Entrapment of plasmid DNA in liposomes

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ABSTRACT

The entrapment of plasmid DNA (pMB9) and high molecular weight DNA into large unilamellar liposomes is described. The entrapment of DNA is specific and due to encapsulation of DNA into the aqueous compartment of liposomes. The entrapped DNA, resistant to deoxyribonuclease treatment, could be re-isolated from liposomes intact and, as has been shown by transformation assay, it remains biologically active. The advantages of our method and possible applications are discussed.

INTRODUCTION

Phospholipid vesicles (liposomes) since their definition by Bangham (1) have been used extensively first, as models for biological membranes and more recently as carriers for the introduction of different molecules into target cells which normally have no intracellular access (2-7). Thus liposomes have been successfully applied as carriers of enzymes, in enzyme replacement therapy (8-9), of anti-tumour drugs in cancer chemotherapy (10), and have also been used as immunological adjuvants (11) and for entrapment of synthetic polynucleotides such as polyuridylic acid (12) and synthetic double-stranded ribonucleic acid, poly(I):poly(C) for induction of interferon synthesis (13).

Recent work in this (14-16) and other laboratories (17-20) has shown that nucleic acids can be entrapped in liposomes and subsequently micro-injected into cultured mammalian cells, where they can express the information which they carry.

In the present paper we demonstrate the entrapment of plasmid DNA (pMB9) in large unilamellar liposomes. The entrapped DNA, resistant to deoxyribonuclease treatment, could be re-isolated from liposomes intact and remains biologically active as has been shown by transformation assay.

MATERIALS AND METHODS

Beef brain phosphatidylserine was obtained from Lipid Products, Redhill

Surrey and after washing with EDTA to remove divalent cations was kept at -30°C under nitrogen until used. ^{32}P -dGTP and ^{32}P -dCTP were from Radio-chemical Centre, Amersham, Bucks. Pancreatic deoxyribonuclease (DNase I) was purchased from Worthington Corporation, Freehold, New Jersey. All other reagents were from BDH Chemicals Ltd or Sigma.

E. coli cells harbouring plasmid pMB9 were kindly provided by J.G. Williams. The plasmid was purified by caesium chloride/ethidium bromide density gradient centrifugation (21). ^{32}P nick translated plasmid was prepared according to Rigby et al (22). The radioactivity was determined by Cherenkov counting in a Beckman LS-250 liquid scintillation spectrometer. Specific activity of radiolabelled plasmid DNA was $10^7\text{cpm}/\mu\text{g}$ DNA.

Large unilamellar liposomes consisting of phosphatidylserine only ($10\mu\text{mol}$ for each preparation) were prepared according to Papahadjopoulos et al (23). Routinely liposomes were prepared in NHTE buffer, pH 7.4 (0.1M NaCl, 2mM histidine, 2mM TES, 0.4mM EDTA, pH 7.4). After formation liposomes were sedimented by centrifugation at $35,000\text{rpm}$ at 20°C for 30min in a Beckman SW60 rotor, and washed two times with phosphate buffered saline free of Ca^{2+} and Mg^{2+} .

Entrapped plasmid DNA was extracted as follows: extensively washed liposomes containing pMB9 were suspended in 1ml DNase medium (2mM Tris, pH 7.4, 0.1M NaCl, 0.5mM MgCl_2) and incubated with 400 units of pancreatic deoxyribonuclease for 30min at room temperature. The enzyme was inactivated by addition of 1mM EDTA containing 0.5% SDS and 0.1mg/ml yeast tRNA. An equal volume of phenol-chloroform (1:1, v/v) was added and the mixture was shaken vigorously. The organic phase was extracted once more, the two aqueous phases combined and the DNA was precipitated by the addition of 2.5vol. ethanol at -20°C overnight. Electrophoresis of DNA was carried out on 1% agarose gels in Tris-acetate buffer (40mM Tris, pH 7.8, 1mM EDTA) at 2volt/cm for 16hr. The gel was stained with ethidium bromide ($2\mu\text{g/ml}$) and photographed in the U.V. light using an orange filter. EcoRI fragments of

DNA, a gift from Dr V. Zogza-Dimitriadis, were used as markers. Separation of liposomes from non-entrapped DNA was carried out on a linear $5\text{--}20\%$ sucrose gradient in TE buffer (0.01M Tris, pH 8.0, 0.001M EDTA). The samples for the sucrose gradient which were treated with deoxyribonuclease were first passed through a Sephadex G-200 column ($0.3 \times 5\text{cm}$) to remove small nucleotides, the fractions constituting the void volume were combined and a portion was overlaid on the sucrose gradient.

Transformation assay was done essentially as described by Lederberg

and Cohen (24).

RESULTS

Entrapment of plasmid DNA in liposomes

Trapping of plasmid pMB9 was assayed radioisotopically and varied from 6–8% of the input DNA, in different experiments (Table I). Entrapment was carried out in two different concentrations of salts, 1x and 1/10x NHTE buffer without any significant alteration in the capture efficiency of the phosphatidylserine liposomes (Table I). This result is an indication that the association of plasmid DNA with the liposomes is not due to unspecific binding because of electrostatic forces but to the sequestration into the internal aqueous phase of liposomes. To demonstrate further that plasmid DNA was in fact entrapped and not adhering to the surface, liposomes containing plasmid DNA were treated with pancreatic deoxyribonuclease and subsequently centrifuged on a linear 5–20% sucrose gradient (see Methods).

Fig 1A shows the centrifugation pattern of liposomes after the entrapment of plasmid DNA and before treatment with deoxyribonuclease. Liposomes containing plasmid DNA remain on the top of the gradient, while the non-entrapped plasmid migrates to below the middle of the gradient. After treat-

TABLE I

Entrapment of DNA in liposomes

Exp	Buffer	Incubated ^{32}P - labelled DNA (cpm)	Entrapped ^{32}P - labelled DNA (cpm)	Entrapment (%)
1	1 x NHTE	601,500	36,730	6.10
2	1 x NHTE	1,370,000	103,560	7.56
3	1 x NHTE	1,890,500	152,340	8.06
4	1/10 x NHTE	620,500	36,920	5.95
5	1/10 x NHTE	1,230,500	97,990	7.96
6	1/10 x NHTE	1,720,000	132,670	7.71

The entrapment of ^{32}P -labelled DNA in liposomes was assayed after treatment of liposomes with deoxyribonuclease, chromatography on Sephadex G-200 and centrifugation on 5–20% sucrose gradient (see Methods). The top of the gradient (liposomes) was collected and the radioactivity of trichloroacetic acid insoluble material was measured. 10 μ moles of phosphatidyl serine were used for each preparation.

Fig 1 5-20% sucrose gradient centrifugation of liposomes containing ^{32}P -labelled DNA. After the entrapment of DNA, liposomes were divided into two equal parts. One portion was applied directly to the gradient (A) and the other was treated with deoxyribonuclease, passed through a Sephadex G-200 column and then applied to the gradient (B). Fractions (0.5ml) were collected and A_{450} (○-○) and radioactivity (●-●) read.

ment of liposomes containing DNA with deoxyribonuclease and chromatography on a Sephadex G-200 column to remove small nucleotides, all the non-entrapped DNA was digested but the entrapped DNA was resistant to deoxyribonuclease and on the sucrose gradient co-migrates again with the liposomes (Fig 1B). To exclude any possibility of non-specific binding of plasmid DNA to liposome surface, preformed liposomes were mixed with radioactive plasmid DNA

only, without using cold plasmid DNA, which would probably saturate non-specific binding sites if such sites do exist. Fig 2A shows that there is no radioactivity in the position of liposomes (Fig 2A) and after treatment of the preformed liposomes and DNA mixture with deoxyribonuclease, the whole DNA was digested (Fig 2B).

Integrity of liposome-entrapped DNA

After entrapment of DNA, liposomes were washed, treated with deoxyribonuclease and run on sucrose gradients. The top of the gradient (liposomes) was collected and the DNA was extracted (see Methods) and electro-

Fig 2 5-20% sucrose gradient centrifugation of preformed liposomes incubated with ³²P-labelled DNA. For details see Fig 1 legend. (A) before, (B) after deoxyribonuclease treatment. A₄₅₀ (○ - ○); radioactivity (● - ●).

phoresed in 1% agarose gels. Figure 3 shows such an electrophoregram. To test the trapping abilities of large unilamellar liposomes we entrapped a crude preparation of plasmid which was highly contaminated with bacterial chromosomal DNA of molecular weight up to $20-30 \times 10^6$ daltons (Fig 3A,B). Figure 3 (slots D,E) also shows that the liposomes sequester all forms of plasmid DNA to the same extent.

Biological activity of entrapped DNA

To examine to what degree the plasmid DNA conserves its biological activity, DNA extracted from liposomes was used for transformation assay. As Table II shows, DNA extracted from liposomes can transform the competent cells with a transformation frequency in the same range as that of the control DNA.

Fig 3 1% agarose gel electrophoresis of DNA extracted from liposomes. (A) after, (B) before deoxyribonuclease treatment of liposomes containing a crude preparation of plasmid pMB9 DNA. (C) EcoRI fragments of phage λ DNA. (D) before, (E) after deoxyribonuclease treatment of liposomes containing purified plasmid pMB9 DNA.

TABLE II

Transformation efficiency of plasmid DNA (pMB9) extracted from liposomes

Exp	Source of DNA	DNA (ng)	Transformants	Transformants/ μ g DNA
1	liposomes	5	252	50,400
2	liposomes	50	2,300	46,000
3	liposomes	100	4,200	42,000
4	control	35	2,050	58,572
5	no DNA	-	-	-

Transformants were selected on agar plates containing tetracycline (15 μ g/ml).

DISCUSSION

The results of the present investigation demonstrate the entrapment of high molecular weight DNA (plasmid pMB9) into large unilamellar liposomes. The entrapment (6-8%) is not due to unspecific binding (via electrostatic forces) of DNA to lipid bilayers but to the capture of DNA by the internal aqueous space of the liposomes. The entrapped DNA is resistant to deoxyribonuclease (Fig 1B) and can be re-isolated intact from liposomes (Fig 3). As transformation assays show the entrapped plasmid DNA conserves its biological activity and can transform the competent cells to the same extent as the control DNA. Hoffman *et al* (17) have also demonstrated entrapment (binding) of high molecular weight DNA into multilamellar liposomes consisting of egg lecithin. But in this case, as these authors found, more than 90% of the DNA was electrostatically bound to the lipids and thus a considerable amount of bound DNA would be wasted if the liposomes were used to microinject DNA into cells. Also using multilamellar liposomes to microinject macromolecules into the cytoplasm has some disadvantages. If we suppose that the multilamellar liposomes fuse with the cells only the outermost compartment, which accounts for approximately 10% of the total trapped volume (25) will merge its contents (DNA) immediately with the cytoplasm while the aqueous compartments remain lipid-bound (6). The intracytoplasmic liposome minus its outer bilayer fuses mainly with lysosomes, the environment of which is not very favourable for maintaining the integrity of the DNA. Moreover, in the particular case of multilamellar liposomes consisting of egg lecithin, the main mechanism of

uptake of liposomes by cells in culture is endocytosis, which means that the whole liposome fuses with the lysosomes (26). The use of large unilamellar phosphatidylserine liposomes is a good alternative to overcome the problems mentioned above, because they carry negative charge, a fact which minimises unspecific binding of nucleic acids, they mainly fuse with the cells (26), introduce their cargo directly to the cytoplasm and finally have a high efficiency of entrapment.

Liposomes containing recombinant DNA molecules can be used to transfer genes from different origins to a foreign cellular environment and to study their expression in the host cells. Such investigations should furnish information about the species-related diversification of molecular processes which contribute to convert the genetic information into the corresponding phenotype (27). Also liposomes containing plasmid DNA or recombinant DNA molecules can be used either to increase the frequency of transformation, as in Bacillus subtilis (28) or for the introduction of phenotypically cryptic plasmids. Of course, this procedure requires removal of the bacterial cell wall before fusion of bacterial protoplasts with liposomes.

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