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Entrapment of nucleic acids in liposomes

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Abstract

The entrapment efficiency of three main methods used in the literature for the encapsulation of nucleic acids in liposomes were studied using 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) liposomes. In particular the reverse phase method, the dehydration/rehydration method, and the freeze/thawing method were compared to each other under standardised conditions, i.e. using in every case the same concentration of guest molecules (DNA, tRNA and ATP as low molecular weight analogue) and equally extruded liposomes. The percentage of entrapment strictly referred to the material localized inside the liposomes, i.e. particular care was devoted to ruling out the contribution of the nucleic acid material bound to the outer surface of the liposomes: this was eliminated by extensive enzymatic digestion prior to column chromatography. Depending on the conditions used, the percentage of the entrapped material varied *between 10 and 54%* of the initial amount. Further, the encapsulation efficiency was markedly affected by the salt concentration, by the size of liposomes, but to a lower degree by the molecular weight of the guest molecules. In general, we observed that the freeze/thawing encapsulation procedure was the most efficient one. In a second part of the work the freeze/thawing method was applied to encapsulate DNA (369 bp and 3368 bp, respectively) using liposomes obtained from POPC mixed with 1–10% charged cosurfactant, i.e. phosphatidylserine (PS) or didodecyldimethylammonium bromide (DDAB), respectively. Whereas PS had no significant effect, the entrapment efficiency went *up to 60%* in POPC/DDAB (97.5:2.5) liposomes. The large entrapment efficiency of DNA permits spectroscopic investigations of the DNA encapsulated in the water pool of the liposomes. UV absorption and circular dichroism spectra were practically the same as in water, indicating no appreciable perturbation of the electronic transitions or of the conformation of the entrapped biopolymer. This was in contrast to the DNA bound externally to the POPC/DDAB liposomes which showed significant spectral changes with respect to DNA dissolved in water. © 1997 Elsevier Science B.V.

Keywords: Liposome; Encapsulation; Nucleic acid; Salt effect; CD spectroscopy

1. Introduction

The entrapment of nucleic acids in liposomes is interesting from two different perspectives. On the one hand, liposomes hosting nucleic acid permit the study of the influence of microcompartmentation on biochemical reactions involving DNA and RNA [1–3] and also provide simple models for protocells [4,5]. On the other hand, DNA-containing liposomes are finding applications in the biomedical area, in particular in gene transfer therapy [6,7].

The procedures for encapsulation are described in various papers [8–12]. However, the overall picture one receives is rather confusing, since DNA entrapment experiments are carried out under quite different conditions [13–16], no comparison is generally

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offered. Furthermore, the liposomes which are used are not well characterized in terms of size and lamellarity. In addition there is in the literature a basic uncertainty concerning the meaning of the term 'entrapped DNA'. In fact, it has not always made clear whether this term refers to DNA which is encapsulated in the internal water pool of the liposomes, and/or to material which is simply bound to the liposome surface. We believe (and this work was performed under this assumption) that for the sake of clarity the notion of 'entrapped DNA' should refer to material which is compartmentalized in the internal water pool. In order to meet this condition, two major experimental difficulties must be solved: (i) one should be able to quantify the DNA entrapped inside the liposomes; (ii) one should work with well-characterized unilamellar vesicles – in fact, in the case of large multilamellar vesicles (LMV), a sizeable part of DNA can be located between the different layers of the LMV and not in the water pool of the liposome compartment.

The main aim of this paper is to determine the best conditions to maximally entrap DNA and RNA in the interior of unilamellar liposomes, and to show that under these conditions the entrapped nucleic acids can be studied directly by spectroscopic experiments. In the first part of this work we will compare some of the techniques most commonly used in the literature to entrap DNA. Once the best procedure has been established, the influence of various parameters (such as the size and the chemical structure of the liposomes) on the entrapment rate is studied. Under the standardized conditions, entrapment yield up to 60% (relative to the initial amount of DNA or RNA) can be obtained.

2. Materials and methods

2.1. Materials

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was purchased from Avanti Polar Lipids Inc. (Birmingham, AL, USA), phosphatidylserine (PS) was obtained from Serva (Heidelberg, Germany). Didodecyldimethylammonium bromide (DDAB), DNA (from herring testes), wheat germ tRNA, cholate and deoxycholate were purchased from Fluka (Buchs, Switzerland). ATP was obtained from Pharmacia (Uppsala, Sweden). Pancreatic DNase I (from bovine pancreas) with a specific activity of 2000 Kunitz units $\times$ mg^{-1}, exonuclease III (*E. coli* with 100 units $\times$ μl^{-1}) and proteinase K (*Tritirachium album* with 20 units $\times$ mg^{-1}) were purchased from Boehringer Mannheim GmbH (Mannheim, Germany). The DNA polymerase for PCR was purchased from Finnzymes Oy (Espoo, Finland), the DNA polymerase I large fragment (Klenow), and the restriction enzymes were bought from New England BioLabs Inc. The oligonucleotides used for the polymerase chain reaction were purchased from Mycrosynth AG (Balgach, Switzerland). The plasmid pSP64-JE was a kind gift of Juan Gomez (University of Zurich, Switzerland). [α-^{35}S]ATP and [α-^{35}S]dATP were purchased from Amersham (England). Bio-beads Bio-Gel A15m were obtained from Bio-Rad Lab. (Richmond, CA, USA). The beads were extensively washed before use with the elution buffer.

2.2. Preparation and radioactive labelling of different DNAs

Plasmid DNA was isolated by the alkaline lysis method and purified by cesium chloride density gradient centrifugation [17] and the purified plasmid was linearized by digestion with EcoRI. The radioactive labelling was carried out by filling the recessed 3′-termini using 8 units of Klenow, 50 μM dCTP, dGTP and dTTP each, 5 μM dATP and 3–5 μCi [^{35}S]dATP. The linearized plasmid DNA was separated from the free [^{35}S]dATP by Sephadex G-50 spin column chromatography as described elsewhere [18]. For the entrapment of plasmid DNA, about 8 μg of linearized DNA were used. The 369-bp DNA fragment was produced/labelled by PCR as described elsewhere [4] and purified by gel filtration chromatography. The experiments using the 369-bp fragment were always carried out with 8–9 μg of sonicated testis DNA (sonication was carried out on ice using a probe sonicator (Sonifier 250 from Branson, 30 min, 20 W, duty cycle 50%); the mean length of the DNA strands was shown by agarose electrophoresis to correspond to approximately 500 bp) and 1–2 μg of PCR-product with about 10 000–20 000 cpm in total. The solutions containing the

DNAs used for our experiments were shown to be precipitable by trichloroacetic acid to 85–95%.

2.3. Preparation of liposomes by the freeze / thawing procedure

The lipids were dissolved in chloroform and the solvent was subsequently removed by evaporation followed by an overnight drying under high vacuum. The dried lipids were dispersed in a buffer solution (50 mM Tris, pH 8.0, containing appropriate amounts of NaCl) and sonicated for 10 min in a bath sonicator (Sonorex RK 100H from Bandelin Electronic). Then the material to be encapsulated was added and the final concentration of lipids was adjusted to 120 mM or 160 mM depending on the experiment. The dispersion was treated by freeze/thawing 10 times. After freezing in liquid nitrogen, the samples were always thawed for 15 min at room temperature. Before extrusion, the liposome dispersion was diluted to a lipid concentration of 40 mM using 50 mM Tris (pH 8.0), containing the appropriate amount of NaCl, and then forced 10 times through two stacked polycarbonate filters with pore sizes of 400 nm in diameter (for extrusion a Liposofast from Avestin Inc. was used). The procedure was repeated with filters with pore sizes of 200, 100 and 50 nm depending on the experiment. The extruded liposomes were loaded on a 'spin column' (Bio-Gel A-15 m, previously equilibrated with the appropriate buffer, pH 8.0) and centrifuged at $165 \times g$ for 2 min as described elsewhere [18]. Usually 22–24 eluates of about 50 μl each were collected: the fractions 2–7 were usually turbid, the others showed no clearly visible turbidity, indicating that they contained no significant number of liposomes.

2.4. Preparation of liposomes by dehydration / rehydration

Vesicle aggregates were prepared as described elsewhere [9]. In brief, vesicle aggregates were produced by dissolving a POPC film in 50 mM Tris (pH 8.0) followed by a sonication treatment for 10 min in a bath sonicator. The nucleic acids – dissolved in 50 mM Tris (pH 8.0) – were added and the dispersion was vortexed. The resulting mixture was kept at room temperature under a constant nitrogen flow until almost dry. For the rehydration step, the required volume of water was added for a final lipid concentration of 120 mM and subsequently diluted to 40 mM. The extrusion of the dispersion and the other procedures were done as described above.

2.5. Preparation of liposomes by reverse phase evaporation

The reverse phase evaporation was in principle performed as previously described [8]. In brief, 10 mM lipid was first dissolved in a 9:1 mixture of diethylether/chloroform. Half of the final aqueous phase volume was added without nucleic acids first, and the two-phase system was sonicated for 1 min with a probe sonicator (Sonifier 250 from Branson, 10 W, duty cycle 50%). The turbid dispersion was then evaporated for 10 min at room temperature under a pressure of 500 mbar; thereafter the solute to be entrapped was added at a lipid concentration of 120 mM. After complete evaporation of the organic solvents (90 min, 40°C, 400 mbar), the remaining lipid dispersion was diluted to 40 mM before extrusion of the vesicle aggregates. The further steps of the procedure have been carried out as described above.

2.6. Encapsulation of DNA into liposomes of various compositions

For encapsulation of nucleic acids into liposomes, 10 μg of linearized 369-bp fragment DNA or 10 μg of plasmid DNA containing ^{35}S-labelled DNA were added to the sonicated liposomes and treated as described above by freeze/thawing, by dehydration/rehydration or by reverse phase evaporation. For digestion of the unentrapped material, 700–800 units of pancreatic DNase I, 200 units of exonuclease III, 5 mM $MgCl_2$, and 0.1 mM DTT were added to the external phase after extruding the liposomal aggregates. This amount of enzyme was shown to be sufficient to digest 10 μg of DNA (for some experiments with higher quantities of DNA the amount of enzymes was proportionally adjusted). After incubating for 3 h at 37°C, the reaction was stopped by adding 7 mM EDTA. The external bulk medium was separated from the liposomes by gel filtration spin column chromatography and the result-

ing fractions were analyzed either by UV/Vis spectroscopy (in this case the liposomes were first solubilized by adding 40 mM cholate) or by measuring the radioactivity using liquid scintillation counting. To test the encapsulation efficiency of unextruded liposomes, they were separated after DNase I/exonuclease III digestion by centrifugation in an Eppendorf centrifuge ($10\,000 \times g$). The pellet was washed three times in the same volume of buffer and the radioactivity of the supernatants as well as of the pellet was determined.

2.7. UV / Vis and circular dichroism spectroscopy

UV/Vis absorption spectra were recorded at 25°C on a Cary 1E spectrophotometer from Varian using quartz cells. Circular dichroism (CD) spectra were measured on a JASCO J-600 spectropolarimeter equipped with a thermostatted cell holder. Quartz cells of 0.05 cm pathlength were used. Samples were scanned 16 times at a rate of 50 nm $\times$ min^{-1}. Molar ellipticities were normalized per nucleotide. For CD or UV/Vis measurements of DNA inside liposomes (extruded through polycarbonate filters with pore sizes of 100 nm in diameter) a much larger amount of DNA (240 μg of sonicated DNA with an estimated mean length of 500 bp) was required and therefore much more pancreatic DNase I (3000 units) or exonuclease III (300 units), respectively, had to be used for the digestion. To avoid a protein signal from these high amounts of digestive enzymes, the liposomal dispersion was treated with proteinase K (0.5–1 units) for 120 min before gel filtration column chromatography was performed. The turbid fractions were pooled and CD measurements were carried out.

2.8. Electron microscopy

For the determination of the mean size and shape of the surfactant aggregates, electron microscopy analysis was carried out by the freeze/fracture method [19]. We analyzed 10 electron micrographs with about 30–40 liposomes each and determined their size. Because only half-shadowed liposomes can be counted directly, we used the correction procedure proposed by Egelhaaf et al. [20] by which direct determination of the size of liposomes is possible.

2.9. Visualization of DNA by polyacrylamide gel electrophoresis (12.5%) and quantification of the obtained DNA bands

DNA encapsulated in POPC/PS and POPC/DDAB liposomes by the freeze/thawing method was recovered from the anionic and cationic liposomes and the extent of such a recovery was determined by gel electrophoresis. To this aim, DNA outside the liposomes was first digested enzymatically and the liposomes were separated from the external bulk medium by gel filtration column chromatography; the liposomes corresponding to fraction number 2–6 were solubilized by adding 1% deoxycholate (W/V) and the solution was immediately extracted 2 times by phenol/chloroform treatment. The aqueous solution containing the DNA was subjected to gel electrophoresis and visualized/quantified using a PhosphorImager® (Molecular Dynamics).

3. Results

3.1. Comparison of various experimental procedures

There are three main methods which are commonly used to entrap nucleic acids in liposomes. The first is based on the reverse evaporation (REV method) [8], according to which the nucleic acids dissolved in water are added to lipids dispersed in organic solvents and the resulting dispersion is subsequently evaporated in order to induce vesiculation. The second procedure is based on a dehydration/rehydration cycle [9]. The nucleic acids are added to a dispersion of SUL (small unilamellar liposomes) which is then dehydrated under a constant nitrogen flow until almost dry. Afterwards the dried material is rehydrated and subsequently vortexed in order to induce the formation of nonextruded liposomal aggregates. The third method is the freeze/thawing procedure [10–12] which involves the addition of the material to be entrapped to a dispersion of SUL, followed by a series of freeze/thawing operations. In contrast to the other two methods, here we have a final sizing down by extrusion. As mentioned in the introduction, these three methods were compared with each other using standardized conditions. To this aim,

we used in all cases liposomes obtained from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) which were generally extruded through filters of a certain pore size. In particular, polycarbonate filters of 50 and 400 nm were used, which yielded liposomes having, under our conditions, an average diameter of 45 and 180 nm, respectively (see Table 1). The same concentration of lipid was used in all cases: starting with an initial concentration of 120 mM, the suspensions were diluted to 40 mM lipid after extrusion.

In the first part of this work different nucleic acid materials, namely yeast tRNAs (consisting of ca. 75 bases), a linearized 369-bp DNA segment and ATP – as low molecular weight reference – have been used. It would have been desirable to apply the same nucleotide concentration of all these compounds, but this was not easy in view of their large difference in molecular weight and in view of the sensitivity assay. However, ATP can be compared with the other two compounds: by using 35 mM ATP and 466.7 μM tRNA (normalized per nucleotide this equals approximately 35 mM) as well as 9 mM ATP and 12.5 μM DNA (a very high concentrated DNA solution of 3 $mg \times ml^{-1}$).

Before looking at the results, consider the meaning of these concentrations. With a 40 mM POPC solution, the 'molar' concentration of 100-nm liposomes (assuming an aggregation number of 8.2×10^4) is around 0.5 μM. This means that the initial concentration of the nucleic acid material is always in significant excess over the liposomes. Consider now that one liposome having a 50-nm diameter has an internal volume of 4.05×10^{-17} ml, which becomes 4.16×10^{-16} ml for those having a 100-nm diameter. This means that under our typical conditions the total volume of the whole liposomal water pool equals 6 and 13%, respectively of the bulk solution. These figures, within the limits of the approximations used for the calculation, should set a theoretical maximal percentage of the guest molecules that can be entrapped (assuming a passive encapsulation, i.e. no specific interactions with the membrane).

Finally, let us consider the details of the experimental protocol used for these studies. Especially critical, as already mentioned in the introduction, was the discrimination between the material entrapped in the water pool of the liposomes from the material which was bound to the outer surface or was present in the bulk solution. Particular care was taken here to destroy the nonentrapped polymeric material by the use of high amounts of pancreatic DNase I/exonuclease III (the enzymes do not penetrate into the liposomes). The amount of digestive enzymes was

Table 1
Comparison of the entrapment yields in POPC liposomes produced with the three selected methods

Solute to be encapsulated in POPC liposomes	Diameter of the liposomes and (in parentheses) of the filter pores used for extrusion (nm)	Method and encapsulation yield (%)		
		Freeze/thaw	Dehydration/rehydration	Reverse phase evaporation
(A)				
ATP	180 (400)	29 ± 5	21 ± 5	16 ± 5
	45 (50)	25 ± 4	N.D.	9 ± 3
tRNA	180 (400)	12 ± 2	9 ± 2	9 ± 3
(B)				
ATP	180 (400)	31 ± 3	23 ± 3	17 ± 3
	45 (50)	24 ± 3	N.D.	9 ± 3
DNA (369 bp)	Unextruded	54 ± 8	43 ± 8	25 ± 5
	180 (400)	30 ± 8	14 ± 5	11 ± 5

(A) The entrapment was performed with 120 mM POPC and 35 mM ATP or 466.7 μM tRNA (corresponds to about 35 mM normalized per nucleotide) by the indicated method. Thereafter the liposome suspension was diluted to 40 mM POPC. After solubilizing the liposomes with 40 mM cholate, the UV absorption was measured and the yield of entrapment was determined. The size of the liposomes was determined by virtue of freeze/fracture electron micrographs. *(B)* The encapsulation was carried out as described above but with 9 mM ATP or 12.5 μM 369 bp DNA (containing ^{35}S-labelled DNA; the 12.5 μM DNA correspond to 9 mM ATP). The analysis of the entrapped material was accomplished by scintillation counting. All the experiments were done at least in triplicate.

such, that in control experiments containing the same amount of liposomes and the same overall concentration of DNA added from the outside, the digestion was complete (more than 98%, see Fig. 1) within 180 min. This suspension was then passed down a spin column. This kind of chromatography was very efficient in separating the liposomes from the lower molecular weight compounds such as ATP, tRNA or the small oligonucleotides which were the products of the DNase I/exonuclease III digestion. By contrast the direct separation between liposomes and the undigested high molecular weight DNA was not successful. In this way, by relying on this combination of extensive enzymatic digestion and an efficient column chromatography, one can be rather confident that the amount of nucleic acid material, eluted together with the liposomes, was really compartmentalized inside the liposomes. To the best of our knowledge, these precautions have not been considered in the literature before.

Results of the entrapment experiments are shown in Table 1 and are expressed in percentage of the initial amount of nucleic acid material. The size of the liposomes, as determined independently by freeze/fracture electron microscopy and by light scattering analysis, is also indicated, together with the pore size of the polycarbonate filters (in parentheses) used for extrusion. The percentage of the entrapment varies from 9–31% for the extruded liposomes (and up to 54% for the nonextruded liposomes), and it is apparent that the entrapment efficiency for a given substance tends to be higher with larger liposomes (consider that the lipid concentration i.e. the total bilayer surface is constant). The dehydration/rehydration method gave high entrapment yields with unextruded liposomes – but these yields became rather modest once the liposomes were extruded. This indicates that by employing the dehydration/rehydration method most of the guest material was entrapped in the various layers of multilamellar liposomes, whereas, relatively little material seemed to go inside the water pool. This phenomenon could also be observed using the other two methods – see the data for the 369-bp fragment – but to a much lower extent. Note also that the dimension of the guest molecule does not seem to play a major role for the entrapment efficiency – the 369 bp DNA was entrapped as efficiently as ATP. Surprising was the relative low yield obtained with tRNA as compared to that of DNA. Extensive studies by varying the initial concentration of the limiting nucleic acid material have not been carried out, however, the experiments reported in Table 1 for different ATP concentrations – as well as experiments which are presented later on in this paper, indicate that the entrapment efficiency does not change noticeably by increasing the amount of solute (this is true under our conditions of significant excess of nucleic acid material over liposomes).

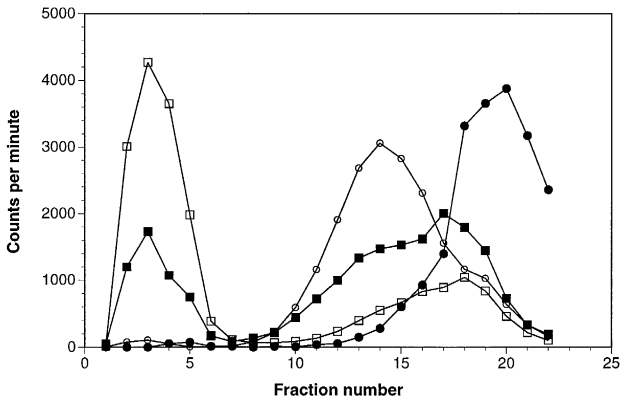


Fig. 1. Digestion of DNA added from the outside to zwitterionic or cationic liposomes. 10 μg of linearized plasmid DNA (^{35}S-labelled) were added externally to POPC (filled circles) or POPC/DDAB (97.5:2.5; open circles) liposomes together with 700 units of pancreatic DNase I and 200 units of exonuclease III, 5 mM $MgCl_2$, and 0.1 mM DTT. The reactions were incubated at 37°C for 3 h, before the reaction mixture was subjected to a Bio-Gel A15m 'spin column' and centrifuged at 165× *g*. Only the fractions number 2–7 showed visible turbidity. For comparison, data of our 'normal entrapment experiments' (filled squares for POPC, open squares for POPC/DDAB liposomes) are also shown.

3.2. The influence of various parameters on the entrapment efficiency of nucleic acids by the freeze/thawing method

Having selected the freeze/thawing method as the most useful one, we set about studying whether and to what extent the entrapment efficiency could be affected by external as well as structural parameters. As a first important external parameter we have chosen the salt concentration. Results are shown in Table 2 and are quite surprising. In all the cases investigated, the presence of 200 mM NaCl in the

Table 2
Entrapment yields depending on the salt concentration

Liposome composition, solute	Type of solute and encapsulation yield (%)		
	Dialysed solution	50 mM Tris (pH 8.0)	50 mM Tris (pH 8.0) with 200 mM NaCl salt
POPC/DDAB 97.5 : 2.5), ATP	32 ± 4	N.D.	12 ± 3
POPC/DDAB 97.5 : 2.5, tRNA	43 ± 4	45 ± 4	5 ± 3
POPC, DNA	N.D.	19 ± 4	4 ± 3
POPC/DDAB 97.5 : 2.5, DNA	51 ± 4	47 ± 4	14 ± 4

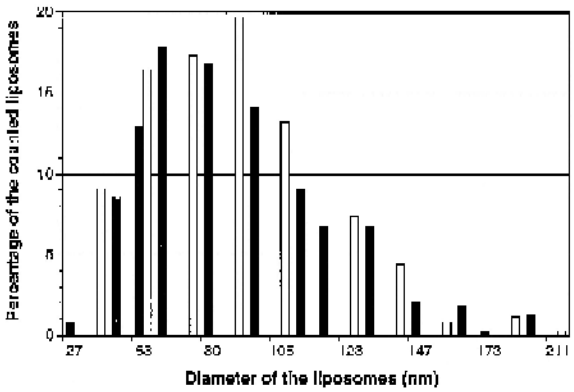
These experiments were carried out with 100 nm extruded liposomes using 160 mM lipids during the freeze/thaw cycles and diluted to 40 mM before extrusion. The same amount of solute was used as in Table 1. The lipid films were dispersed in a buffer solution containing 50 mM Tris (pH 8.0) with/without 200 mM NaCl. In order to remove any salt possibly present in the commercial preparations the suspensions containing the solutes were dialyzed prior to the addition of 200 mM NaCl (left column). All experiments were done at least in triplicate.

stock solution remarkably decreased the entrapment efficiency with POPC liposomes as well as with POPC/DDAB (97.5 : 2.5) liposomes. We argued initially that this might be due to the influence of the salt ions on the dimensions of the liposomes, resulting in smaller liposomes during the freeze/thawing cycles [21,22] at higher salt concentrations. Therefore the size distributions of liposomes in the presence or absence of 200 mM NaCl was determined and, as shown in Fig. 2A, no significant differences can be observed. The obtained dimensions of the liposomes prepared without NaCl corresponded to approximately 85 nm, the liposomes prepared in the presence of 200 mM NaCl had a mean diameter of about 81 nm; this slight difference can not be the reason for this decrease of the entrapment yields in the presence of salt. An alternative explanation for this salt effect is suggested by the observation that the salt induces formation of multilamellar structures as documented in Fig. 2B. Clearly, these 'onion-like structures' re-

Table 3
Entrapment yields depending on the length of DNA fragments and the diameter/composition of the liposomes

Liposome composition	Diameter (nm) of the liposomes after extrusion (pore size of the filters indicated in parentheses)	Linearized plasmid DNA (3368 bp) (%)	PCR fragments (369 bp) (%)
POPC	180 (400)	27 ± 5	30 ± 8
	70 (100)	9 ± 4	22 ± 4
	45 (50)	5 ± 4	9 ± 4
POPC/PS 9 : 1	N.D. (400)	26 ± 5	32 ± 5
	65 (100)	9 ± 3	25 ± 3 [a]
	45 (50)	6 ± 3	11 ± 3
POPC/DDAB 97.5 : 2.5	120 (400)	N.D.	61 ± 5
	80 (100)	N.D.	55 ± 4
	N.D. (50)	N.D.	48 ± 4
POPC/DDAB 99 : 1	120 (400)	50 ± 4	N.D.
	80 (100)	50 ± 4	N.D.
	N.D. (50)	17 ± 4	N.D.

These experiments were carried out using 160 mM lipids during the freeze/thaw cycles and the liposomal suspensions were diluted to 40 mM before extrusion. 10 μg of DNA (containing radioactive DNA) were used for the encapsulation. The size of the liposomes was estimated by virtue of freeze/fracture electron micrographs. All experiments were at least done in triplicate. [a] In these experiments we also varied the initial amount of DNA between 0.8 and 100 μg (10 nM to 1.25 μM) and obtained practically the same result as indicated for 10 μg of DNA.


A
Percentage of the counted liposomes
20
15
10
5
0
27
53
80
105
128
147
173
211
Diameter of the liposomes (nm)

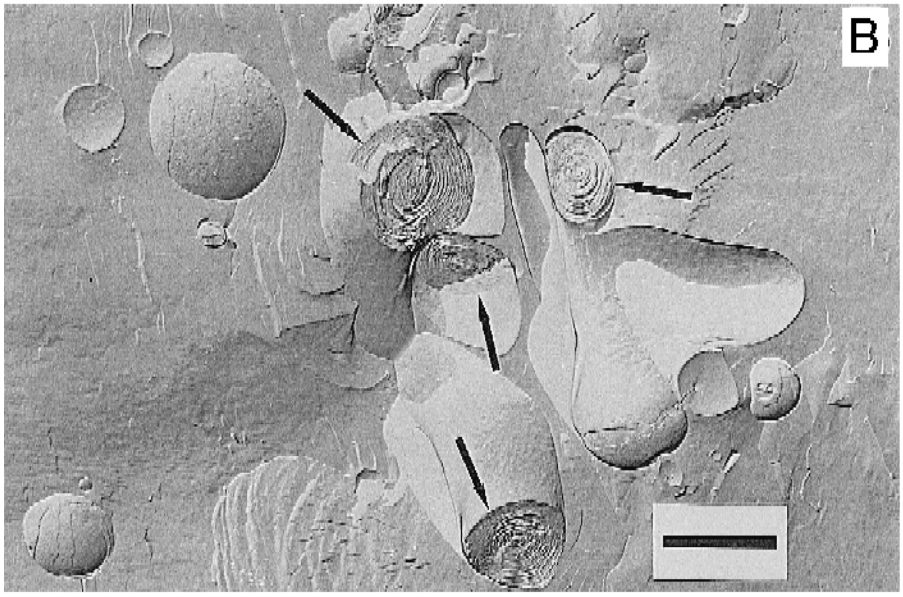

B

Table 4
Efficiency of the digestion of DNA added *externally* to cationic liposomes of various compositions

DDAB content (%) of total lipid amount	DNA in the fractions containing the liposomes after separation on a spin column (%)	
	Linearized plasmid DNA (3368 bp)	PCR fragments (369 bp)
10	32	16
5	17	5
2.5	9	1
1	2	0.3

All these data were obtained as described in Section 2. The liposomes were extruded through polycarbonate filters with a pore size of 400 nm before 10 μg of DNA, 700–800 units of DNase I and 200 units of exonuclease III were added. The suspension was incubated for 3 h at 37°C before subjected to a Bio-Gel column. The DNA coeluting with liposomes (fraction number 2–7) was determined by scintillation counting.

duce the amount of lipid which is available for the entrapment. We believe that this higher lamellarity partly persists also after the extrusion thus causing an overall decrease of the liposomal water pool (as it has been described in the literature using similar lipids [23]).

Another parameter, which is important for the entrapment efficiency, is the chemical structure of the liposomes, in particular the charge and charge density. To this aim, POPC liposomes containing either the negatively charged phosphatidylserine (PS) or the positively charged didodecyldimethylammonium ions (DDAB) were prepared (see Table 3). The percentages of PS and DDAB were kept relatively low, in particular POPC/PS 90:10 and POPC/DDAB from 99:1 to 90:10 were used, but for the final encapsulation studies the amount of DDAB was limited to 2.5%. There were good reasons for maintaining the percentage of PS or DDAB so low: one was to keep the structure of the mixed liposomes as close as possible to the one of the POPC liposomes [24]; the other was that it was not possible to digest reliably all DNA outside the liposomes possessing a larger amount of positively charged surfactant. This was checked by using radiolabelled DNA (the 369-bp segment) which was added externally to a POPC/DDAB (90:10) liposomal preparation (for details see experimental part). Even after a prolonged digestion with DNase I and exonuclease III (up to 48 h) the eluted liposomes showed a considerable amount of radioactivity coeluting with the liposomes. Some results are presented in Table 4. In fact, about 20% of DNA was coeluted with POPC/DDAB (90:10) liposomes, indicating that about 20% of the initial amount of DNA could not be digested. In contrast, radioactive ATP added to POPC/DDAB (90:10) could be separated completely by column chromatography. In conclusion, the positively charged liposomes seem to bind longer DNA fragments so tightly, that a complete enzymatic digestion is not possible. For this reason, as stated initially, the amount of DDAB was limited to a value allowing a complete digestion of the DNA added externally. This observation is important also for reading and interpreting some of the data

Fig. 2. (A) Size distribution of POPC/DDAB (97.5:2.5) liposomes in the presence or absence of 200 mM NaCl. The liposomes were prepared in 50 mM Tris (pH 8.0) with/without 200 mM NaCl by freeze/thaw cycles as described in Section 2. As solute we used tRNA (11.6 mg/ml). The suspensions were diluted to a lipid concentration of 40 mM and then extruded through 100-nm filter pores. The mean diameter, as estimated by virtue of freeze/fracture micrographs, was 85 nm for the resulting liposomes prepared without 200 mM NaCl (340 liposomes counted, filled bars) and 81 nm for the ones prepared in presence of 200 mM NaCl (388, open bars). (B) Electron micrographs showing the lipidic aggregates (40 mM POPC) after several cycles of freeze/thaw and before extrusion in the presence of 200 mM NaCl. The arrows show typical 'onion-like' structures not observed to that extent in the liposomal suspension prepared without NaCl (bottom micrograph). The bar corresponds to 1 μm.

presented in the literature concerning the use of cationic surfactants or cosurfactant for the 'encapsulation' of DNA.

Even using only 2.5% DDAB, the influence of the charge density is very clear, as shown in Table 3. In general, the entrapment efficiency for DNA could be significantly increased by using DDAB, whereas the presence of the negatively charged cosurfactant PS did not seem to affect the encapsulation yields. In the case of 2.5% DDAB, the entrapment efficiency went *up to 61%*, which was indeed remarkable especially if one takes into account that the average size of a 400 nm-extruded POPC/DDAB (97.5:2.5) liposome is clearly reduced in comparison to POPC liposomes (about 120 nm in diameter instead of 180 nm, both without NaCl during the preparation). Finally, notice the influence of the DNA chain length: the two DNA species used differ in length by an order of magnitude at about the same molar nucleotide concentration. Consider that the length of the linearized 3368 bp plasmid, if it existed as a linear rod, would be (approximately) 1400 nm, to be compared to the mean diameter of the liposomes that varies in our experiments between 45 and 120 nm. Clearly, the entrapped DNA material must somehow be 'superpackaged' (as also evidenced by our CD measurements).

An important question was whether and to what extent the percentage of entrapment changed by varying the initial amount of DNA. Some typical data are also reported in Table 3 and they indicate no substantial difference between 0.8 and 100 μg of DNA. We also examined whether the encapsulated DNA could be recovered and whether its molecular weight was decreased during this relatively long period used for the entrapment. The encapsulated DNA could be almost quantitatively recovered (up to 80%), and visualized/quantified by gel electrophoresis and the PhosphorImager® technique (data not shown). This was also the case for the entrapment of DNA within the water pool of cationic liposomes.

3.3. Spectroscopic observations on the encapsulated DNA

The relatively large amount of DNA entrapped in liposomes gave us the possibility to carry out direct spectroscopic analysis of the compartmentalized biopolymer. Fig. 3 shows the CD spectra of DNA entrapped within liposomes. Despite the significant scattering due to the liposomes, the UV and CD absorption spectra of DNA could be clearly assessed, and they showed no substantial difference with respect to DNA dissolved in water. In other words, the entrapment procedure did not seem to affect the electronic transitions of the biopolymer. This is at variance with our earlier studies with the entrapment of DNA in the water pool of reverse micelles [25], where considerable spectroscopic and structural changes have been observed that are ascribed to the supercondensed form of DNA – in fact under those conditions the CD spectra acquired the form of a Ψ-spectrum. Since the Ψ-spectrum of DNA is usually ascribed to intermolecular interactions producing a condensed association of DNA molecules, our data indicate that the compartmentalization of DNA in liposomes does not induce this kind of intermolecular packaging. Fig. 3 also shows the CD spectrum of DNA added externally to the POPC/DDAB liposomes (20% DDAB). In this case, significant perturbations of the spectrum relative to water were observed, which increase with increasing DDAB content (data not shown) and which are reminiscent of

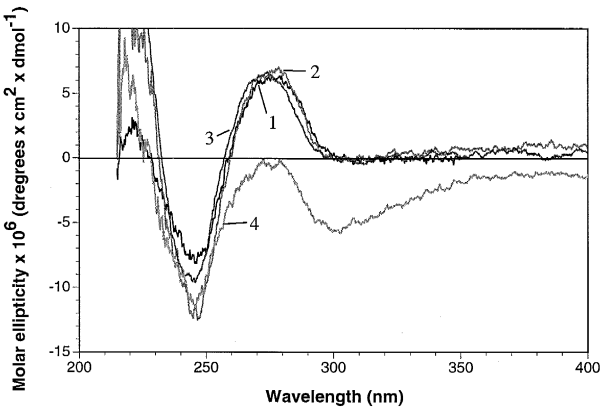


Fig. 3. Circular dichroism spectra of DNA entrapped in liposomes of various compositions. The liposomes used for these measurements were prepared in 50 mM Tris (pH 8.0) by freeze/thaw cycles as described above and diluted to a lipid concentration of 40 mM. (1) DNA in 50 mM Tris (pH 8.0). (2) DNA inside POPC/DDAB (97.5:2.5) liposomes. (3) DNA inside POPC/PS (90:10) liposomes. (4) DNA added externally to POPC/DDAB (80:20) liposomes after extrusion. Only the spectrum of DNA added externally to positively charged liposomes shows a significant perturbation.

the Ψ-spectrum as obtained for example from the interaction of DNA with positively charged polyelectrolytes. Thus, binding of DNA to the external positively charged surface of the liposomes induced some kind of condensation of the DNA, if the charge density was increased above the value used for our entrapment experiments.

4. Discussion

The present work was partly inspired by the uncertainty in the literature on the subject of DNA entrapment in liposomes, and it has been shown that it is possible, by careful choice of the experimental procedure, to prepare unilamellar liposomes in which the nucleic acid material is properly localized in the vesicular water pool. Particularly important for the determination of the amount of material localized in the inner aqueous phase is the elimination of nucleic acids bound to the external surface of the liposomes as well as the material bound between the various lamellae of multilamellar, nonextruded liposomes.

This work also shows the relevance of a number of parameters on the entrapment efficiency: the molecular weight of the guest molecules, the relative concentration of liposomes and guest molecules, the size of the liposomes, their charge and charge density and, particularly important, the salt concentration. The rationalization of the results is far from being simple. It is not, for example, clear why the size of the liposomes (assuming a constant total water pool volume) should play a role whereas the size of the guest molecules does not. It is also not clear why the entrapment efficiency of L-shaped tRNA should be smaller than that for the larger and more rigid 369-bp DNA fragment. The relatively high entrapment percentage obtained with the uncharged (zwitterionic) POPC liposomes, i.e. higher than the volume percentage of the liposomes in the solution and in the absence of apparently significant interaction forces (6% for 50 nm and 13% for 100 nm-liposomes) is caused by the fact that the freeze/thawing procedure can be realized employing 160 mM POPC during the freeze/thawing process – something which is not possible with other entrapment procedures – and therefore the final entrapment yield is much higher than the calculated 6 and 13%, respectively (see Section 3). It could also be shown that it is possible to encapsulate up to 60% of the total DNA into POPC liposomes containing 2.5% DDAB despite the fact that these cationic liposomes were shown to be significantly smaller than the zwitterionic ones. This shows that mainly electrostatic interactions are responsible for the DNA encapsulation, and not the size of the inner aqueous pool.

The salt effect is also very interesting. It might be due to a change in the nature of the counterion of the solute molecules and/or on the charged bilayer, which would alter the electronic interaction between them. It has been known for a long time that increasing the salt concentration may cause multilamellarity [26] and decrease the entrapment efficiency of solutes [10]; but to the best of our knowledge, there is no systematic investigation with nucleic acids on this effect and it needs to be further investigated. We have evidence that the encapsulation efficiency is not linearly decreased with increasing amounts of salts (data not shown). Also the role of the different cations and anions needs to be further exploited.

The compartmentalized DNA appears to be unperturbed as far as the structure and conformation are concerned – the interesting occurrence of Ψ-spectra of condensed DNA has not been observed, but on the other hand the maintenance of the native structure is important for numerous biological studies.

Further work is in progress along these lines focusing on the compartmentalization of nucleic acid material possessing more specific functional properties, such as ribosomes and/or messenger RNA.

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References

[1] P. Walde, A. Goto, P.-A. Monnard, M. Wessicken, P.L. Luisi, J. Am. Chem. Soc. 116 (1994) 7541–7547.

[2] A.C. Chakrabarti, R.R. Breaker, G.F. Joyce, D.W. Deamer, J. Mol. Evol. 39 (1994) 555–559.
[3] T. Oberholzer, R. Wick, P.L. Luisi, C.K. Biebricher, Biochem. Biophys. Res. Commun. 207 (1995) 250–257.
[4] T. Oberholzer, M. Albrizio, P.L. Luisi, Chem. Biol. 2 (1995) 677–682.
[5] R. Wick, P.L. Luisi, Chem. Biol. 3 (1996) 277–285.
[6] G.J. Nabel, E.G. Nabel, Z.-Y. Yang, B.A. Fox, G.E. Plautz, X. Gao, L. Huang, S. Shu, D. Gordon, A.E. Chang, Proc. Natl. Acad. Sci. USA 90 (1993) 11307–11311.
[7] N.J. Caplen, E.F.W.W. Alton, P.G. Middleton, I.R. Dorin, B.J. Stevenson, X. Gao, S.R. Durham, P.K. Jeffery, M.E. Hodson, C. Coutelle, L. Huang, D.J. Porteous, R. Williamson, D.M. Geddes, Nature Med. 1 (1995) 39–46.
[8] F. Szoka Jr., D. Papahadjopoulos, Proc. Natl. Acad. Sci. USA 75 (1978) 4194–4198.
[9] D.W. Deamer, G.L. Barchfeld, J. Mol. Evol. 18 (1982) 203–206.
[10] U. Pick, Arch. Biochem. Biophys. 212 (1981) 186–194.
[11] M.J. Hope, M.B. Bally, G. Webb, P.R. Cullis, Biochim. Biophys. Acta 812 (1985) 55–65.
[12] C.J. Chapman, W.L. Erdahl, R.W. Taylor, D.R. Pfeiffer, Chem. Phys. Lipids 55 (1990) 73–83.
[13] R.M. Hoffman, L.B. Margolis, L.D. Bergelson, FEBS Lett. 93 (1978) 365–368.
[14] R. Fraley, S. Subramani, P. Berg, D. Papahadjopoulos, J. Biol. Chem. 255 (1980) 10431–10435.
[15] J.-Y. Legendre, F.C. Szoka Jr., Pharmacol. Res. 9 (1992) 1235–1242.
[16] C. Puyal, P. Milhaud, A. Bienvenue, J.R. Philippot, Eur. J. Biochem. 228 (1995) 697–703.
[17] J. Sambrook, E.F. Fritsch and T. Maniatis (1989) in: Molecular Cloning: A Laboratory Handbook (N. Ford and C., Nolan, Eds.), Cold Spring Harbor Laboratory Press, New York.
[18] A. Chonn, S.C. Semple, P.R. Cullis, Biochim. Biophys. Acta 1070 (1991) 215–222.
[19] M. Müller, N. Meister, Mikroskopie (Wien) 36 (1980) 129–140.
[20] S.U. Egelhaaf, E. Wehrli, M. Müller, M. Adrian, P. Schurtenberger, J. Microsc. 184 (1996) 214–228.
[21] R.C. MacDonald, F.D. Jones, Biochim. Biophys. Acta 1191 (1994) 362–370.
[22] N. Oku, R.C. MacDonald, Biochemistry 22 (1983) 855–863.
[23] R. Schubert, H. Wolburg, K.-H. Schmidt, H.J. Roth, Chem. Phys. Lipids 58 (1991) 121–129.
[24] P. Mitrakos, P.M. Macdonald, Biochemistry 35 (1996) 16714–16722.
[25] V.E. Imre, P.L. Luisi, Biochem. Biophys. Res. Commun. 107 (1982) 538–545.
[26] D. Papahadjopoulos, A. Portis and W. Pangborn (1978) in: Liposomes and their Uses in Biology and Medicine (D. Papahadjopoulos, Ed.), Vol. 308, pp. 50–66, The New York Academy of Science, New York.