QUANTITATIVE ASPECTS OF NUCLEIC ACIDS SEQUESTRATION IN LARGE LIPOSOMES AND THEIR EFFECTS ON PLANT PROTOPLASTS

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Received 23 January 1981

1. Introduction

Large liposomes have been shown to entrap RNA and DNA molecules ([1–5] and references therein). When incubated with living cells, nucleic acids sequestered in lipid vesicles were also shown to be efficiently transferred into cellular compartments where they became functionally expressed. For instance, poliovirus RNA included in L-α-phosphatidyl–L-serine (PS) large unilamellar liposomes (LUV) transfected mammalian cells at high frequency [1]. Similarly, the *Escherichia coli* β-lactamase gene isolated from the plasmid pBR322 and encapsulated in PS small unilamellar liposomes was shown to be incorporated and apparently expressed in mammalian cells [2]. Thus, liposomes seem to constitute promising vectors for the genetic engineering of animal cells.

Although transformation in plant cells still awaits final proof, it appears that here also, liposomes have considerable potential in achieving this goal. Indeed, previous studies suggest that DNA sequestered in large liposomes can under certain conditions be transferred to plant protoplasts nuclei in a rather undegraded form [4–8]. However, DNA uptake by higher plant protoplasts presents specific problems [4,5] and hence, several parameters regarding, i.e., DNA trapping efficiency in liposomes and lipid cytotoxicity must be determined in order to promote high DNA transfer values in the absence of major cellular damage. This paper describes the sequestration of linear and covalently closed circular DNA and single-stranded plant viral RNA in different types of liposomes. Some of their effects on cowpea and carrot protoplasts are also investigated.

2. Materials and methods

2.1. *Nucleic acids*

Cowpea chlorotic mottle virus (CCMV) RNA was isolated as in [11] and was a gift from Dr S. Wyatt (Department of Plant Pathology, WSU). Col E_1 [^{3}H]DNA (800 cpm/mg) was isolated from *E. coli* JC 411 after chloramphenicol amplification and was a gift from R. Calza (Program in Genetics, WSU). Low sequestration values obtained with PS-LUV and PS multilamellar vesicles (MLV) were confirmed by using [^{3}H]DNA of 10-fold higher specific radioactivity. *Agrobacterium tumefaciens* C58 pTi [^{3}H]DNA (1000 cpm/mg) was a gift from Dr F. van Vliet (Genetics Laboratory, State University of Ghent). R6K, pCR1 and pBR322 plasmid DNAs were isolated as in [10]. Salmon sperm DNA (Sigma) needle-sheared to M_r 5 × 10^6 was complexed with ethidium bromide (EtBr) as in [4] prior to encapsulation in liposomes.

2.2. *Liposomes*

Neutral MLV composed of L-α-phosphatidyl choline (lecithin) (Sigma, type V-E from egg yolk) were produced by mechanical shaking as in [4] except that shaking of the lipid film in the presence of DNA dissolved in 'liposome buffer' [4] was reduced to 15 s. Negatively charged MLV composed of L-α-phosphatidyl–L-serine (Sigma) or lecithin plus increasing amounts of dicetyl phosphate (Sigma) were similarly produced. Positively charged MLV composed of lecithin and increasing amounts of stearylamine (Sigma) were also obtained by mechanical shaking. Positively charged LUV of identical composition were obtained by ether infusion as in [5]. Negatively charged PS-LUV were generated from sonicated liposomes as in [9]. All liposomes were spun down at

15 000 rev./min for 15 min in a Sorvall SS 34 rotor and resuspended in 'liposome buffer' [4] prior to analysis.

2.3. *Nucleic acids analysis*

In experiments involving CCMV RNA, liposomes containing the RNA were treated with 2 units/ml micrococcal nuclease (Miles) for 30 min at room temperature in 0.05 M sodium borate buffer containing 5 mM $CaCl_2$ (pH 9.0). After centrifugation, liposomes were resuspended in 0.02 M Tris–HCl buffer (pH 7.5) containing 0.05 M EGTA and lysed with 2% Triton X-100 (Sigma). RNA was then precipitated with 2 M LiCl, collected by centrifugation and analyzed by polyacrylamide gel electrophoresis [11]. In DNA trapping experiments, liposomes were treated with pancreatic deoxyribonuclease as in [4] unless otherwise stated. Liposomes were disrupted with 2% Triton X-100 or 2% sodium deoxycholate (Sigma). DNA binding values were then estimated by fluorimetric measurement of the released DNA–EtBr complex [4] or by liquid scintillation counting where [^{3}H]DNA was used. In some cases, sequestered DNA was analyzed by agarose gel electrophoresis as in [4] except that it was kept at 15 V. pBR322 sequestration was also determined by transformation. After trapping in lecithin MLV, lecithin/stearylamine (10:1, w/w) MLV, PS MLV and PS LUV (1.2 mM lipid in all cases) and DNase treatment [4], liposomes were resuspended in 0.005 M Tris–HCl containing 0.001 M EDTA (pH 8.0) and lysed with 2% sodium deoxycholate. Lipids were extracted with a 9:1 mixture of chloroform–methanol and the aqueous phase collected. Salmon sperm DNA (40 μg/ml final conc.) was added, the solution was made 0.25 M in NaCl and DNA was precipitated overnight at −15°C with 2 vol. of ethanol. Precipitated DNA was collected by a 45 min centrifugation at 15 000 rev./min in a Sorvall SS 34 rotor. Pelleted DNA was redissolved in 0.2 ml, 0.1 M $CaCl_2$. Competent *E. coli* SK1592 cells were produced by overnight incubation at 0°C in 0.1 M $CaCl_2$. Aliquots of those cells were then incubated for 10 min at 0°C with DNA recovered, from lysed liposomes, further incubated for 5 min at 37°C and finally allowed to grow for 2 h at 37°C after addition of 2 ml L-broth. Aliquots were then plated on medium containing ampicillin and tetracyclin at 10 μg/ml. Transformants were scored after overnight incubation at 37°C.

2.4. *Isolation of protoplasts*

Cowpea protoplasts were prepared as in [10]. Carrot protoplasts were derived from carrot cells cv. Amsterdam growing in suspension culture. In the latter case, cell wall digestion proceeded for 4 h at 27°C in 0.45 M mannitol and 0.05 M potassium citrate buffer (pH 4.8) containing 2% Driselase (Kyowa Hakko Kogyo), 1% Cellulose Onozuka R-10 and 0.5% Macerozyme R-10 (Yakult Biochemicals Co.). Protoplasts were filtered through a stainless steel sieve (106 μm openings), harvested and washed in the above buffer devoid of enzymes. Protoplasts were incubated for 15 min at 21°C with increasing amounts of liposome suspensions originally formed in the presence of 20 μg/ml salmon sperm DNA. After washing, protoplasts were counted and the ratio between spherical and burst protoplasts calculated.

In other experiments, carrot protoplasts were incubated for 15 min at 21°C with a given amount of liposomes, washed and incubated for 15 h in buffered mannitol in the presence of 60 μCi/ml carrier free $H_3{}^{32}PO_4$ (NEN). In some cases, the incubation with liposomes was followed by the addition of 10% final polyethylene glycol 6000 (Sigma) in 0.01 M $CaCl_2$. Following progressive dilution [12] and washing, protoplasts were incubated with $H_3{}^{32}PO_4$ as above. After incubation, protoplasts were washed and harvested by centrifugation, lysed with 2% final sodium dodecylsulfate and mixed with 2 vol. cold 10% trichloroacetic acid. The precipitates were collected by filtration though Whatman GF/A filters, dried and counted by Cerenkov effect.

3. Results

3.1. *Nucleic acids encapsulation*

It has been suggested [5,6] that positively charged liposomes have a much larger affinity for nucleic acids than neutral or negatively charged ones. Since the sizes of lecithin (neutral) MLV and LUV on the one hand, and those of lecithin/stearylamine (positively charged) MLV and LUV on the other hand, are not significantly different (P.F.L., unpublished), it may be assumed that the electric charge of these liposomes plays an important role in the nucleic acid binding and sequestration.

Fig.1A shows the total binding capacity of lecithin (neutral) and PS (negatively charged) MLV for a preformed DNA–EtB complex as a function of lipid

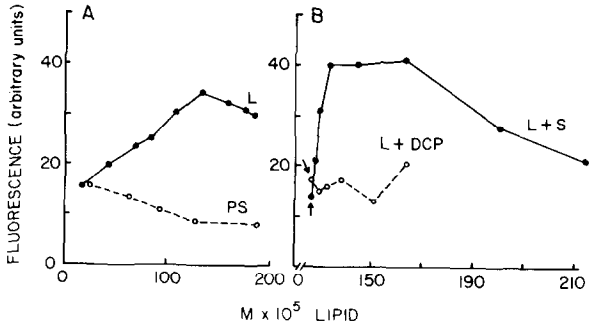

Fig.1. Entrapment of a preformed DNA–EtBr complex in liposomes as a function of lipid concentration. (A) (•) lecithin MLV; (○) L-α-phosphatidyl–L-serine MLV. (B) (•) lecithin/stearylamine MLV; (○) lecithin/dicetyl phosphate MLV. The arrows designate data points corresponding to liposomes composed of lecithin only. Lecithin was kept at 1.2 mM and the concentrations of stearylamine and dicetyl phosphate were progressively increased. DNA was kept constant at 40 μM. Excitation was at 310 nm and emission at 588 nm.

concentration. It can be seen that an increase in the ratio between lecithin and DNA leads to higher binding values displaying an optimum, whereas such a phenomenon is not observed with PS liposomes. Similarly, the presence of increasing amounts of stearylamine in lecithin-based liposomes greatly enhances DNA binding, whereas the addition of dicetylphosphate, resulting in the production of negatively charged vesicles, does not significantly modify the affinity of liposomes for DNA (fig.1B). However, entrapment and/or binding of a DNA–acridine orange complex by negatively charged MLV can be visualized by fluorescence microscopy (not shown). Thus, the low fluorescence values detected with those MLV do not necessarily mean that no DNA binding occurs.

Fig.2 further shows that contrary to all other liposome preparations positively charged ones are not saturated by as high as 200 μM DNA. However, aggregation occurs at high [DNA], probably reflecting neutralization of liposome surface charge. Indeed, this phenomenon occurs even at low [DNA] if preformed lecithin/stearylamine liposomes are incubated with DNA. We also found that at a comparable DNA/lipid ratio, PS LUV bind 50% less DNA than corresponding MLV. In our hands, such LUV are considerably smaller (~1/10th the size) than PS MLV.

DNA sequestration was also determined with Col E1 [^{3}H]DNA. The results are described in table 1. It can be seen that here also, positively charged MLV are most efficient in trapping and protecting DNA

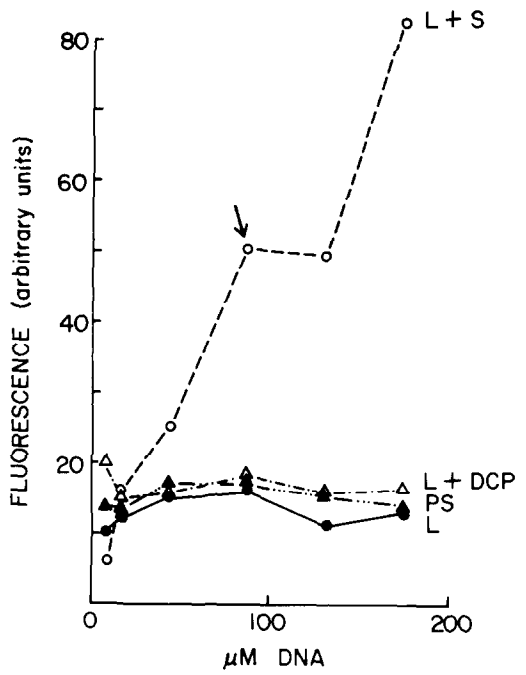

Fig.2. Entrapment of a preformed DNA–EtBr complex in liposomes as a function of DNA concentration; (○) lecithin/stearylamine (21:1 w/w) MLV; (△) lecithin/dicetyl phosphate (15:1, w/w) MLV; (▲) PS MLV; (•) lecithin MLV; lipid was 1.2 mM.

from deoxyribonuclease action. Again, negatively charged MLV and LUV show very poor sequestration capacity. These results confirm the observation [2] that DNA of $M_r > 3 \times 10^6$ is encapsulated at low efficiency.

Fig.3 shows that positively charged MLV can sequester pBR322 (2.8×10^6 M_r) and its multimers,

Table 1
Sequestration of Col E1 [^{3}H]DNA in liposomes[a]

	Liposome composition			
	Lecithin	Lecithin/ stearylamine	PS MLV	PS LUV
Input DNA	4.72	4.35	4.75	4.59
Unbound	4.38	2.43	4.70	4.55
DNase-sensitive	0.10	0.81	0.03	0.03
Sequestered	0.23	1.11	0.01	0.007
	(4.9)	(25.5)	(0.2)	(0.15)

[a] Lipid is 1.4 mM; DNA is expressed in μg

Figures in parentheses represent % input DNA sequestered in liposomes; the lecithin/stearylamine ratio is 10:1 (w/w)

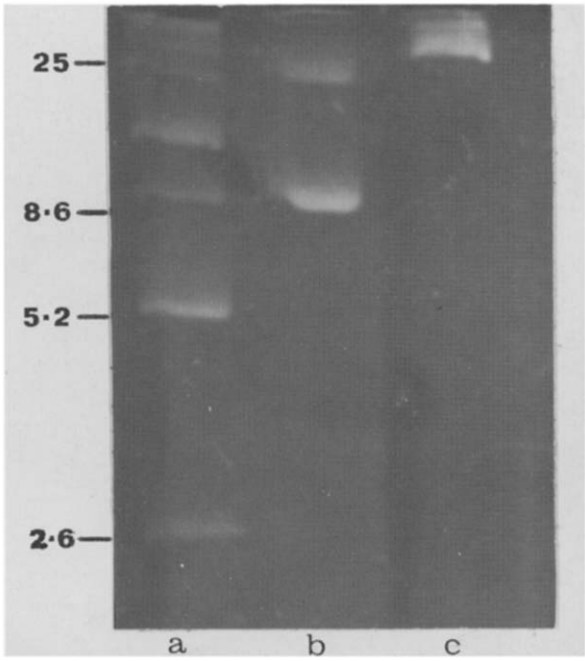

Fig.3. Agarose gel electrophoresis of plasmid DNA recovered from DNase-treated lecithin/stearylamine (10:1, w/w) MLV: (a) pBR322 monomer and multimers; (b) pCR1; (c) R6K. All plasmid preparations contained a certain proportion of open circular DNA. Supercoiled and open circular DNA were not well-resolved in the R6K slot. M_r values are $\times 10^{-6}$.

pCR1 (8.6×10^6 M_r) and R6K (26×10^6 M_r) plasmids. Those molecules are then highly protected from DNase attack. Results obtained by agarose gel electrophoresis and trapping of radiolabeled DNA were confirmed by transformation assay in *E. coli*. pBR322 DNA (3.6 μg) was sequestered in lecithin, lecithin/stearylamine, PS MLV and PS LUV and the liposomes were treated with DNase as above. After lysis and lipid extraction, the pBR322 DNA was incubated with competent *E. coli* cells and transformants were scored on ampicillin–tetracyclin plates. The extract from lecithin/stearylamine MLV yielded 75 colonies, that from lecithin MLV 12 colonies, that from PS LUV 5 colonies and finally, 0 colonies were found in an extract from PS MLV. The low transforming efficiency was due to the presence of carrier salmon sperm DNA and that of residual lipids, especially in the case of PS liposomes. Nevertheless, it turns out that here also, positively charged liposomes show much higher DNA binding efficiency. Plasmid DNA of much higher M_r can also be bound to positively charged liposomes. Indeed, pTi C58 [^{3}H]DNA also becomes DNase-resistant after trapping by both lecithin/stearylamine MLV and LUV. Entrapment values of DNase-resistant pTi C58 DNA of 25–65% of input were obtained when 5 μg plasmid were sequestered in such liposomes formed with 10 mg lipids as in [5]. However, in this case, it is not clear whether this large plasmid was actually located inside the liposomes. Indeed, outside binding to positively charged liposomes also rendered DNA resistant to DNase [6].

High M_r plant viral RNA can also be sequestered in lecithin/stearylamine liposomes. Fig.4 shows an electrophoretic pattern of CCMV RNA recovered from such liposomes after treatment with micrococcal nuclease. In this experiment, 56 μg CCMV RNA were trapped in MLV formed with 1 mg lecithin and 0.1 mg stearylamine in 1 ml. Treatment with micrococcal nuclease only released 10% of UV-absorbing material and it can be seen that the sequestered RNA suffered minimal damage. Moreover, all 4 subunits of M_r 1.2, 1.0, 0.8 and 0.3×10^6 were entrapped to a similar degree. Under the same conditions, sequestration in neutral MLV was too low to be detected by polyacrylamide gel electrophoresis and scanning at 254 nm or by staining with toluidine blue.

3.2. *Effects of cowpea and carrot protoplasts*

Liposomes are known to cause protoplasts bursting and to impair their regeneration [5,6]. If DNA-loaded liposomes are to be used in transformation experiments, it is imperative to determine conditions

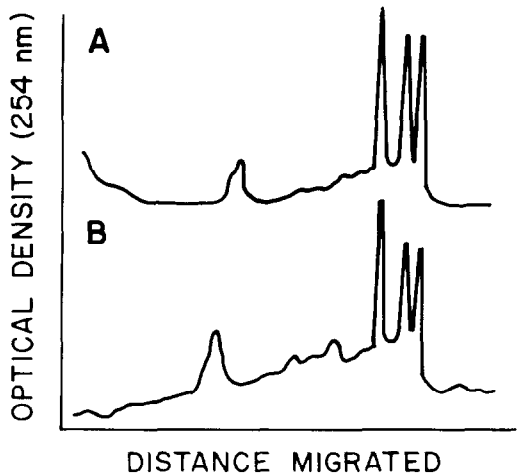

Fig.4. Polyacrylamide gel electrophoresis of CCMV RNA recovered from nuclease-treated lecithin/stearylamine (10:1, w/w) MLV: (A) control RNA; (B) encapsulated RNA; direction of migration is from right to left.

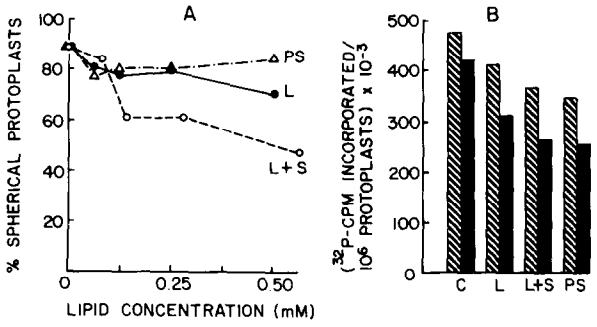

Fig.5. Structural and metabolic effects of liposomes on carrot protoplasts. (A) Protoplast bursting as a function of liposome concentration: (△) PS MLV; (●) lecithin MLV; (○) lecithin/stearylamine (10:1, w/w) MLV. (B) Effect of liposomes on $H_3{}^{32}PO_4$ incorporation into carrot protoplasts (acid-insoluble radioactivity): C, untreated control; L, protoplasts preincubated with lecithin MLV; L + S, protoplasts preincubated with lecithin/stearylamine (10:1, w/w) MLV; PS, protoplasts preincubated with PS MLV; filled bars, values obtained with protoplasts preincubated with both liposomes and polyethylene glycol; hatched bars, with liposomes only.

under which those deleterious effects are minimized. Fig.5A shows that lecithin/stearylamine (10:1, w/w) liposomes cause more carrot protoplast bursting than lecithin and PS MLV at high concentration. However, lipid at <0.09 mM induces little visible structural damage. At a given lipid molarity, the damage is inversely proportional to protoplast concentration in the mixture. Virtually identical observations were made with cowpea protoplasts.

However, fig.5B indicates that liposomes can exercise toxic effects even when little structural damage is visible. For example, when 10^6 carrot protoplasts in 1 ml medium were preincubated with 0.1 mM lipid in liposome form, it was observed that the incorporation of $H_3{}^{32}PO_4$ into acid-precipitable compounds was always decreased. Nevertheless, this decrease did not exceed 37% of the control value. Since polyethylene glycol was found to promote the transfer of DNA from liposomes to plant protoplasts [4,7], the above experiment was repeated after incubation of protoplasts with both liposomes and 10% polyethylene glycol. As expected, polyethylene glycol added to the toxic effect of liposomes, but the lowest incorporation value, found with PS MLV, was still equal to 60% of the control value.

4. Discussion

This paper clearly shows that lecithin/stearylamine positively charged MLV bind and/or sequester much more DNA and RNA than neutral MLV and negatively charged MLV and LUV. Sequestration in lecithin/stearylamine liposomes very efficiently protects nucleic acids against nuclease degradation. Thanks to their high binding capacity, such liposomes can be used at low concentration in nucleic acids transfer experiments and thus induce minimal damage in plant protoplasts. The nature of the interactions between liposomes and plant protoplasts is not known. Therefore, the use of neutral and negatively charged LUV and MLV in gene transfer experiments should not be precluded on the basis of nucleic acids sequestration capacity only.

Experiments to determine the effects of liposomes on the regeneration of higher plant protoplasts are presently underway.

Acknowledgements

This work was supported by USDA/SEA competitive research grant 59-2531-0-1-465-0 and by funds provided for medical and biological research by the State of Washington Initiative Measure no. 171. We thank Dr S. Wyatt for his help in performing the polyacrylamide gel electrophoresis analysis of CCMV RNA and Dr J. Magnuson for the use of his spectrofluorimeter.

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