treatment was employed. Phospholipids were purchased from Avanti Polar Lipids. The vesicles (at a phospholipid concentration of 1 mg/ml in 30 mM-Tris/HC1 buffer, pH 7.0, containing 0.1 M-NaCl) were prepared by sonication for 30 min in a water-bath sonifier at temperatures above the phase transition of the corresponding phospholipid. The concentration of α-sarcin was determined by absorbance measurements and considering its extinction coefficient at 280 nm [4]. α-Sarcin–phospholipid complexes were prepared by adding the protein to freshly prepared vesicles and incubating at 37°C for 60 min. The proteolytic treatment was performed using N-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin at an enzyme/substrate weight ratio of 1:50. Aliquots were taken at different hydrolysis times, reduced with 3% (w/v) 2-mercaptoethanol and analysed by 0.1% (w/v) SDS/polyacrylamide-slab-gel electrophoresis [4% (w/v) and 15% (w/v) acrylamide for the stacking and running gels, respectively]. The absorbance scans at 550 nm (on a Beckman DU-8 spectrophotometer) of the vacuum-dried gels (stained with Coomassie Blue) were used to calculate the remaining intact protein after the trypsin treatment. The results obtained are given in Fig. 1. The protein was completely hydrolysed after 24 h of trypsin treatment at 37°C. However, phospholipid vesicles provided an effective protection for α-sarcin against proteolysis. The percentage of hydrolysis reached a plateau at about 30% protein hydrolysis after about 10 h of trypsin treatment. Thus, about 70% of the α-sarcin molecules in saturated protein–phospholipid complexes were protected against proteolysis. The total number of lysine plus arginine residues in α-sarcin represents about 15% of the total amino acid residues, which enhances the importance of the protection of the whole polypeptide chain against proteolysis caused by the lipid vesicles.

The described interaction between α-sarcin and phospholipid vesicles may be involved directly in the molecular mechanism by which the protein enters tumour cell membranes.


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Trapping drug efficiency in liposomes produced by extrusion of freeze–thaw multilamellar vesicles

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Introduction

Liposomes have applications as models of biological membranes and as drug-delivery vehicles for applications in vitro [1] and in vivo [2]. Since liposomes have been used to entrap a wide range of drugs [3], the higher ratio of encapsulated molecules/lipid is an interesting topic in liposome preparation. Multilamellar vesicles (MLVs), prepared by mechanical dispersion, exclude solutes during their formation [4] resulting in osmotic imbalances between the exterior and interior environments. Equilibrium solute distributions can be achieved by techniques involving organic solvent [4], but this largely limits the procedure. Mayer et al. [5] have shown that repetitive freeze–thaw cycles of MLVs (FT-MLV) can also result in a transmembrane equilibrium of solute concentrations with increased trapping efficiencies. In an attempt to increase the yield of encapsulation in large unilamellar vesicles (LUV), we have compared the encapsulation efficiency of several molecules with different molecular mass and structure in liposomes obtained by extrusion through polycarbonate filters of preformed MLVs (LUV) and FT-MLVs (FT-LUV).

Materials and methods

The encapsulated material was (molecular mass): carboxyfluorescein (CF; 376.3 Da); protein A (40 kDa) and pN2-α-1 AT plasmid (9.3 kbp; a gift from Dr Y. Yamada, Bethesda, U.S.A.). The phospholipid concentration was determined to evaluate the encapsulation efficiency [6]. The entrapped CF was evaluated by fluorimetry. The amount of protein A encapsulated was determined by the inclusion of 125I-labelled protein A [7] as tracer. Liposomes containing the pN2-α-1 AT plasmid were disrupted with chloroform and the recovered DNA was evaluated by fluorimetry, using the DNA-binding dye Hoechst 33258 [8].

Liposomes were prepared by mechanical reuspension of a previously freeze-dried lipid mixture. This included egg phosphatidylcholine (PC; 30 μmol) and cholesterol (30 μmol) in 2 ml of aqueous phase containing CF (20 μmol) or protein A (400 μg) or plasmid (50 μg). Four fractions of 0.5 ml were established. In the first one (MLV), the non-entrapped material was removed by several centrifugations. The second fraction was extruded through polycarbonate filters of 0.2 μm pore size to obtain LUV, under N2 pressure, and the liposomes were subsequently purified in a Sepharose 4B column. The rest (1 ml) was subjected to five freeze–thaw cycles, utilizing liquid N2 and a 40°C water bath. The third and fourth fractions were then processed as above, to obtain purified FT-MLV and FT-LUV, respectively. In all cases, liposomes containing protein A or plasmid were respectively incubated (1 h at 37°C) with pronase (1 mg/ml) or DNAase I (50 μg/ml in 5 mM-MgCl2) before purification, to remove the material associated with the liposome surface.

Results and discussion

FT-MLV were more efficient (2-fold) than MLV in encapsulating CF (Fig. 1a). In addition, we have observed that the yield of CF entrapped in FT-LUV was significantly increased (3-fold) compared with the LUV. These results are in accordance with previous reports in which an increase in the trapped volume was also observed in both FT-MLV [5] and FT-LUV [9]. This suggests that the transmembrane equilibrium of solutes, mediated by the freeze–thaw procedure, could produce an appreciable increase in the aqueous trapped volume as well as in the CF-trapping efficiencies.
That increased encapsulation of CF is related to particular properties of CF is supported by two additional series of experiments using protein A and the pN2-α-1 AT plasmid (Fig. 1b). Protein A was also more efficiently encapsulated (2-fold) in FT-MLV than in MLV, but no significant differences were observed between LUV and FT-LUV. In contrast, the freeze-thaw procedure failed to increase the yield of DNA encapsulation in both FT-MLV and FT-LUV liposomes.

In summary, our results suggest that the freeze-thaw procedure increases the encapsulation efficiencies of small molecules such as CF in multi- and uni-lamellar liposomes, but the yield of drug encapsulation may be principally limited by the size and/or structure of the molecules.

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Influence of lipid characteristics on the encapsulation efficiency and stability of liposomes

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Entrapment efficiency and stability are two main points to be considered as far as the practical uses of loaded liposomes are concerned. Carboxyfluorescein has been extensively used as a reference molecule to check these two parameters [1]. Nevertheless, the evidence accumulated over the past few years shows that it is difficult to generalize and results can vary from one type of liposome to the other, and are highly dependent on the physicochemical characteristics of the lipids and of the encapsulated drug. In a previous paper [2], we found that opioid molecules gave important differences in encapsulation efficiencies despite their structural similarities.

This led us to undertake a systematic and comparative study of the encapsulation efficiency and stability in dehydration–rehydration vesicles (DRV), prepared with saturated and unsaturated phospholipids (PL) containing two different drugs: gentamicin and morphine. This investigation was carried out for two different molecules: morphine hydrochloride and gentamicin sulphate.

Egg phosphatidylcholine was purchased from Merck (Frankfurtur Strasse 250, D-6100 Darmstadt 1, R.F.A.) and purified by column chromatography. Hydrogenated egg phosphatides were from Asah! (Tokyo 100, Japan). Cholesterol was from Aldrich (D-7924 Steinheim, F.R.G.). Phosphate salts employed for the preparation of phosphate-buffered saline (PBS) solutions were quality pro analysis (Merck). The pH of the PBS solutions was always 7.4. Gentamicin sulphate was kindly supplied by Infavet (Macia Vila I, 43201 Reus, Spain). Morphine hydrochloride was kindly supplied by Federacion Farmacutica (Barcelona) (Aussia Mare 101, Barcelona, Spain).

DRV were prepared according the description given in [3]. The ratio of PL to drug was 12.5:1 (w/w).

The entrapment and encapsulation efficiencies, given as mg of drug:mmol of PL and percentage of the initial amount of drug incorporated into the liposomes, as well as the encapsulation capacity (volume of entrapped aqueous phase/mg of PL), are given in Table 1.

It is clear that under the same conditions of initial concentration (w/w) and PL/drug ratio (w/w), the main para-

![Graph](image-url)