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The cryopreservation of liposomes: 3. Almost complete retention of a water-soluble marker in small liposomes in a cryoprotectant containing dispersion after a freezing/thawing cycle

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Summary

Small liposomes (mean particle size, 0.14 μm; bilayer composition, PL100H/dicetyl phosphate 10:1) showed almost complete carboxyfluorescein (CF) retention after a freezing/thawing (F/T) cycle in the presence of cryoprotectants. Retention of CF exceeded 95% for liposomes in dispersions containing trehalose or saccharose after storage for 45 min at -50 or $-75\,^{\circ}$C. At a storage temperature of $-25\,^{\circ}$C, only 50% CF retention was found. Dispersions with larger vesicles showed less marker retention after an F/T cycle. Independent of size and storage temperature mean liposome sizes measured by dynamic light scattering showed only slight, if any, alterations after an F/T cycle when the liposomes were dispersed in cryoprotectant-containing dispersions. Aliquots of 0.25 ml liposomes (mean size 0.13 μm) in 10% saccharose, frozen and stored in boiling liquid nitrogen for 7 days, still contained 100% CF encapsulated in liposomes after thawing. Marker retention after freezing in liquid nitrogen for 15 min and storage afterwards at $-25\,^{\circ}$C depended on storage time. During the first 4 days, a gradual release of CF was found until a marker retention of 40–45% was reached that remained constant upon prolonged storage. In addition to the presence of a cryoprotectant and the choice of optimal process parameters, liposome size and storage temperature need particular attention, as they proved to be important parameters for the successful cryopreservation of liposomes.

Introduction

Liposomes are currently under investigation for their possible use as delivery systems for drugs (Juliano and Layton, 1980; Gregoriadis, 1984; Ostro, 1987; Roerdink et al., 1987). Stability problems, as demonstrated by particle size alterations and/or leakage of incorporated drugs, might hamper or delay introduction of liposomal dispersions as drug delivery systems in therapy (Crommelin and Storm, 1987). In particular, liposomal dispersions containing water-soluble, non-bilayer interacting, drugs pose shelf-life problems (Özer et al., 1988).

In earlier studies, the freezing/thawing (F/T) behaviour of liposomal dispersions (bilayer composition: PL100H/dicetyl phosphate) was investigated with differential scanning calorimetry (DSC) (Talsma et al., 1991a,b). The liposomal dispersions showed partly crystallization below

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$-40\,^{\circ}C$, as demonstrated by an exothermal heat flow. Small liposomes ($< 0.2\ \mu m$) showed a relative large crystallization enthalpy at temperatures below $-40\,^{\circ}C$ and up to 50% carboxyfluorescein (CF) retention after an F/T cycle in the DSC. Nucleation at the lowest possible supercooling temperature (homogeneous nucleation temperature; Mackenzie, 1977) was correlated with a relatively high retention of 50% CF for the small liposomes. Dispersions containing liposomes with an average diameter above $0.6\ \mu m$ showed only a relatively low crystallization enthalpy below $-40\,^{\circ}C$ and low retention (about 10% CF) upon an F/T cycle. For these dispersions relatively large liposome size alterations were found after an F/T cycle.

In the literature, successful prevention of aggregation of liposomes after an F/T cycle was reported when cryoprotectants were used (e.g. Crowe et al., 1986; Fransen et al., 1986). The objective of the present study was to improve the stability at low temperature of small liposomes even further by using the appropriate cryoprotectants and to obtain data concerning the long-term stability of these liposomes in frozen condition. Trehalose has been described as an effective cryoprotectant for the stabilization of organisms and liposomes (e.g. Crowe et al., 1984, 1985). Therefore, trehalose was chosen as cryoprotectant. Because different trehalose concentrations were used in stabilization studies and a 5% (w/w) solution would produce an acceptable 'cake' upon freeze-drying, a concentration of 5% was chosen rather arbitrarily. Trehalose is expensive and it is questionable whether it will be allowed as an additive in parenteral pharmaceutical preparations by regulatory authorities. Therefore, the cryoprotective potential of trehalose was compared with that of 10% (isotonic) saccharose solution. In the well-controlled DSC environment, small samples of $25\ \mu l$ were used (Talsma et al., 1991a). The question of whether larger samples would show a different behaviour was also addressed. To this end, 0.25 ml samples in glass vials were frozen in a mixture of acetone/dry ice and in boiling liquid nitrogen in order to determine whether, under these conditions, the same CF retention could be achieved. Finally, the stability on storage of dispersions frozen in boiling liquid nitrogen and stored at $-25\,^{\circ}C$ in a freezer was studied.

Materials and Methods

Phospholipon 100H (hydrogenated soybean phosphatidylcholine; PL100H) was a gift from Nattermann (Köln, Germany). Dicetyl phosphate (DCP) was supplied by Sigma Chemicals (St. Louis, MO, U.S.A.). Trehalose was obtained from Janssen Chimica (Beerse, Belgium). Carboxyfluorescein (CF) was obtained from Eastman Kodak (Rochester, NY, U.S.A.) and purified according to the procedure described by Ralston et al. (1981). All other chemicals were of analytical grade. Negatively charged liposomes were prepared by the classical 'film method' as described by Fransen et al. (1986), except that 5 mM instead of 50 mM CF was incorporated. All vesicle preparations had the same bilayer composition (PL100H/DCP in a molar ratio of 10:1) and were prepared in 10 mM Tris buffer pH 7.4. Cryoprotectant concentrations are expressed as % (w/w) of the total dispersion mass; they were present both inside and outside the vesicles. Lipid phosphorus was determined according to the procedure of Fiske and Subbarow (1925). Narrow vesicle size distributions were obtained by extrusion of the vesicles repeatedly through polycarbonate filters with a defined pore size (Uni-pore, Bio-Rad, Richmond, CA). A Netzsch low-temperature DSC (system 200) was used (Netzsch-Gerätebau, Selb, Germany). Temperature scale and heat flux were calibrated with gallium and mercury.

Liposome size was determined by dynamic light scattering with a Malvern 4700 system, using the automeasure vsn 3.2 soft-ware (Malvern Ltd, Malvern, U.K.). In the calculation of the particle size from the light scattering data the viscosity and refractive index of pure water were used for the dispersions without cryoprotectant. A viscosity of 1.16 mPa s for 5% trehalose and 1.26 mPa s for 10% saccharose and a refractive index of 1.334 for 5% trehalose and 1.348 for 10% saccharose were used. After liposome preparation, vis-

cosity of the dispersions was determined by light scattering according to the method described by De Smidt (1990).

Non-encapsulated CF after liposome preparation was removed by gel-permeation chromatography over a Sephadex G-50 column. Retention of CF after an F/T cycle was measured fluorimetrically as described earlier (Talsma et al., 1991b).

Performance of F / T cycle in the DSC (25 μl)

Aliquots of 0.25 μl of the dispersions (duplicate measurements) were cooled, stored and heated in small aluminium pans placed in a DSC. Cooling rate down to the storage temperature was 10 °C/min. The dispersions were stored for 45 min. After storage the dispersions were immediately heated to 20 °C (approx. 8–20 s), at the highest possible heating rate of the DSC equipment.

Performance of the F / T cycle of 0.25 ml samples

Aliquots of 0.25 ml liposome dispersion, containing 10% saccharose as cryoprotectant, were put into glass vials. The dispersion in the vials was frozen by immersion of the total vial in a mixture of acetone and dry ice (temperature approx. −70 °C). It was stored for 15 min in this mixture and immediately thereafter thawed by placing the vials in a water bath at 20 °C for at least 5 min.

Results and Discussion

Retention of CF in small liposomes embedded in cryoprotectant containing solution after an F / T cycle

The retention of carboxyfluorescein as a function of particle size, presence of cryoprotectant and storage temperature of the dispersions is shown in Fig. 1. When the liposome dispersions were stored at −25 °C only a very slight influence, if any, of liposome size on CF retention was observed. The dispersion in 5% trehalose or 10% saccharose stored at −25 °C showed improved CF retention; from approx. 10% (no cryoprotectant) up to 50–60% retention was reached after a freezing/thawing cycle.

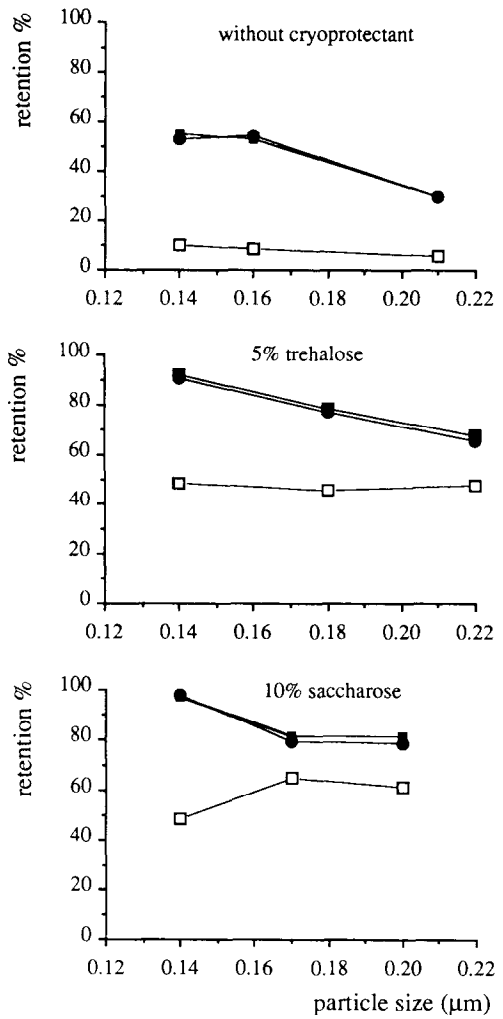

Fig. 1. CF retention vs liposome size for different freezing temperatures and different cryoprotectants. Cooling rate, 10 °C/min; storage time at −25, −50 or −75 °C: 45 min. After storage, the dispersions were immediately heated to 20 °C ($n = 2$). (□——□) −25 °C, (●——●) −50 °C, (■——■) −75 °C.

If the dispersions were stored at −50 or −75 °C an effect due to liposome size was shown. In general, the smaller the liposomes, the higher was the CF retention after a freezing/thawing cycle. When trehalose or saccharose was present in the dispersion, no difference in CF retention was evident after storage at −50 or −75 °C for the investigated liposome sizes.

The presence of both 5% trehalose and 10% saccharose improved carboxyfluorescein retention after a freezing/thawing cycle in comparison with the dispersions without cryoprotectant. For the smallest liposomes (0.14 μm) in the 10% saccharose containing dispersion, 99% retention of marker was determined for dispersions stored at -50 or $-75\,^{\circ}$C. For the 0.14 μm liposomes in 5% trehalose dispersion, retention above 90% was found under comparable conditions. For liposomes stored at temperatures of -50 and $-75\,^{\circ}$C, the addition of trehalose/saccharose clearly induced a dramatic improvement in CF retention compared to the dispersions without cryoprotectant.

Alterations of liposome size after storage at different temperatures

Mean particle sizes were determined after an F/T cycle and storage for 45 min at -25, -50 and $-75\,^{\circ}$C. The dispersions without cryoprotectant showed a size-dependent aggregation/fusion behaviour, especially at $-25\,^{\circ}$C. For the dispersions containing 5% trehalose or 10% saccharose the same particle size was found before and after an F/T cycle (results not shown).

Retention of carboxyfluorescein after a freezing/thawing cycle of 0.25 ml samples

The possibility of F/T of larger quantities (0.25 ml instead of 25 μl) of dispersion with subsequently almost complete carboxyfluorescein retention was investigated. Only one cooling temperature (approx. $-70\,^{\circ}$C) was used. Table 1 lists the CF retention data for different liposome sizes.

TABLE 1

Carboxyfluorescein retention in 0.25 ml samples after a freezing/thawing cycle

Liposome size (μm)	CF retention (%)
0.20	51
0.18	84
0.13	96
0.12	98

Composition: PL100H/DCP 10:1 in 10 mM Tris buffer containing 10% (w/w) saccharose. Sample volume, 0.25 ml; lipid concentration, 30 μmol/ml ($n = 2$); F/T conditions, see text.

A clear influence of liposome size was observed. For the smaller particle sizes almost complete marker retention after a freezing/thawing cycle was obtained as had earlier been found with 10-fold smaller sample volumes in the DSC.

Influence of freezing protocol

A liposome dispersion with a mean particle size of 0.12 μm in 10% saccharose was frozen in two different ways: by immersion in boiling liquid nitrogen, or in an acetone/dry ice mixture. The vials were stored in these media for 15 min and immediately afterwards thawed in a water bath of $20\,^{\circ}$C for 5 min. A retention of 97% in liquid nitrogen and 96% in acetone/dry ice after the F/T cycle was found.

Influence of storage temperature on marker retention after – initially – complete crystallization of the water in the dispersion

Complete crystallization of water in the cryoprotectant-containing liposome dispersions might render stable dispersions independently of storage conditions afterwards. Dispersions (0.25 ml) with different particle sizes were frozen in acetone/dry ice and thereafter stored at two different temperatures: in a freezer at $-25\,^{\circ}$C and in boiling liquid nitrogen. After 65 h of storage the samples were quickly thawed in a water bath at $20\,^{\circ}$C and kept there for 5 min. The results are shown in Table 2.

Comparison of Tables 1 and 2 shows that no significant difference in retention was found be-

TABLE 2

Carboxyfluorescein retention after 65 h of storage under different conditions

Liposome size (μm)	Retention of CF (%)	
	Freezer ($-25\,^{\circ}$C)	Liquid nitrogen
0.20	22	47
0.18	41	84
0.13	51	94
0.12	55	97

Composition: PL100H/DCP 10:1 in 10 mM Tris buffer containing 10% (w/w) saccharose. Lipid concentration, 30 μmol/ml ($n = 2$); F/T conditions, see text.

tween storage in acetone/dry ice for 15 min and an additional storage time of 65 h in boiling liquid nitrogen. As observed before, marker retention was liposome size dependent. Storage at −25 °C clearly showed significant marker loss in comparison with storage in boiling liquid nitrogen.

Influence of freezing protocol on long-term storage stability at moderately low temperatures

The influence of direct nucleation of the internal volume at the homogeneous nucleation temperature vs 'slow' nucleation at higher temperatures was investigated for dispersions containing 10% saccharose. Two different freezing protocols were used: one, in which the dispersion was frozen in boiling liquid nitrogen for 15 min and afterwards stored at −25 °C, and another, in which the dispersion was frozen by placing it directly into a freezer at −25 °C. Before analysis the dispersions were thawed quickly by transfer to a water bath at 20 °C and left there for 5 min. CF retention and particle size alterations are shown in Fig. 2A and B.

The data for retention at time 0 were obtained after 15 min freezing in boiling liquid nitrogen or at −25 °C. It was observed that the dispersions frozen in boiling liquid nitrogen were not stable during storage at −25 °C, but slowly released part of their contents until the same parent retention had been reached as for the dispersion frozen at −25 °C. After reaching 45% retention both dispersions remained stable for the rest of the storage time. Only small particle size alterations were evident during storage. Reference dispersions that were stored for 7 days in a refrigerator at 4 °C or in boiling liquid nitrogen showed both 100% marker retention and no particle size alterations at all after thawing.

Influence of particle size and addition of cryoprotectant on marker retention

Marker retention was particle size dependent. Small liposomes in cryoprotectant-containing dispersions showed almost complete marker retention. Crowe et al. (1985) and Madden et al. (1985) reported that F/T and freeze-drying of liposomes with almost complete marker retention of water-soluble, non-bilayer interacting markers was possible in trehalose and other carbohydrate-containing dispersions. In their studies, unilamellar liposomes with small mean particle size were used (< 0.1 μm). Other authors (Özer et al., 1988) reported incomplete recovery of the encapsulated water-soluble, non-bilayer interacting drugs in the presence of cryoprotectants after an F/T cycle or freeze-drying of liposomes. The size of liposomes used in the literature was not recorded in every case reported. However, the available information suggests that in the unsuccessful studies, vesicle sizes larger than 0.1 μm and sometimes multilamellar vesicles were used. The importance of particle size for the successful cryopreservation of liposomes is usually not rec-

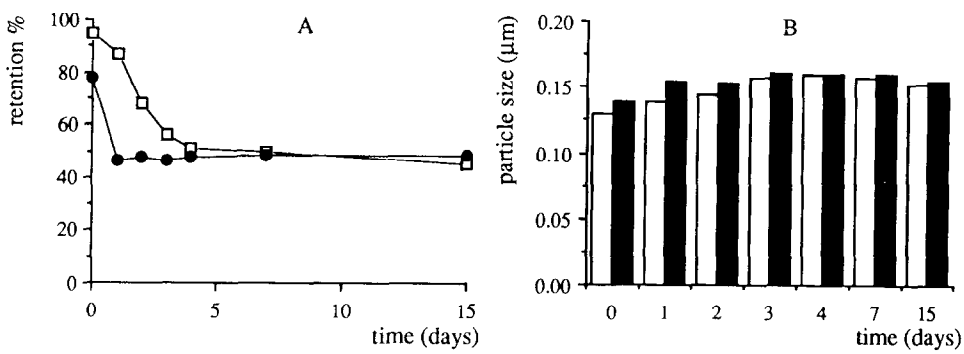

Fig. 2. CF retention (A) and particles size (B) of liposome dispersions in 10% saccharose vs storage time after different freezing protocols and storage at −25 °C ($n = 2$). Time 0: the percent retention after freezing for 15 min and immediate heating to 20 °C. (A) (□ ——— □) Dispersion frozen in liquid nitrogen, (● ——— ●) dispersion frozen at −25 °C; (B) (□) dispersion frozen in liquid nitrogen, (■) dispersion frozen at −25 °C.

ognized in the literature; this might explain part of the discrepancy in the reported data mentioned above.

The question as to whether the choice of the cryoprotectant itself is critical cannot be answered as yet. Both 5% trehalose and 10% saccharose showed high marker retention for small particles. For 5% trehalose and 10% saccharose stable mean particle sizes were found. These stabilizing effects on the size of the vesicles are in agreement with the results of Womersley et al. (1986). They reported inhibition of dehydration-induced fusion of liposomal membranes by carbohydrates and stable mean particle sizes of liposomes after dehydration of liposomal dispersions by means of vacuum drying or after an F/T cycle.

When the dispersions are cooled to -50 or $-75\,^{\circ}C$, a heat flow below $-40\,^{\circ}C$ is recorded by DSC (Kristiansen and Hvidt, 1990; Talsma et al., 1991a). It was suggested that this heat flow is associated with the internal nucleation of ice inside the liposomes. Particle size and cooling rate both influence the time point of nucleation of ice inside the liposomes. At cooling rates of $10\,^{\circ}C$/min the internal volume of dispersions with particle sizes below approx. 0.15 μm almost completely nucleated at the homogeneous nucleation temperature. For liposome dispersions in 5% trehalose and 10% saccharose, a crystallization heat flow between -40 and $-45\,^{\circ}C$ was determined (results not shown). Apparently, under these conditions, crystallization of the internal volume of liposomes at the homogeneous nucleation temperature does not damage the bilayer structure considerably, since over 90% recovery of encapsulated CF was achieved.

Storage at $-25\,^{\circ}C$ showed marker leakage even when cryoprotectants were present. At least two possible mechanisms for such marker leakage can be given: osmotic forces and ice crystal growth. In the first instance, incomplete crystallization of water in the dispersion occurs. During cooling, ice nucleates outside the vesicles, yielding concentrated cryoprotectant/liposome/additive(s) dispersions in channels inside the ice matrix. If carbohydrates are used as cryoprotectants, this channel structure appears as a 'cake' after freeze-drying under appropriate conditions. Because internal nucleation is delayed, an osmotic force exists over the liposome bilayer until the homogeneous nucleation temperature is reached. To compensate for this osmotic force water will be transported from the internal volume of the liposome towards the outside medium. Rapid rewarming of the liposome dispersion might introduce a fast decrease in the osmotic force of the external medium by redistribution of the cryoprotectant in the medium. This might introduce (instantaneous) hypertonicity in the internal volume of the liposomes and could induce marker leakage by rupture of the liposomes. The bilayer will burst open under osmotic forces and part of the internal volume will be ejected, immediately followed by resealing of the bilayer. This phenomenon, described in the literature as popping (Siminovitch and Chapman, 1971), would not significantly alter liposome size after an F/T cycle.

Another possible explanation is that during storage at subambient temperatures, ice crystals might grow and cause damage to the bilayers by mechanical forces. After thawing recovery of the bilayer will take time and during recovery marker might leak out of the liposomes. Small particles are apparently more resistant against osmotic and/or mechanical stress.

Crystallization of the internal volume of the liposomes at the homogeneous nucleation temperature appears to be essential to obtain almost complete marker retention. The high crystallization enthalpy of water liberated during freezing of the external medium might interfere with homogeneous nucleation of the internal volume of the liposomes. Therefore, sample parameters, such as sample size and container, might influence the retention. Freezing of 0.25 ml samples in 1 ml glass vials was performed in boiling liquid nitrogen or a mixture of acetone/dry ice. Both cooling systems provide relatively high cooling rates in the critical temperature range in conjunction with the possibility for absorption of large heat quantities. Retention data obtained for the liposomes in 10% saccharose (Table 1) were similar to those of the 25 μl samples frozen in the DSC, except for the vesicles with a size of 0.2 μm; they showed lower marker retention after an

F/T cycle in boiling liquid nitrogen. Again, clear liposome size dependent marker retention after an F/T cycle was shown. Additional storage for 65 h in liquid nitrogen did not show significant higher marker loss (Table 2). Storage at $-25\,^{\circ}C$ after initial freezing in boiling liquid nitrogen demonstrated considerable marker loss for all liposome sizes. A possible explanation might be bilayer damage due to ice crystal growth and/or (re)crystallization during storage of the liposome dispersions at $-25\,^{\circ}C$ after initial cooling in boiling liquid nitrogen. This hypothesis is supported by the results shown in Fig. 2. For the dispersions frozen in boiling liquid nitrogen, damage as a result of osmotic forces is unlikely; in the first instance, complete marker retention is found. The small particle size alterations upon thawing after keeping the dispersions at $-25\,^{\circ}C$ might be due to bilayer damage as a result of ice crystal growth, causing limited fusion/aggregation during thawing and relatively high leakage rates. The slow change in marker retention is remarkable. Apparently, at $-25\,^{\circ}C$ the system is unstable, continuing to change even after starting the freezing procedure by cooling in boiling liquid nitrogen. After reaching 45% marker retention, leakage stopped (also for the dispersion frozen at $-25\,^{\circ}C$). Apparently, for reasons that are currently unclear, part of the dispersion (the smallest particles?) is not affected.

In this study the influence of particle size and storage temperature on marker retention after an F/T cycle was clearly demonstrated. Cryoprotectant-containing dispersions with small liposomes of mean particle size below approx. 0.15 μm showed considerable storage stability at temperatures below $-50\,^{\circ}C$. Since storage at $-50\,^{\circ}C$ is impractical for drug delivery systems, the next step, i.e. the influence of freeze-drying on marker retention of liposome dispersions containing small particles, will be studied in a subsequent article.

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