stryer, Biochemistry, W.H. Freeman and Co., San Francisco, 1975, pp. 482.
[17] T. Oberholzer, P.L. Luisi, The use of liposomes for constructing cell models, J. Biol. Phys. 28 (2002) 733–744.
[18] W.O. Wilkison, R.M. Bell, *sn*-Glycerol-3-phosphate acyltransferase from *Escherichia coli*, Biochim. Biophys. Acta 348 (1997) 3–9.
[19] J. Coleman, Characterization of the Escherichia coli gene for 1-acyl-*sn*-glycerol-3-phosphate acyltransferase (pIsC), Mol. Gen. Genet. 232 (1992) 295–303.
[20] P.K. Schmidli, P. Schurtenberger, P.L. Luisi, Liposome-mediated enzymatic synthesis of phosphatidylcholine as an approach to self-replicating liposomes, J. Am. Chem. Soc. 113 (1991) 8127–8130.
[21] H.H. Zepik, E. Blochliger, P.L. Luisi, A chemical model of homeostasis, Angew. Chem. Int. Ed. Engl. 40 (2001) 199–202.
[22] C. Yanish-Perron, J. Vieira, J. Messing, Improved M13 phage cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors, Gene 33 (1985) 103–119.

Please cite this article in press as: G. Murtas et al., Protein synthesis in liposomes with a minimal set of enzymes, Biochem. Biophys. Res. Commun. (2007), doi:10.1016/j.bbrc.2007.07.201