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Sterile filtration of liposomes: retention of encapsulated carboxyfluorescein

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Abstract

Multilamellar vesicles incorporating carboxyfluorescein (CF) were calibrated through polycarbonate membranes of 0.1, 0.2 or 0.4 μm pore size. After removal of unencapsulated material, the vesicles were passed through 0.2 μm sterilizing filters constituted of polycarbonate, cellulose acetate or polyvinylidene fluorure (PVDF). The liposomes were readily filtered without significant change in the vesicle size and loss of phospholipids. The leakage of encapsulated material was only slight, lower than 5% in all cases, except for vesicles of 300 nm sterilized through polycarbonate membranes of 0.2 μm pore size. However, the same preparation was easily passed through 0.2 μm acetate cellulose membranes without significant leakage of the incorporated dye. In spite of the small size of the liposomes, trapping efficiencies up to 33% were achieved, depending on the vesicle mean diameter, lipid concentration, bilayer composition and inclusion of freeze-thawing cycles.

Keywords: Liposome; Carboxyfluorescein; Sterilization; Filtration; Drug retention

1. Introduction

Pharmaceutical liposomal formulations for parenteral use should be produced in a manner that ensures their sterility. Since degradation products can be formed during heat (Kikuchi et al., 1991) or ionizing irradiation (Konings, 1984) treatments, these methods are not easily applicable for the sterilization of liposomes. Thus, sterile filtration appears to be the method of choice for liposomes smaller than 200 nm (Freise, 1984). The aim of this study was to investigate in more detail the sterilization of liposomes by a filtration procedure, especially when hydrophilic drugs are incorporated in the vesicles. Carboxyfluorescein was used as a solute marker to monitor the leakage from the liposomes during passage through sterilizing-grade membranes.

2. Materials and methods

Lipids used were soybean phosphatidylcholine (SPC) (Epikuron 200 S, Lukas Meyer Inc., Hamburg, Germany), dimyristoylphosphatidylglycerol (DMPG) (Phospholipon MG-Na, Nattermann Phospholipid GmbH, Köln, Germany), and

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cholesterol (CH) (99% GC grade, Sigma Co., St Louis, MO). Triton X-100 was obtained from Sigma and 4(5)-carboxyfluorescein (CF) from Fluka (Buch, Switzerland). All other chemicals were of analytical grade.

2.1. Preparation of liposomes

Liposomes constituted of SPC were prepared at various lipid concentrations (10, 100 and 200 μmol/ml) using an extrusion technique similar to that described by Olson et al. (1979). Other lipid formulations studied were SPC/CH 7:3 (molar ratio), SPC/DMPG 9:1 (molar ratio) and SPC/DMPG/CH 6:1:3 (molar ratio) at 200 μmol/ml of total lipids. Briefly, the lipids were dissolved in methanol, chloroform or a chloroform/methanol mixture (2:1). The organic solvent was slowly removed under reduced pressure on a rotary evaporator at 30°C. The thin film of dried lipids was hydrated at room temperature with a buffer solution (pH 7.4) containing 15 mg/ml of CF (300 mosmol/kg). The resulting liposome dispersion was vortexed for 5 min and a part of the preparation was subjected to five consecutive freeze-thawing cycles (F-T) at $-196/+60$°C. The vesicles were calibrated by extrusion through polycarbonate membrane filters of 0.1, 0.2 or 0.4 μm pore size (Nuclepore Corp., Pleasanton, CA) using the Extruder (Lipex Biomembranes Inc., Vancouver, Canada). The vesicles were extruded as follows: three times through 0.2 μm followed by five times through 0.1 μm; or 10 times through 0.2 μm; or 10 times through 0.4 μm. Free CF was removed by applying 1 ml of liposome dispersions on a fine Sephadex G-50 column (Pharmacia, Uppsala, Sweden) equilibrated with phosphate-buffered saline (PBS pH 7.4). Volumes of 2, 3 and 5 ml of purified vesicles were recovered when the initial dispersions contained 10, 100 and 200 μmol/ml of lipids, respectively.

2.2. Sterile filtration

After removal of unencapsulated material, the liposomes were passed through sterilizing-grade membranes (0.2 μm pore size) constituted of polycarbonate (Nuclepore), cellulose acetate (Minisart® 16 535 K, Sartorius GmbH, Goettingen, Germany), or polyvinylidene fluorure (PVDF) (Millex®-GV, Millipore Corp., Bedford, MA) using a plastic syringe. Whereas Millex® and Minisart® systems are sterile disposable filtration units, polycarbonate membranes were placed in Millipore filter holders of 25 mm diameter and autoclaved.

2.3. Vesicle size determination

The vesicle size distribution was measured by quasi-elastic light scattering using a laser particle sizer (Model N4 SD, Coulter Electronics Ltd, Luton, UK). This apparatus provides a mean diameter (MD) associated with a standard deviation ($\pm$SD).

2.4. Phospholipid content

Phospholipid concentrations were determined according to the procedure of Stewart (1980) before and after sterilizing filtration. When DMPG was included in the formulations, the lipid phosphorus was quantified by first extracting the lipids according to the method of Bligh and Dyer (1959) and then performing the assay described by Rouser et al. (1970).

2.5. Leakage of incorporated CF

The percentage of CF released from the vesicles upon sterilizing filtration was monitored using an SLM 800 spectrofluorimeter (SLM Instruments Inc., Urbana, IL) at excitation and emission wavelengths of 490 and 515 nm, respectively. The fluorescence of the liposome dispersions diluted in PBS was measured before (F_i) and after (F_t) addition of Triton X-100 (0.1% final concentration). Leakage of the incorporated dye was calculated from the equation:

$$\text{Leakage}(\%) = \frac{(F_i/F_t) - (F_{i,0}/F_{t,0})}{1 - (F_{i,0}/F_{t,0})} \times 100$$

where $F_{i,0}$ and F_i are the initial fluorescence intensities before and after sterilizing filtration,

respectively, and $F_{t,0}$ and F_t denote the total fluorescence intensities following detergent lysis with Triton X-100.

2.6. Control of sterility

Blank liposomes constituted of SPC (10 μmol/ml) were prepared in PBS alone and extruded through polycarbonate membranes of 0.2 μm pore size. The preparation was filtered under aseptic conditions in a laminar flow hood through polycarbonate, cellulose acetate or PVDF sterilizing-grade membranes. Aliquots were then filtered through 0.2 μm polycarbonate membranes and sterility was assessed by incubation of the filter discs in tryptocasein soya agar or thioglycollate resazurin broth USP (Diagnostic Pasteur, Marnes la Coquette, France) for 7 days at 20 and 37°C, respectively.

3. Results and discussion

3.1. Percentage of trapping efficiency

Multilamellar vesicles made of increasing amounts of SPC (10, 100 and 200 μmol/ml) were down-sized by extrusion through polycarbonate membranes of 0.1 or 0.2 μm pore size, producing vesicles of approx. 130 (±35) and 180 (±40) nm mean diameter, respectively. As shown in Fig. 1, trapping efficiencies were markedly improved by increasing the lipid concentration and subjecting the liposomes to five consecutive freeze-thawing cycles (F-T). Furthermore, entrapped volumes were greater for liposomes extruded through 0.2 μm membranes than smaller vesicles calibrated through 0.1 μm membranes. These results are in accordance with those reported previously by others (Hope et al., 1985, Mayer et al., 1985, 1986).

3.2. Leakage of incorporated CF

The liposome dispersions were readily filtered through polycarbonate sterilizing filters (Nuclepore 0.2 μm) without significant change in the vesicle size or loss of phospholipids. The leakage of incorporated material was only slight (Fig. 2), generally lower than 2% except for vesicles calibrated through 0.2 μm membranes which were not subjected to F-T cycles; CF release was about 4% in that case. It may be noted that the leakage was not dependent on the lipid concentration at which the liposomes were prepared. Moreover, the retention was improved by using cellulose acetate (Sartorius) or PVDF (Millipore) membranes (Fig. 3).

3.3. Vesicles extruded through 0.4 μm membranes

Liposomes calibrated through 0.4 μm membranes produced vesicles of about 300 (±40) nm

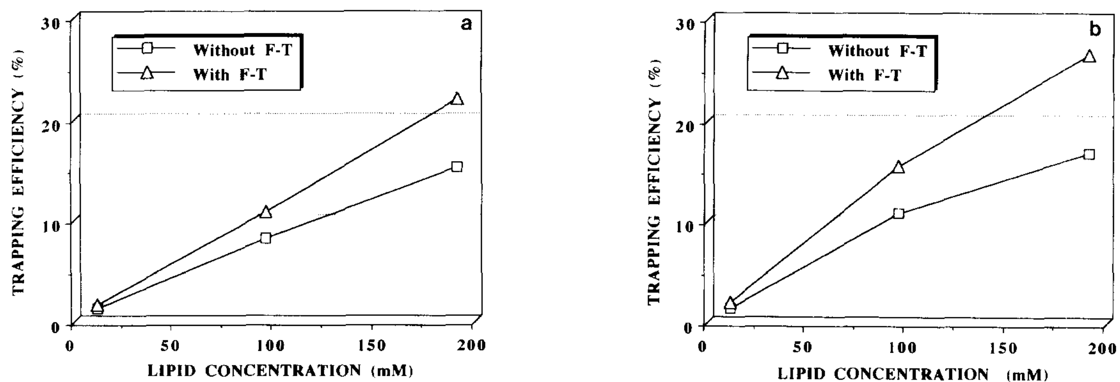

Fig. 1. Percentage of trapping efficiency of SPC vesicles extruded through 0.1 μm (a) or 0.2 μm (b) polycarbonate membranes. Liposomes incorporating CF were prepared with and without freeze-thawing (F-T) cycles at various lipid concentrations. Results are the mean of three measurements (±SD).

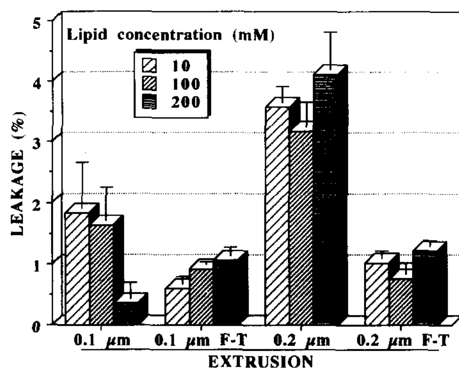

Fig. 2. CF leakage from SPC liposomes during sterilizing filtration through polycarbonate membranes of 0.2 μm pore size. Vesicles were prepared with and without freeze-thawing (F-T) cycles at various lipid concentrations and calibrated through membranes of 0.1 and 0.2 μm pore size. Results are the mean of three measurements (±SD).

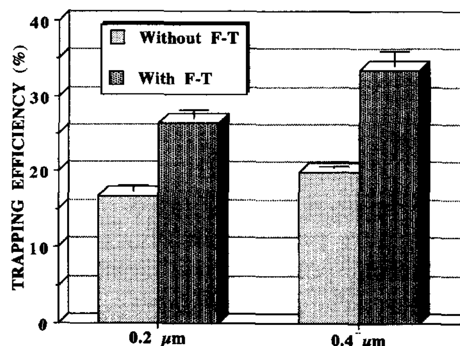

Fig. 4. Percentage of trapping efficiency of SPC vesicles prepared at 200 μmol/ml of lipids with and without freeze-thawing (F-T) cycles and extruded through 0.2 or 0.4 μm polycarbonate membranes. Results are the mean of three measurements (±SD).

mean diameter. The amount of encapsulated material was markedly improved in comparison with vesicles extruded through 0.2 μm membranes and trapping efficiencies up to 33% were achieved when freeze-thawing cycles were performed (Fig. 4). As shown in Fig. 5, substantial leakage ranging from 20 to 25% of the incorporated dye occurred when 300 nm vesicles were sterilized through 0.2 μm polycarbonate filters (Nuclepore); in addition, CF release was accompanied by a significant reduction of the vesicle mean diameter to 240 (±35) nm. Thus, a single passage through the sterilizing filter produced breakage of the integrity of the vesicle membrane and consequently leakage of entrapped solute. On the other hand, 300 nm liposomes were filtered through 0.2 μm cellulose acetate membranes (Sartorius) without significant change in vesicle size and no leakage was observed in this case (Fig. 5). These differences may be explained by different structures of polycarbonate membranes (with

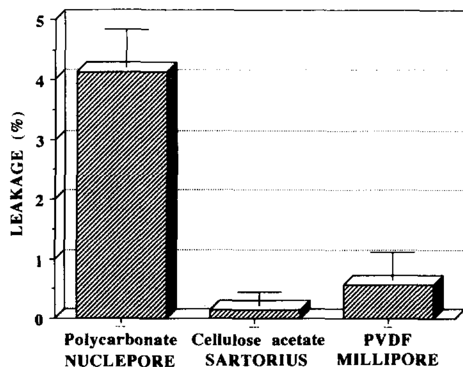

Fig. 3. CF leakage from SPC liposomes during sterilizing filtration through membranes of 0.2 μm pore size constituted of polycarbonate, cellulose acetate or polyvinylidene fluorure (PVDF). Liposomes were prepared at 200 μmol/ml of lipids without F-T cycles and calibrated through 0.2 μm polycarbonate membranes. Results are the mean of three measurements (±SD).

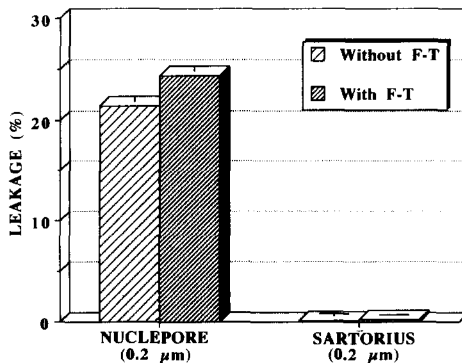

Fig. 5. Leakage of incorporated CF from SPC vesicles during sterilizing filtration through membranes of 0.2 μm pore size constituted of polycarbonate (Nuclepore) or cellulose acetate (Sartorius). Liposomes were prepared at 200 μmol/ml of lipids with or without F-T cycles and calibrated through 0.4 μm polycarbonate membranes. Results are the mean of three measurements (±SD).

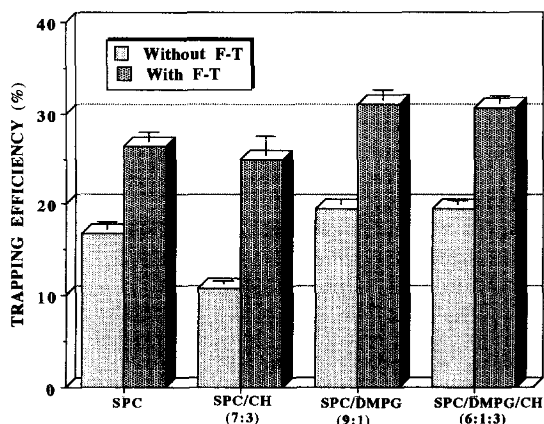

Fig. 6. Percentage of trapping efficiency of different liposome formulations prepared at 200 μmol/ml of lipids with and without freeze-thawing (F-T) cycles and extruded through 0.2 μm polycarbonate membranes. Results are the mean of three measurements (±SD).

straight-through pores) and cellulose acetate filters (with tortuous pores).

3.4. Influence of lipid composition

Incorporation of negatively charged lipids (DMPG) increased the trapping efficiencies up to 30% for vesicles calibrated through 0.2 μm membranes and subjected to F-T cycles (Fig. 6). On the other hand, cholesterol decreased slightly the amount of encapsulated material. The percentage of CF released upon sterilizing filtration was not influenced by the lipid composition: it was lower than 1.5% in all cases, except for SPC vesicles which were not subjected to F-T cycles, as shown previously (Fig. 7). Thus, liposomes of various lipid composition can readily be sterilized by filtration through sterilizing grade membranes without significant leakage of entrapped CF. However, problems of clogging would probably appear for vesicles constituted of phospholipids which are at the gel state at room temperature (Nayar et al., 1989). These formulations should probably be heated above the phase transition temperature to allow their passage through the pores of sterilizing membranes.

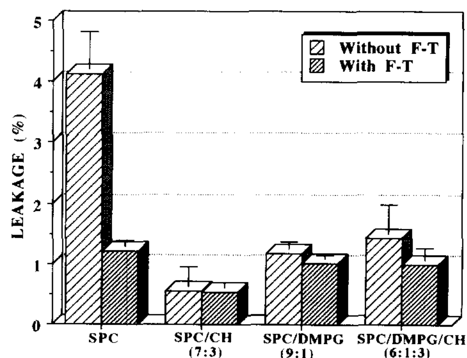

Fig. 7. Leakage of incorporated CF from different liposome formulations during sterilizing filtration through 0.2 μm polycarbonate membranes. Vesicles were prepared at 200 μmol/ml of lipids with or without F-T cycles and calibrated through 0.2 μm polycarbonate membranes. Results are the mean of three measurements (±SD).

3.5. Sterility

Liposome preparations filtered through Millex® and Minisart® filtration units were always sterile when the procedure was carried out under aseptic conditions. However, when polycarbonate membranes were used in standard filter holders, the liposome dispersions were not sterile in every case. This seems to be due to problems of tightness of the filter holders, especially when a substantial pressure was required to pass the vesicles through the pores of the membrane. Thus, the use of filtration units or tight filter holders are required for reliable sterilization of liposome preparations.

4. Conclusion

This study has shown that liposomes smaller than 300 nm mean diameter can readily be sterilized by simple filtration through 0.2 μm bacterial filters. When carried out under aseptic conditions and using reliable filtration units, this operation produced sterile liposome dispersions. No significant leakage of the entrapped solute was detected for all the preparations tested, except for vesicles of 300 nm mean diameter filtered through polycarbonate membranes of 0.2 μm pore size. However, filters constituted of cellulose acetate were more appropriate for this procedure, since

liposomes larger than the pore size of the membrane were filtered without leakage. Trapping efficiencies up to 33% were achieved for the formulations studied, depending on vesicle size, bilayer composition, lipid concentration and performance of freeze-thawing cycles.

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