Polymerase chain reaction in liposomes

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Background: Compartmentalization of biochemical reactions within a spherically closed bilayer is an important step in the molecular evolution of cells. Liposomes are the most suitable structures to model this kind of chemistry. We have used the polymerase chain reaction (PCR) to demonstrate that complex biochemical reactions such as DNA replication can be carried out inside these compartments.

Results: We describe the first example of DNA amplification by the PCR occurring inside liposomes composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), or of a mixture of POPC and phosphatidylserine. We show that these liposomes are stable even under the high temperature conditions used for PCR. Although only a very small fraction of liposomes contains all eight different reagents together, a significant amount of DNA is produced which can be observed by polyacrylamide gel electrophoresis.

Conclusions: This work shows that it is possible to carry out complex biochemical reactions within liposomes, which may be germane to the question of the origin of living cells. We have established the parameters and conditions that are critical for carrying out this complex reaction within the liposome compartment.

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Introduction

It has often been suggested that liposomes and membrane-enveloped vesicles may have been the precursors of the earliest cells [1–4]. It has been only recently, however, that the first attempts to carry out biochemical reactions inside these compartments have been made [5–7]. Here we report that DNA synthesis can be carried out in liposomes, using the polymerase chain reaction (PCR) in liposomes composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) or of a mixture of POPC and phosphatidylserine (PS).

The PCR is undoubtedly important both in clinical applications and in basic research [8–10]. This technique may also be relevant to the study of the origin of life, because it has been suggested [11,12] that life may have originated in an environment comparable to a hydrothermal vent, where a form of primordial PCR may have occurred [13]. It is therefore of interest to study this reaction in liposomes, not only because of the biological relevance of these molecular structures, but also because of the intrinsic interest of the microcompartment chemistry that occurs in a liposome. Four different macromolecules (a thermostable DNA polymerase, a DNA template and two single-stranded oligonucleotides), four different deoxyribonucleotides, and Mg^{2+} ions must all be present in one liposome for the PCR to proceed. Furthermore, the liposomes must be stable under the high temperatures (up to 95 °C) used for the PCR and under a layer of mineral oil, which is used to avoid water evaporation. The encapsulated DNA polymerase, which is not a membrane protein, must remain active despite the exposure to the high lipid concentration that is essential for the process of encapsulation.

It is clear that these constraints impose a difficult operational compromise on experiments designed to examine the possibility that the PCR could occur in liposomes. We report here that it is, in fact, possible to optimize conditions such that a small but significant PCR activity is found inside the liposomes. We show that the liposomes are stable at temperatures up to 95 °C, maintaining the integrity of the contents of their aqueous pool during the long time course of the experiment.

Results and discussion

Thermal stability of POPC liposomes

We first determined whether POPC liposomes remain stable at the high temperatures required for the PCR to occur, and under the mineral oil layer that is currently used in the PCR technique. There are reports in the literature of attempts to sterilize liposomes by autoclaving. Often aggregates have been observed after heat sterilization [14]; however, there are conflicting reports about whether solutes leak from heat-sterilized liposomes [14,15]. The heat stability of lipid components of liposomes at 72 °C has been shown to be pH dependent [16].

The structural integrity of the POPC liposomes produced in this study following thermal cycling (25 cycles, each cycle at 60 °C for 2 min, 72 °C for 1 min, 95 °C for 1 min) was determined by freeze-fracture electron microscopy (EM) [17] and by measuring the leakage of radioactive dATP entrapped within them. The analysis of ~500 liposomes by EM showed no significant structural damage following the temperature cycles, and no formation of aggregates of the type described previously [14]. Two typical electron micrographs are shown in Figure 1. It is surprising that the only effect of the thermal cycles

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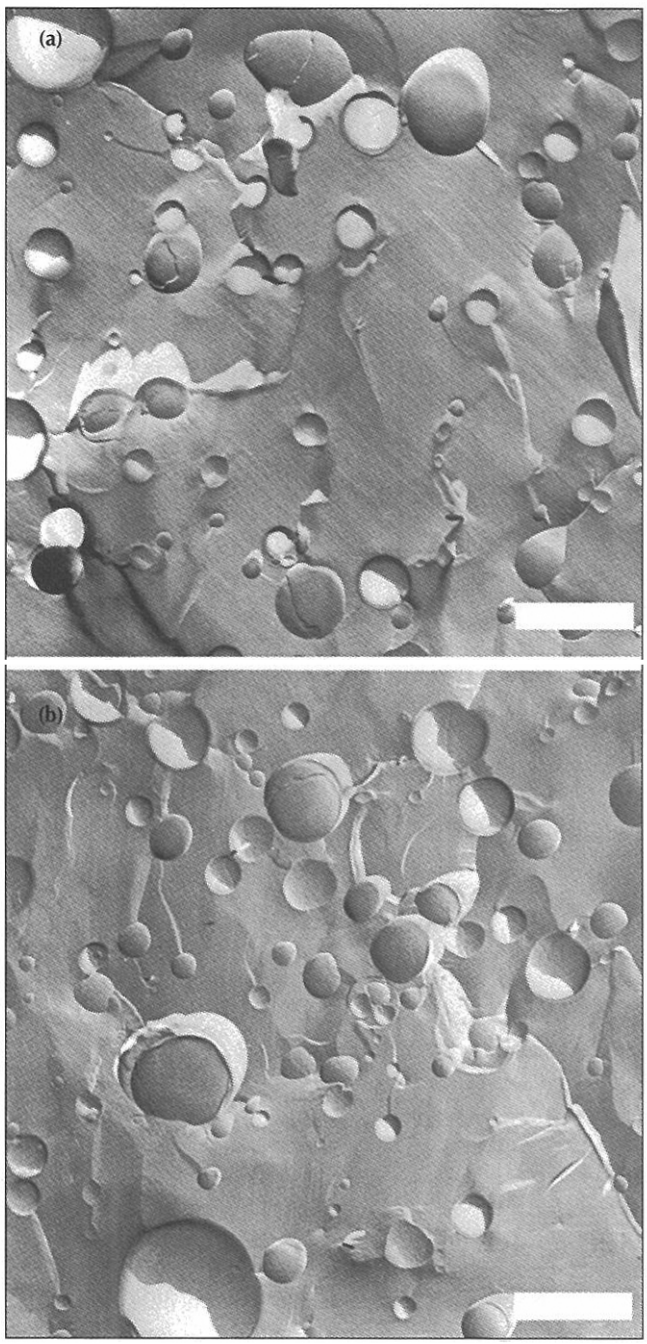

Fig. 1. POPC liposomes are stable at high temperature. Freeze-fracture electron microscopy of POPC liposomes **(a)** before and **(b)** after the temperature cycles used for PCR. The liposome dispersion was prepared the same way as for the PCR experiments (see Materials and methods) but without radioactive nucleotides. The dispersion was divided into two aliquots and overlaid with mineral oil; one aliquot was taken before (a) and one after (b) the temperature cycles, and freeze-fracture electron microscopy [17] was carried out. Bar, 500 nm.

was a shrinkage of the liposomes from, typically, 185 nm to 175 nm in diameter.

To test for leakage of the liposomes during thermal cycling, all reagents normally used for the PCR (except for the DNA polymerase) including radioactive dATP were encapsulated into liposomes and a gel-filtration column chromatography step was performed. The turbid eluates containing the liposomes were collected and divided into two fractions. One was incubated at room temperature; the other was subjected to 25 rounds of thermal cycling as above, to simulate the treatment given to permit the PCR to take place. Following incubation, both samples were subjected to an additional gel-filtration chromatography step and the radioactivity of all eluted fractions, each containing ~50 μl, was determined and compared. Figure 2 demonstrates that the amount of radioactivity that was eluted with fractions 2–6 (the turbid fractions containing the liposome dispersion; for details see Materials and methods) was nearly the same for the heat-treated liposomes as for those incubated at room temperature. The elution profile of the heat-treated liposome dispersion was somewhat sharper than that of the control, and only the elution profile of fractions 15–20 can be interpreted as showing a certain degree of leakage. We estimate from these data that the amount of leaked material was in the range of 3 to 5 % of the total encapsulated radioactivity.

Polymerase chain reaction within POPC liposomes

The thermal stability of the POPC liposomes gave us the confidence to carry out the PCR experiments. The low permeability of the bilayer membrane makes it difficult to achieve a sufficient concentration of all the PCR reagents within the inner aqueous core of the liposomes.

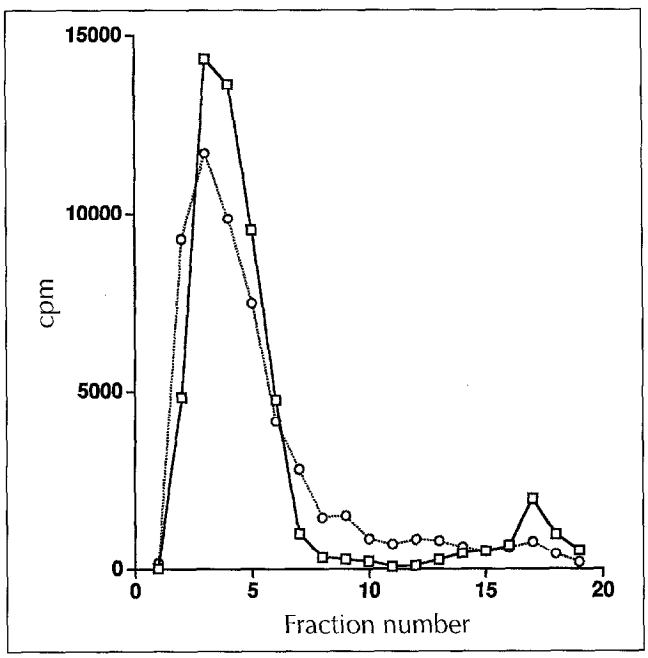

Fig. 2. POPC liposomes show minimal leakage at high temperature. Liposomes containing [α-^{35}S]dATP were purified by gel filtration column chromatography. The turbid fractions (containing ~50 μl each) were collected, divided into two samples and incubated with thermal cycling (25 cycles, each cycle at 60 °C for 2 min, 72 °C for 1 min, 95 °C for 1 min) or at room temperature for 2.5 h under a layer of mineral oil. After removing the mineral oil, both liposome dispersions were again subjected to gel filtration column chromatography. The fractions were collected and their amount of radioactivity was determined by liquid scintillation counting. ○, liposomes incubated at room temperature; ❑, liposomes incubated with thermal cycling.

In principle, one might carry out the reaction by first entrapping all of the macromolecular components — enzyme and DNA components in this case — and then adding the deoxyribonucleotides in excess to permeate into the liposome from the external aqueous milieu. We were unable, however, to find conditions under which such a permeation was possible with POPC liposomes, and we had to resort to the alternative method of entrapping all the reagents in the liposomes. When one takes this approach, the ratio of liposome number to the concentrations of the limiting macromolecular reagents becomes critical. For practical reasons, one is limited by the operational concentration of lipids and of enzyme (the enzyme stock solution cannot be concentrated over a certain limit and the concentration of liposomes must be high enough to permit a reliable chromatographic separation), thus the percentage of filled liposomes cannot be easily increased.

To entrap the PCR reagents we have used two types of liposomes, obtained from POPC or from a 9:1 (w/w) mixture of POPC and PS [18]. For both POPC and POPC/PS liposomes, the procedure was the same (Fig. 3). All of the components were added to a lipid film in a buffered solution: 25 nM plasmid DNA (linearized or nonlinearized), 10 nM DNA polymerase, 4.7 μM oligonucleotide primers A3 and A5, 1 mM of dATP, dGTP, and dTTP, and 0.2 mM dCTP (for the POPC liposomes) or 0.32 mM dCTP (for the POPC/PS liposomes). We typically used a 40 mM concentration of lipid, corresponding to a concentration of POPC or POPC/PS liposomes of about 130 nM. To calculate this value we have assumed a monodisperse, unilamellar liposome population of 185 nm in diameter (as estimated from the freeze fracture electron micrographs), giving a mean volume of 3.3×10^{-18} l.

By assuming a concentration of the solutes inside the liposomes that equals the concentration outside, and by assuming a Poisson distribution of the materials, we have estimated that only ~0.1 % of all POPC liposomes could contain all four macromolecular components. This calculation is based on the assumption that the components are passively encapsulated, in other words that there is no chemical interaction between the lipid bilayer and the dissolved components. This assumption does not, however, hold true for the negatively-charged POPC/PS liposomes. Calculation of the percentage of POPC/PS liposomes containing all four macromolecular components is thus more difficult, but it is reasonable to assume that we are not very far from the value of 0.1 % calculated for POPC liposomes. We therefore expect that under the conditions used here, only a very small fraction of liposomes contain all of the reactants required for the PCR to occur. It may be possible to increase this number, but even the most extreme efforts would be unlikely to increase the percentage of liposomes that are PCR-competent to more than 1–2 %. We have not, therefore, pursued the search for optimal conditions further, as this is not our main aim; instead, we show that even under the unfavorable conditions of this assay, a clear indication of PCR activity in liposomes can be found. Note, however, that under the conditions we have used a linear rather than an exponential amplification of DNA can be expected. This would be the case even in aqueous solution with a similar concentration of PCR reactants. Indeed, control reactions in 50 μl of aqueous solution using the same conditions as those for PCR in liposomes gave 200 ng of the 369-base-pair (bp) fragment after two temperature cycles, 550 ng after three cycles, 1.6 μg after four cycles and 3.5 μg after seven cycles. This linear amplification may be due to the unfavorable ratio of oligonucleotides to template DNA.

The dispersed liposomes were purified by gel filtration, thermal cycling was carried out and the products were separated by electrophoresis in a polyacrylamide gel. A ^{32}P-labelled product of 369 base pairs was observed with

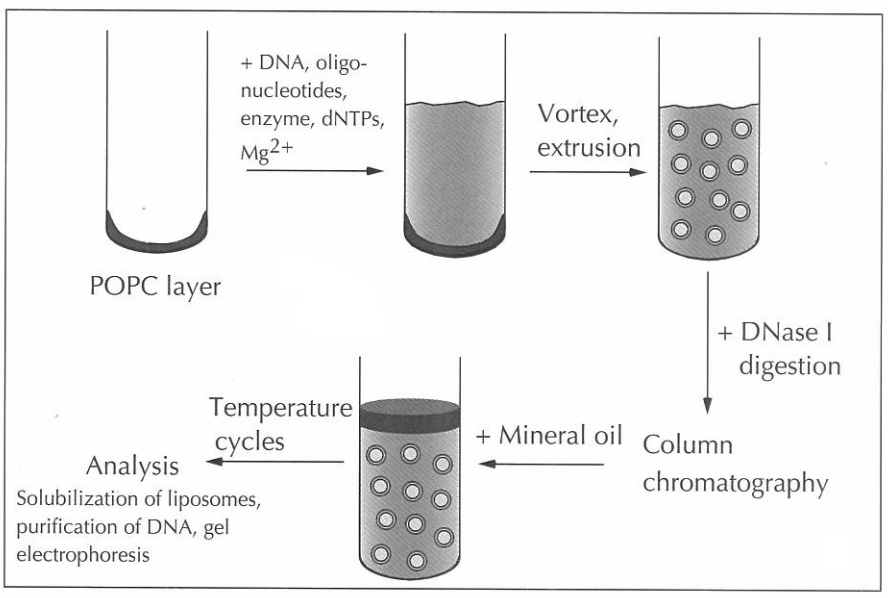

Fig. 3. Schematic drawing illustrating the experimental procedure.

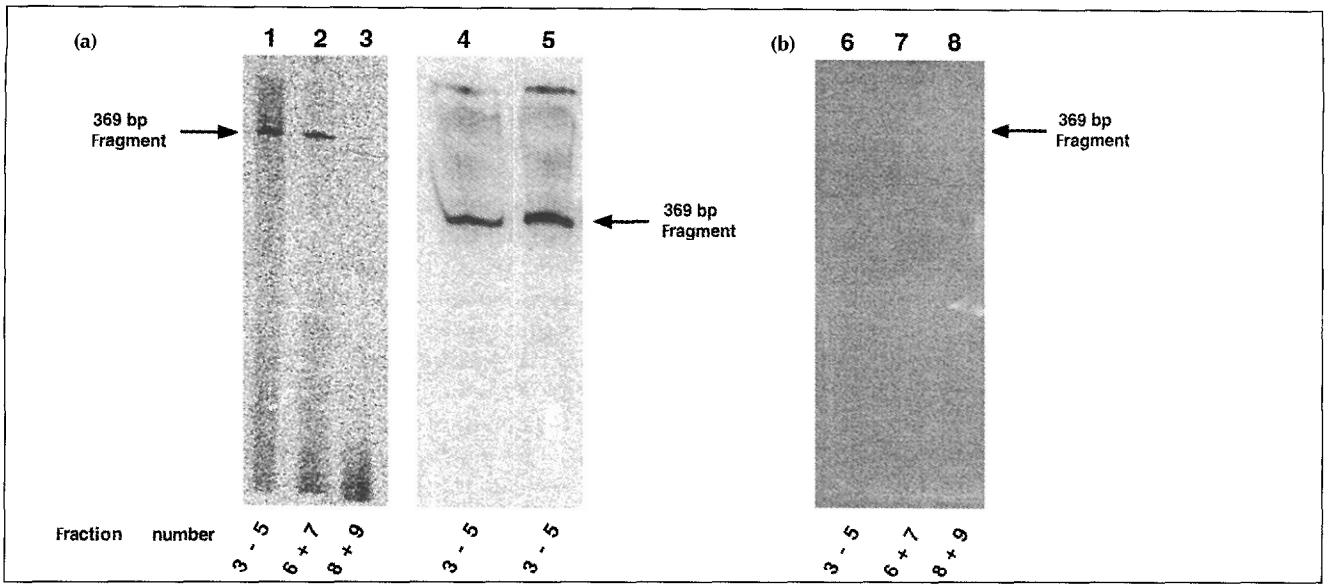

Fig. 4. DNA replication within liposomes. **(a)** Polymerase chain reaction inside POPC liposomes (lanes 1–3) or inside POPC/PS liposomes (lanes 4 and 5). Liposomes were produced in a buffered solution containing all reagents used for PCR, purified by gel filtration column chromatography and fractions 3–8 were subjected to 12–25 cycles at different temperatures (for details see Materials and methods). After liposome solubilization in 1 % deoxycholate and a phenol/chloroform extraction of the solution, the DNA was purified and analyzed on a 7.5 % acrylamide gel. The indicated fractions were combined and loaded onto the acrylamide gel. The PCR experiments shown in lanes 1–3 and 5 were carried out using linearized plasmid DNA, lane 4 shows the same experiment using the corresponding nonlinearized plasmid DNA. The arrows indicate the position of the 369-bp fragment produced by PCR. **(b)** PCR outside liposomes (lanes 6–8). To test whether the leaked deoxyribonucleotides, oligonucleotides and Mg^{2+} ions that were not removed by gel filtration could be responsible for the DNA product obtained, POPC liposomes were produced and treated as for a PCR experiment inside liposomes but without DNA polymerase and DNA template. The liposomes were purified by gel filtration column chromatography. Fractions 3–5, 6 and 7, 8 and 9 were combined again, DNA polymerase and DNA template were added to the external aqueous medium and the samples were subjected to the temperature cycles as described above. The same results were obtained using POPC/PS liposomes (data not shown).

POPC liposomes (Fig. 4a, lanes 1–3). This experiment has been repeated 5 times. A much stronger band was detected following PCR in POPC/PS liposomes (Fig. 4a lanes 4,5); we estimate that the efficiency of the PCR within these liposomes was 6–8 times higher than that within POPC liposomes. The reasons for this increased efficiency are unclear at present.

We estimated the total amount of DNA produced within liposomes and compared this amount to the value calculated by assuming that PCR is occurring in 0.1 % of our average sized liposomes. The amount of DNA produced in the POPC liposomes was 70 000 phosphorimager counts, and we estimate that this corresponds to ~150–250 pg. The value after two temperature cycles in aqueous solution (calculated for a volume corresponding to 0.1 % of the liposomal volume) was in the same range. It is interesting to note that the amount of PCR product we obtained with the nonlinearized plasmid was ~65 % of the value obtained with the linearized plasmid (compare lanes 4 and 5, Fig. 4a).

Several precautions were taken to ensure that the PCR products that we observed did not result from a reaction taking place outside the liposomes. First, we added pancreatic DNase I to the suspension of dispersed liposomes prior to gel filtration, to digest the external DNA template. As shown in Figure 5, the digestion was very effective, with 99 % of the trichloroacetic acid-precipitable radioactivity added to the external aqueous medium digested. Second, the chromatographic separation removes virtually all of the DNA substrate from the bulk aqueous phase of the liposome suspension (data not shown). At most, there is substrate present from the 3–5 % leakage described above (see Fig. 2). We set up a control experiment based on the assumption that all of the macromolecular components had leaked out of 3–5 % of the liposomes. We encapsulated this estimated amount of oligonucleotides and deoxyribonucleotides (in the absence of radioactive dCTP) within liposomes using the same buffer as used for PCR, and treated the liposome dispersion as before. After the gel filtration column chromatography, the same fractions were collected and DNA polymerase, DNA template and [α-^{32}P]dCTP were added. No DNA product could be detected when POPC (Fig. 4b) or POPC/PS (data not shown) liposomes were used. We have therefore excluded the possibility that the polymerase chain reaction could also have occurred outside the liposomes.

Significance

We have shown that PCR can take place in a small membrane-enveloped compartment. Liposomes composed of POPC or of a mixture of POPC and PS are very stable at high temperature, but only a

small fraction of the liposomes could be filled with all of the reagents necessary for PCR. It is probable that these two features are linked; the high stability of the POPC or POPC/PS bilayer may cause low permeability, which in turn makes it impossible to furnish the substrates from the outside. This feature, however, also prevents the leakage of the materials from inside the liposome to the external medium.

Even with a low percentage of liposomes containing all of the necessary reagents, we have shown that a compartmentalized, complex biochemical reaction can take place under extreme conditions, such as high temperature and the requirement for eight different reagents. This is consistent with the hypothesis that liposomes may have been the primordial microreactors that were important in the transition from a prebiotic self-replicating system such as the 'RNA world' to cellular life.

Materials and methods

Materials

Salmon-sperm DNA was purchased from Fluka (Buchs, Switzerland). The plasmid pSP64-JE was a kind gift of Juan Gomez (Department of Biochemistry, University of Zürich, Switzerland). It derives from the cloning vector pSP64 (from Promega) and contains a 369 bp fragment of the JE-gene (from nucleotide 2161 downstream) [19]. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was obtained from Avanti Polar Lipids, Inc., phosphatidylserine (PS) was purchased from Serva (Heidelberg, Germany), the oligonucleotide primers A3 and A5 (140 pmol μl^{-1}, A3 = 5'-GAATTCAGATCTCCT-GACTG-3' and A5 = 5'-TGCAAGGTGTGGATCCATTT-3') were purchased from Microsynth (Balgach, Switzerland), and the DNA polymerase (5 U μl^{-1}, with an estimated 250 000 U mg^{-1}) used for PCR experiments was obtained from Finnzymes Oy (Espoo, Finland). The pancreatic DNase I (2000 Kunitz U mg^{-1}) was purchased from Boehringer Mannheim, the Bio-Gel A-15 m column chromatography material from Bio-Rad Lab. (Richmond, VA), [α-^{35}S]dATP (> 1000 Ci $mmol^{-1}$) and [α-^{32}P]dCTP (> 3000 Ci $mmol^{-1}$) were obtained from Amersham, the deoxyribonucleotides from Pharmacia (Uppsala, Sweden), and the mineral oil and the deoxycholate from Fluka (Buchs, Switzerland).

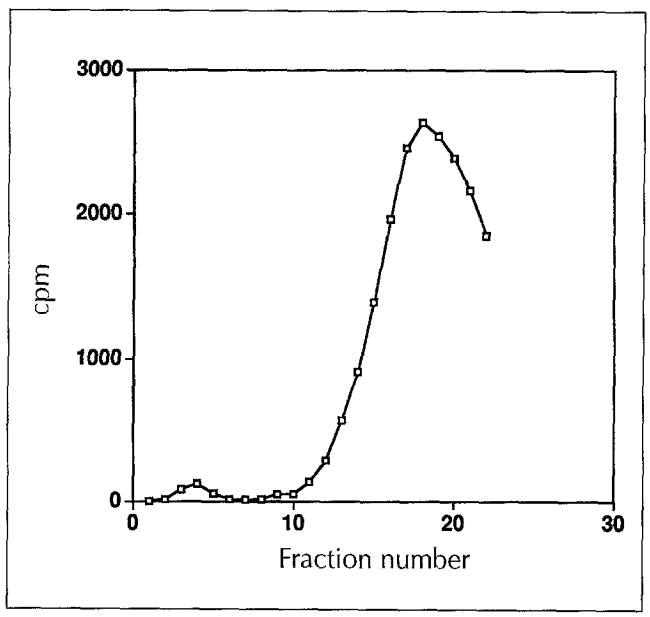

Fig. 5. Digestion of external DNA template by pancreatic DNAse I outside liposomes. 15 nM plasmid DNA (linearized) and 20 000 cpm of [α-^{35}S]dATP-labelled 369 bp DNA were added to the external aqueous medium of a liposome dispersion (40 mM POPC) in 50 mM Tris (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$. The DNA was digested by 600 U of pancreatic DNAse I for 1.5 h, the reaction mixture was subjected to gel filtration column chromatography and the amount of radioactivity in each fraction was determined. Note that undigested DNA co-elutes with the liposomes, but shows a broader elution profile.

Stability of the POPC liposomes

Liposomes were prepared by dissolving a POPC film in a buffered solution containing 50 mM Tris (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM dCTP, dGTP, and dTTP, 0.2 mM dATP, 5 μCi [α-^{35}S]dATP and 15 μg of salmon-sperm DNA. After the dispersion was freeze–thawed five times [20,21], 15 μl of DNA polymerase stock solution (20 mM Tris (pH 7.4), 0.1 mM EDTA, 1 mM DTT, 100 mM KCl, 0.1 % Triton X-100, 160 $\mu g\ ml^{-1}$ BSA, 50 % glycerol) was added. After two additional freeze–thaw cycles, the dispersion was forced five times through two polycarbonate filters with pore sizes of 400 nm (for extrusion a Liposofast from Avestin Inc. was used) [22]. The extruded dispersion was applied to a gel filtration column (using Bio-Gel A-15 m) and eluted with 50 mM Tris (pH 8.4) as previously described [23]. The turbid fractions, containing 50–60 μl each, were combined, divided into two tubes, overlaid with 60 μl of mineral oil, and incubated at room temperature or at temperatures normally used for PCR experiments (60 °C/72 °C/95 °C). After incubating for 25 cycles (every cycle at 95 °C/1 min, 60 °C/2 min, 72 °C/1 min), both samples were applied to an additional gel filtration column (again using Bio-Gel A-15 m) and the amount of radioactivity in each fraction was quantitated by liquid scintillation counting using a PicoAqua™ cocktail and a tri-carb® 2200 CA scintillation counter (both from Canberra Packard S.A.). In an additional experiment, everything was done as described above but without [α-^{35}S]dATP. After having carried out the gel filtration column chromatography, samples were analyzed by freeze-fracture electron microscopy [17] and the size distribution of the liposome dispersion was determined for each experiment.

Polymerase chain reaction inside POPC liposomes

Liposomes were prepared by dissolving a pure POPC or a 9:1 POPC/PS (w/w) film in a buffered solution containing 50 mM Tris (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM each of dATP, dGTP and dTTP, 0.2 mM (for POPC liposomes) or 0.32 mM (for POPC/PS liposomes) dCTP, 10 μCi [α-^{32}P]dCTP, 4.7 μM of oligonucleotide primers A3 and A5, and 25 nM of pSP64-JE DNA (linearized by *Eco*RI or nonlinearized) so that the final lipid concentration was about 40 mM. The resulting dispersion was shock frozen in liquid nitrogen and thawed at room temperature four times, then 15 μl of DNA polymerase (containing 75 U, delivered in 20 mM Tris (pH 7.4), 0.1 mM EDTA, 1 mM DTT, 100 mM KCl, 0.1 % Triton X-100, 160 $\mu g\ ml^{-1}$ BSA and 50 % glycerol) was added, and one additional freeze–thaw cycle was carried out. After extrusion of the dispersion through two stacked filters (400 nm pore size) five times, 750 U of pancreatic DNase I

were added and incubation was performed at 37 °C for 3 h. The incubated liposomes were separated by gel filtration column chromatography (eluted with 50 mM Tris, pH 8.4). Usually 10 fractions of 50 µl each were collected; fractions number 2–6 or 7 were normally turbid, and other fractions showed no clearly visible turbidity. Fractions number 3–5, 6 and 7, 8 and 9, containing about 50 µl each, were combined, overlaid with 60 µl of mineral oil and subjected to 25 cycles (POPC liposomes) or 12 cycles (POPC/PS liposomes) of the PCR using 95 °C for 1 min for denaturing, 60 °C/2 min for annealing and 72 °C/1 min for primer extension (denaturing time for the first cycle was 2 min, and the primer extension of the last cycle took an additional 2 min). The cycles were performed with a DNA thermal cycler (MJ Research).

The liposome dispersions were solubilized in 1 % deoxycholate and the aqueous phase was extracted two times with 1 volume phenol/chloroform 1:1 to remove all lipids and proteins. The nucleic acids in the supernatant were precipitated and the PCR products analyzed on a 7.5 % denaturing acrylamide gel and visualized using a PhosphorImager® (Molecular Dynamics).

Control experiments

To test whether pancreatic DNAse I is able to digest the template DNA outside liposomes, 15 nM of plasmid pSP64-JE and [α-^{35}S]-labelled PCR product (containing 20 000 counts per minute (cpm) precipitable by trichloroacetic acid) were added to a liposome dispersion (40 mM POPC) dissolved in 50 mM Tris (pH 8.4), 50 mM KCl, and 2.5 mM $MgCl_2$. After extrusion as described above, 500 U of DNase I was added and the liposome dispersion was incubated at 37 °C for 1.5 h. After digestion, gel filtration column chromatography was carried out and the amount of radioactivity in each eluted fraction was analyzed (undigested PCR product was normally eluted in fractions 3–12).

To test whether PCR occurs only inside the liposomes, a POPC or POPC/PS film was dissolved in the same solution as used for a PCR experiments inside liposomes but without DNA polymerase, plasmid DNA and [α-^{32}P]dCTP. The encapsulation process, DNase I digestion and purification of the liposomes were carried out as described above. After the gel filtration chromatography, fraction numbers 3–5, 6 and 7, 8 and 9 were combined and for each sample 2 U of DNA polymerase, 25 ng of pSP64-JE DNA, and 1 µCi of [α-^{32}P]dCTP were added for each reaction. The liposome dispersions were then subjected to the temperature cycles and the DNA was analyzed as described above.

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