Effect of Freezing Rate on the Stability of Liposomes During Freeze-Drying and Rehydration

Ewoud C. A. van Winden,[1,3,4] Wei Zhang,[1,2] and Daan J. A. Crommelin[1]

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Purpose. In the present study we examined the effect of the freezing protocol on carboxyfluorescein (CF) retention in liposomes after freeze-drying and rehydration.

Methods. Liposomes were frozen slowly at 0.5°C/min, or quickly by submerging the samples in boiling nitrogen before freeze-drying. The thermal behaviour of the frozen dispersions was analysed by Modulated Temperature Differential Scanning Calorimetry (MTDSC). The dried cakes were analysed by SEM, MTDSC and FTIR. The % encapsulated CF of the (re)hydrated liposomes was determined by fluorimetry after GPC, their vesicle size was measured by the Dynamic Light scattering Technique and their bilayer transition was studied by DSC.

Results. Slow freezing resulted in a markedly higher CF retention after freeze-drying and rehydration as compared to quick freezing. The effect of the freezing rate depended on the lipid composition and was most pronounced for rigid liposomes. The damage caused by quick freezing did not occur after a freezing/thawing cycle. The freezing protocol did not influence the interaction between the phospholipids and the lyoprotectants (sucrose, trehalose or glucose) in the freeze-dried state. However, analysis by DSC of dipalmitoylphosphatidylcholine (DPPC): dipalmitoylphosphatidylglycerol (DPPG) = 10:1 and DPPC liposome dispersions showed that the freezing protocol affected the bilayer melting characteristics of these liposomes after freeze-drying and rehydration.

Conclusions. A proper design of the freezing protocol is essential to achieve optimal stability of rigid liposomes during a freeze-drying and rehydration cycle.

KEY WORDS: freeze-drying; liposomes; lyoprotection; bilayer transition; modulated temperature differential scanning calorimetry (MTDSC).

INTRODUCTION

Freeze-drying is presently used to achieve long term stability of liposomes as drug carrier systems (1). Under appropriate lyoprotective conditions, liposomes retain their vesicle size and hydrophobic drugs will remain associated with the bilayer upon freeze-drying and rehydration. Retention of hydrophilic compounds during this process is more difficult, and depends on vesicle size, the lyoprotectant and the bilayer composition (2–7). In recent years, growing insights have been gained into processes that damage liposomes during freeze-drying, and measures to protect these vesicles during these processes have been described (lyoprotection). Lyoprotectants, preferably disaccharides, are added to the liposome dispersions to form a glassy matrix during freezing. This prevents fusion of the vesicles and provides protection against ice formation. Another mechanism of lyoprotection is related to the bilayer transition temperature (T_m) of the liposomes. Drying of liposomes in the absence of lyoprotectants results in a rise of T_m. When the temperature at which the vesicles are rehydrated is above the T_m for hydrated liposomes, and below the T_m for freeze-dried liposomes, a bilayer transition will occur during rehydration. This can result in an increased bilayer permeability and leakage of the encapsulated compound from the liposomes. However, the presence of e.g. disaccharides during drying can suppress such a rise of T_m, preventing leakage during rehydration. This suppression of T_m has been ascribed to hydrogen bond formation between the sugar molecules and the phospholipid headgroups in the dry state, and has been subject of a number of studies, as reviewed by Crowe and Crowe (3).

Previously, we studied the process of freezing of lyoprotected liposomes in boiling nitrogen, which will be referred to as 'quick' freezing, and subsequent thawing at room temperature (7). Liposomes with saturated DPPC and unsaturated EPC as the main bilayer component retained circa 95% and 80% of the encapsulated CF upon freezing/thawing, respectively. No changes in liposome size were observed. From this, we concluded that freezing according to this protocol hardly caused any damage to the liposomes. This was in line with an earlier study (8), where it was found that high freezing rates minimised the damage to liposomes without lyoprotectant. However, CF retention decreased after freeze-drying and rehydration. The drop depended on the lipid composition (7). Apparently, CF leakage was induced during drying or rehydration of the liposomes, but only to a limited extent during freezing. The observed leakage caused by freeze-drying and rehydration could not be explained by current theories on lyoprotection.

Until now, the influence of the process parameters of the freeze-drying process such as freezing rate, drying temperature, etc., on the liposome integrity after rehydration has not been investigated. In this study we focused on the influence of freezing rate on the retention of the water soluble marker carboxyfluorescein (CF) after freeze-drying and rehydration. We found that major improvements in CF retention can be achieved by manipulation of the freezing protocol. To shed light on the mechanism behind this phenomenon we studied the interaction between the lyoprotectant and the phospholipid headgroups by Fourier transform infrared spectroscopy (FTIR), the texture of the freeze-dried cake by scanning electron microscopy (SEM) and the thermal properties of the frozen, dried, and rehydrated liposome dispersions by modulated and conventional differential scanning calorimetry (DSC). Modulated Temperature DSC (MTDSC) is a novel extension of DSC (9,10).

MATERIALS

Dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (sodium salt) (DPPG), Egg phosphatidylcholine (EPC) and egg phosphatidylglycerol (EPG) were a gift from Lipoid (Ludwigshafen, Germany). Cholesterol (CHOL)

[1] Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences, Utrecht Institute for Drug Exploration, Utrecht University, PO BOX 80082, 3508 TB, Utrecht, The Netherlands.

[2] Current affiliation: Dalian Institute of Chemistry and Physics/Membrane R&D Center, PO BOX 110, Dalian, China.

[3] To whom correspondence should be addressed. (e-mail: octoplus@pi.net)

[4] Current affiliation: Octoplus B.V., P.O. Box 722, Leiden, The Netherlands.

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and trehalose were purchased from Sigma Chemicals (St. Louis, MO, USA). Dipalmitoylphosphatidylserine (sodium salt) (DPPS) and brain phosphatidylserine (sodium salt) (BPS) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Carboxyfluorescein (CF) was obtained from Eastman Kodak (Rochester, NY, USA) and purified according to the procedure described by Ralston (11). Sucrose and glucose were obtained from Merck (Darmstadt, Germany). All other chemicals were of commercial grade and solutions were prepared in water purified by reverse osmosis.

METHODS

Liposome Preparation

Mixtures of phospholipids and cholesterol were dissolved in a mixture of chloroform and methanol in a round bottom flask. The solvent was removed by rotary evaporation at 40–45°C followed by blowing nitrogen over the lipid film for 0.5 h. The lipids were hydrated with solutions containing 5 mM CF, and 10 mM Hepes, 10% carbohydrate (w/w) pH 7.4 at 50–60 °C to obtain a phospholipid concentration of 60 mM. Carbohydrates were omitted from the hydration solutions when indicated under 'Results'. For DPPC liposomes 1 mM EDTA was added to the medium in order to minimise aggregation. The dispersions were subsequently extruded several times at elevated temperatures through polycarbonate membranes with pore sizes of 0.2, 0.1 and 0.05 μm, respectively (Uni-pore, Bio-Rad, Richmond, CA) until the desired vesicle size of circa 0.1 μm was obtained. The external phase was removed by gel-permeation chromatography (GPC) over a pre-equilibrated Sephadex G-50 fine column (Pharmacia, Uppsala, Sweden) using media with the same composition as the hydration media, but without CF, as an eluent. The liposomal fraction was diluted to obtain a phospholipid concentration of 30 or 45 mM, corresponding to carbohydrate/phospholipid ratios (CH/PL) of 4.4 or 3 g/g, respectively. All results were obtained with small (0.1 μm) vesicles unless mentioned otherwise. The preparation of larger vesicles with and without reduced lamellarity is described elsewhere (12).

Freeze-Drying of Liposomes

Liposomes were freeze-dried in aliquots of 0.4 ml in 13.5 ml freeze-drying vials. The rubber freeze-drying caps (type V 9172 DZ, Helvoet Pharma, Belgium) were dried at 100°C for 1 day prior to use. The vials were frozen according to 2 different protocols. 'Quickly' frozen samples were submerged in boiling nitrogen for 10 min and placed on the plate of a Leybold GT4 pilot-production freeze-dryer at a temperature of −40°C. 'Slowly' freezing of samples positioned on the plate was achieved by decreasing the plate temperature at 0.5°C/min to −40°C. In the first step of the freeze-drying process, the plate temperature was maintained at −40°C and the chamber pressure at 10–13 Pa for 30 h, followed by additional drying steps at plate temperatures of −30°C, −16°C and + 20°C, each for 5 hours at a pressure of circa 1 Pa. The condenser temperature ranged between −55 and −60°C. At the end of the freeze-drying process the chamber was filled with nitrogen gas and the vials were closed with rubber caps and stored at 4°C until analysis.

Particle Size Determination

Samples of the dispersion were diluted with a solution containing 10 mM Hepes pH 7.4 and a NaCl concentration which was adjusted to the same osmolarity as the intraliposomal medium. The Z-average size and polydispersity was determined at 25°C by dynamic light scattering with a Malvern 4700 system, using the automeasure vsn 3.2 software (Malvern Ltd, Malvern, UK). The values of the viscosity and refractive index used in the calculation of the particle size of the light scattering data were 0.8905 g/m·s and 1.333, respectively. Average size measurements of 100 nm standard particles (Polymer Laboratories Ltd., Shropshire, UK) deviated less than 5% from the size indicated by the manufacturer.

CF Retention

CF in- and outside the liposomes in samples of 20–100 μl was separated over 1 ml Sephadex G-50 fine (Pharmacia, Uppsala, Sweden) columns (length: 0.5 cm). Both the liposomal and the free fraction were collected in pre-weighed tubes, to which 3–7 ml of 1% Triton X100, 10 mM TrisHCl pH 7.4 (21 °C) was added. The tubes were heated to 90°C and cooled down to ambient temperature and weighed again. The fluorescence of the clear solutions was measured with a Perkin-Elmer LS50 fluorimeter with a well plate reader at an excitation wavelength of 491 nm and an emission wavelength of 521 nm. Thus, the retention of CF in the liposomes as the percentage of the total amount of CF present in the dispersion could be calculated, after correction for the weight of the diluted fractions.

Residual Moisture Content

The residual moisture content of the freeze-dried cakes was determined with the Karl-Fisher method using a Mitsubishi moisture meter model CA-05 (Tokyo, Japan) as described elsewhere (13).

(MT)DSC Analysis

Analysis of frozen samples and freeze-dried cakes by MTDSC has been described elsewhere (14). All scans were recorded with a DSC 2920 (TA Instruments, Inc., New Castle, DE, USA), equipped with a liquid nitrogen cooling accessory. For a 2 point temperature calibration indium and gallium were used as a standard, and the heat flow calibration was performed with indium. Aliquots of 25 μl of aqueous dipersions were frozen in boiling nitrogen as described under freeze-drying, or cooled at 0.5°C/min to −80°C on the DSC furnace before scanning. Dry samples (2.5–5 mg) of the freeze-dried cakes were punched out and transferred into aluminium pans, which were closed tightly. This sampling procedure was performed in a dry nitrogen gas environment in order to minimise attraction of water by the hygroscopic samples. Scans were recorded under 'heating only' conditions with 1 modulation/°C using the following settings: average heating rate (q) = 2°C/min, period (p) = 30 s, temperature amplitude (T_a) = 0.159°C. The Thermal Analist software vs. 8.6 was used for data evaluation. The T_g-values were determined by taking the half height between the baseline below and above the temperature range of the glass transition (reversing heat flow). T_m and $T_{g,onset}$ values were determined as the point of intersection of the tangents of the

baseline and the peak or shift in base line (non-reversing heat flow). For DSC scans of rehydrated liposomes 25 μl samples were heated in closed DSC pans with 2°C/min (T_a = 0°C).

Fourier Transform Infrared Spectroscopy (FTIR)

Samples of freeze-dried cakes were spread out between two ZnSe windows in a cabinet flushed with dry nitrogen gas. Spectra were recorded with 2 cm^{-1} optical spectral resolution and represented an average of 16 scans. The measurements were performed in a Biorad FTS-25 spectrophotometer (Bio-Rad Lab., Inc., Cambridge, MA, USA) at 25°C. Spectra were analysed using Win-IR software (Bio-Rad Lab., Inc.). Absorption characteristics in the asymmetric phosphate vibration region of all DPPC containing samples were compared after a 2-point baseline correction at 1291 and 1176 cm^{-1} and adjusting the baseline offset to zero.

Phosphate Determination

Lipid phosphate was determined by the colorimetric method of Rouser *et al.* (15).

Scanning Electron Microscopy (SEM)

Samples (n = 2) of the freeze-dried cakes were coated with gold prior to analysis and examined with a Philips scanning electron microscope (voltage: 13 kV).

RESULTS

Freeze-drying resulted in porous cakes with no signs of shrinkage, except for the glucose cakes. The residual water contents (±s.d.) were 0.4 ± 0.2 (quickly frozen) and 0.7 ± 0.3% slowly frozen) (n = 14) for the sucrose and trehalose containing liposome cakes, and circa 2.5% in the glucose cakes.

Effect of Freezing Rate on CF Retention and Vesicle Size

In Figure 1A, the results of liposomes with DPPC as the main bilayer component are displayed. For all these lipid compositions the retention after freeze-drying and rehydration was higher when using the 'slow' freezing (cooling rate: 0.5°C/min to −40°C) instead of the 'quick' freezing protocol (submerging vials in boiling nitrogen). With both protocols, the CF retention still depended on the bilayer composition of the vesicles, but the differences were smaller for slowly frozen than for quickly frozen, dried and rehydrated vesicles. The same experiment was done with EPC containing liposomes (Figure 1B). For these dispersions, hardly any differences in CF retention after drying and rehydration were found between slowly and quickly frozen samples.

In Figure 2 we plotted the increase in retention after freeze-drying and rehydration when applying 'slow freezing' instead of 'quick freezing', as a function of the leakage observed in quickly frozen samples. It is clear that the higher the leakage was when using the quick freezing protocol, the more CF retention is improved when slow freezing is applied. Interestingly, a straight line could be drawn through the points of the DPPC containing liposomes which were freeze-dried in the presence of trehalose or sucrose. The line passes through the intersection of the y- and x-axis. This implies that the percentage of leakage after

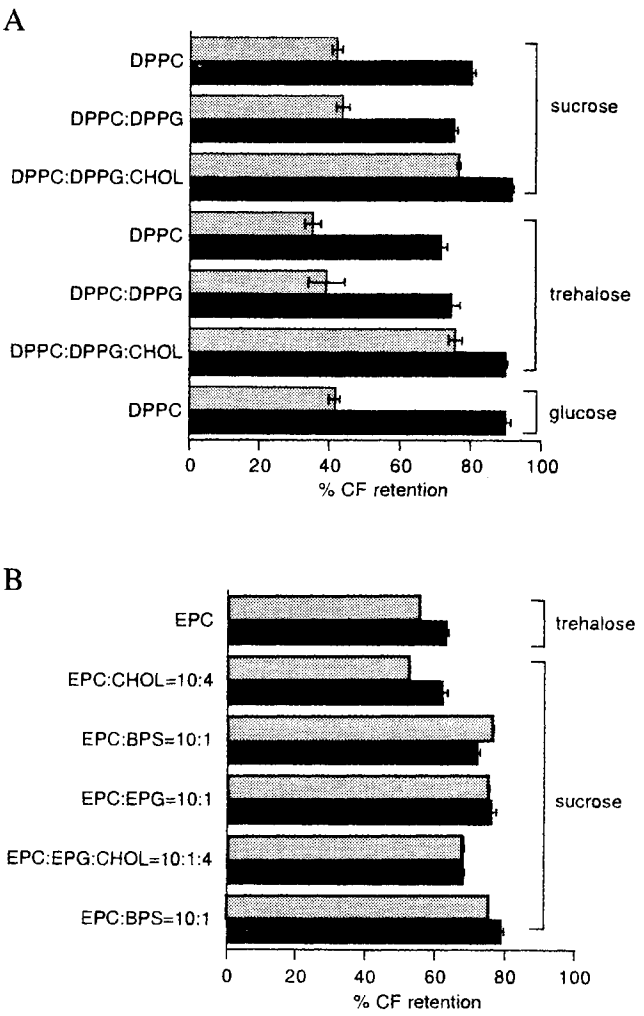

Fig. 1. CF retention in DPPC (A) and EPC (B) containing liposomes after freeze-drying and rehydration using the quick freezing (▒) and slow freezing (■) protocol. Average vesicle size: 0.11–0.14 μm. 2A: All data represent the average of 2 independent experiments (with different 5 vials per lipid composition) and the error bars indicate the pooled standard deviation. The mean values of both experiments differed less than 8% of the full scale. 2B: Error bars indicate the standard deviation of replicates within 1 experiment (n = 1). Lyoprotectants: see Figure. Molar ratio's: PC:PG or PC:PS = 10:1, and PC:CHOL = 10:4.

freeze-drying and rehydration which is prevented by using slow freezing instead of quick freezing, is the same for all DPPC containing lipid compositions. Apparently, independent of how much CF a certain DPPC-vesicle type leaked during the process, slow freezing will prevent leakage in these samples by approximately 60%. From this we conclude that quick freezing in boiling nitrogen induces a stress vector for the DPPC containing liposomes which only surfaces in combination with the drying and rehydation process. The resistance of liposomes to this stress vector depends on their lipid composition, e.g. the presence of cholesterol in the bilayer. In addition, the stress vector is reduced by lowering the freezing rate.

For the other samples too few data points are available to draw firm conclusions. However, it is clear that for e.g. EPC containing liposomes, which showed minimally 25% CF leak-

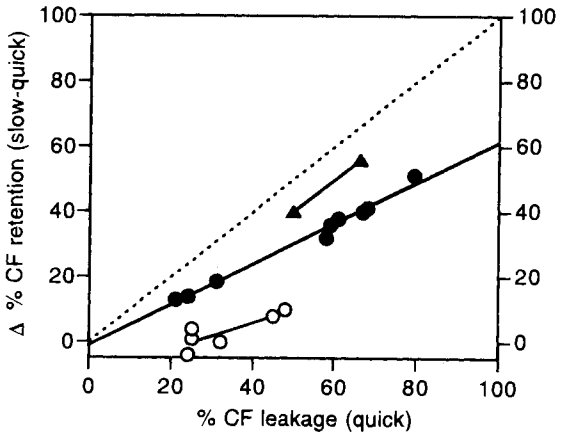

Fig. 2. Difference in CF retention after freeze-drying and rehydration obtained with 2 different freezing protocols. The data in this Figure were replotted from Fig. 1A and B and were obtained in 2 independent experiments. Each data point represents the values obtained with a single batch of liposomes. The lines were calculated by linear regression. Lipid compositions: DPPC containing liposomes (●,▲), EPC containing liposomes (○). Lyoprotectant: sucrose or trehalose (●,○), or glucose (▲). Average vesicle size: 0.11–0.14 μm. The dashed line indicates complete prevention of leakage obtained with the quick freezing protocol by application of the slow freezing protocol.

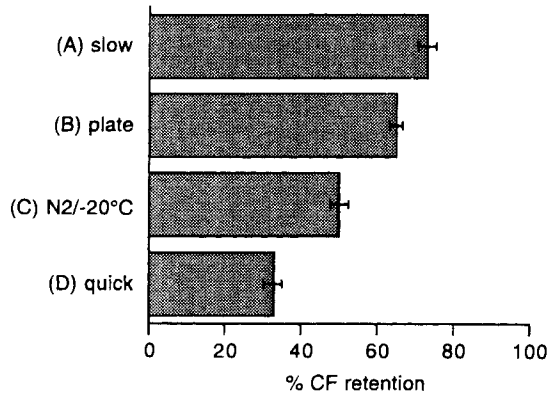

Fig. 3. Influence of freezing protocol on the CF retention in DPPC liposomes after freeze-drying and rehydration. Protocols: (A) samples were cooled with 0.5°C/min to −40°C, (B) samples (25°C) were placed on the freeze-dryer plate with a temperature of −40°C, (C): samples were frozen in boiling nitrogen (10 min) stored at −20°C for 3 h and cooled with 0.5°C/min to −40°C, (D) samples frozen in boiling nitrogen for 10 min, and placed on the freeze-dryer plate at −40°C.

age in this study, a decrease in freezing rate did not improve the stability during the freeze-drying and rehydration cycle in the presence of trehalose or sucrose. This suggests that a different type of damage occurred in these samples, which can not be prevented by a change in freezing protocol.

Analysis of the lipsome size data showed that the two freezing protocols described above did not result into different vesicle sizes in the freeze-dried and rehydrated samples (data not shown). However, a small, but consistent effect of bilayer composition on the change in vesicle size caused by freeze-drying and rehydration was observed. Liposomes containing DPPC exhibited a small average size increase ranging from 0 to 20 nm, whereas the average size of EPC-like liposomes seemed to slightly decrease. Although it is not clear to us what exactly causes these differences, it is an indication that EPC and DPPC liposomes differ in their response to stress vectors existing during the freeze-drying and rehydration process.

Figure 3 shows the influence of 4 different freezing protocols on the CF retention in DPPC liposomes after freeze-drying and rehydration in the presence of trehalose. In this experiment, samples that were placed on the freeze-dryer plate which had been equilibrated at −40°C, retained only slightly less CF (65%) than samples cooled at 0.5°C/min (73%). In two other protocols, samples were frozen in boiling nitrogen. Subsequently, one group of vials was kept at −40°C on the freeze-dryer plate, whereas other vials were first kept at −20°C for 3 h, and then placed on the plate at −40°C. All samples were freeze-dried under identical conditions. The 3 h incubation at −20°C had increased the retention from circa 33 to 55%, which proves that at least part of the leakage did not occur during freezing in boiling nitrogen. Thus, the formation of ice crystals during cooling can not be solely held responsible for the induced leakage. This is in line with the low leakage observed when freezing the liposomes in boiling nitrogen followed by immediate thawing.

Effect of Freezing Rate on the Characteristics of Frozen Dispersions

The thermal profiles of the MTDSC heating scans of a slowly and quickly frozen sample, depicted in Figure 4, showed two main differences. As expected, the glass transition after quick freezing occurs at lower temperatures than after slow freezing. This can be explained by a further progressed freeze-concentration under slow freezing conditions, before the freeze-concentrate solidifies and the growth of ice crystals ceases (16,17). Under such conditions, the amount of water that remains in the sugar glass is smaller, resulting in a higher glass transition temperature (T_g).

The exothermal peak observed at temperatures above T_g can be ascribed to devitrification processes (18). At these temperatures, water from the sugar glass crystallises, indicating that the freeze-concentration of the sugar solution that had been achieved during cooling was incomplete. As for T_g, the onset temperature of the devitrification process (T_{dev}) was lower

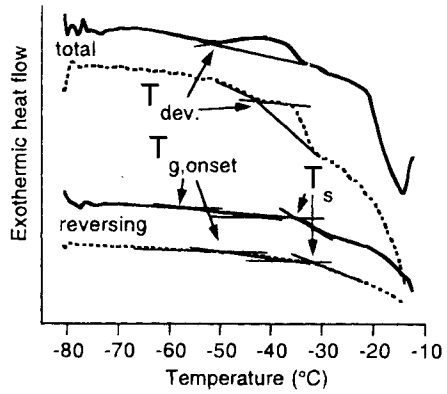

Fig. 4. MTDSC heating profiles of DPPC liposomes with sucrose after quick freezing to −196°C (▬) or slow freezing to −80°C (▬). q = 1°C/min, p = 30 s, T_a = 0.159°C. $T_{g,onset}$: onset temperature glass transition, T_s: softening temperature, T_{dev}: onset temperature devitrification process.

(−49°C) in quickly frozen than in slowly frozen samples (−42°C). From these MTDSC profiles it can be concluded that in liposome dispersions frozen in boiling nitrogen devitrification occurred after the samples were put on the freeze-dryer plate at −40°C. In the slowly frozen samples, devitrification processes may be initiated as well, although the major part of devitrification process was observed at higher temperatures in the MTDSC heating scan. It is presently unknown to what extent devitrification processes influence the stability of frozen liposomes.

The third thermal process, here denoted as T_s, is the 'softening', or collapse temperature, also referred to in literature as T_g (19). The exact nature of this transition is still subject of discussion in literature (17–21). However, it is considered a critical parameter for the freeze-drying process. When the sample temperature exceeds T_s during the removal of ice crystals in the primary drying process, the cake will collapse and the surface area will be strongly reduced.

Effect of Freezing Rate on the Characteristics of Freeze-Dried Cakes

One possible explanation for the beneficial effect of slow freezing and enhanced freeze-concentration on the CF retention could involve a different (freezing rate dependent) bilayer transition temperature (T_m) of liposomes in the freeze-dried state. When the liposomes are rehydrated at a temperature between T_m in the dry state and T_m in the rehydrated state, a phase transition will occur during rehydration. This can result into an increased bilayer permeability and leakage of the encapsulated compound (22), as discussed above.

The T_m of the liposome bilayer after freeze-drying in the presence of sugars can be above or below the T_m in the fully hydrated state, depending on the experimental conditions (see references (13,23–25)). Therefore, we compared the T_m in the dried cakes of quickly as well as slowly frozen dispersions by means of MTDSC analysis. No pronounced differences were observed between samples that had been prepared by quick or slow freezing (data not shown). A complex endotherm consisting of two peaks was observed, which can be ascribed to the melting of phospholipids (13). In both samples the two overlapping peaks had onset temperatures of about 40 and 60°C.

Since suppression of T_m is ascribed to the interaction of the sugars with the phospholipid headgroups in the dried state, we also studied the asymmetric phosphate-diester stretching vibrations ($\nu_{as}(PO^{2-})$) of the phospholipids by FTIR. These vibrations appear as absorption bands between 1260 and 1220 cm^{-1}($\nu_{as}(PO^{2-})$), depending on the hydrogen bond formation between the phosphate moiety of the phospholipids and water or sugar molecules. Enhanced hydrogen bond formation (stronger interaction) is reflected by an increased absorption at lower frequencies (26). In order to detect any differences in the maximal degree of interaction between the lyoprotectant and the phospholipids, we subjected the samples to an "annealing" treatment by heating the cakes to 100°C for 30 min. Such an annealing treatment is known to enhance the interaction between these molecules and further suppress T_m of the phospholpids, probably by a temporarily increase of the molecular mobility of the carbohydrate and phospholipid molecules (see ref. (13,23,25).

The absorption patterns in the $\nu_{as}(PO^{2-})$ vibration region of liposomes freeze-dried with trehalose after quick and slow freezing were nearly identical, as well as before as after annealing (data not shown). For none of the lipid compositions differences were found between the interaction characteristics of freeze-dried liposomes which were quickly or slowly frozen. Based on these data we conclude that the freezing protocol did not influence the interaction between sugar and lyoprotectant.

The structure of the dried cakes was examined with Scanning Electron Microscopy (SEM). The dried liposome/sugar cakes which were frozen in boiling nitrogen had a highly ordered structure (Figure 5A, B and C), with channels directed upwards from the bottom of the vial. At the bottom side, larger holes were observed, reflecting a different crystallisation behaviour in this part of the sample. Apparently, the freezing behaviour in the sample was inhomogeneous despite the small sample size (0.4 ml). Noteworthy was the 'skin' which covered the cake and was interrupted by cracks. This skin gives the cakes the smooth appearance when observed from the top, and probably reduces the sublimation rate during the primary drying process.

In contrast, the holes in dried cakes of the slowly frozen samples were much larger and had a disordered orientation throughout the sample (a typical example is given in Figure 5D). No covering 'skin' was observed, which accounts for the rough appearance of the cake surface as observed macroscopically. The holes represent the space which had been occupied by the ice crystals. Apparently, slow freezing had allowed a more extended growth of the ice crystals, which obviously resulted in a reduced surface area of the cake and an decreased surface/mass ratio of the sugar glass.

Bilayer Melting Characteristics After Freeze-Drying and Rehydration

Leakage of the encapsulated compounds could also be related to the bilayer properties after freeze-drying and rehydration. In a previous study in our group changes in the melting behaviour of small DPPC/DPPG liposomes were observed after freeze-drying and rehydration in the presence of the sucrose (27). The melting endotherm consisted of at least two overlapping peaks, suggesting the existence of separated phases in the liposomal bilayer. Therefore, we examined the melting behaviour of the freeze-dried and rehydrated vesicles prepared with the quick and slow freezing protocol. First, in Figure 6A the DSC profiles of the control samples (untreated (not frozen or dried) and frozen/thawn) of DPPC:DPPG = 10:1 liposomes with sucrose are displayed. The untreated liposomes showed an endotherm with the peak position at 42.7°C (line 2, see Fig. 6B). Freezing/thawing had no effect on the melting properties of the bilayers, and no differences were observed between the first and the second heating scan. However, the same liposomes after quick freezing, drying and rehydration exhibited pronounced changes, as displayed in Figure 6B. In the first scan, two additional peaks appeared in the thermogram, at around 40.7°C [1] and one at circa 46°C [3]. Moreover, the heating profile was changed in the second scan. Peak 3 had disappeared, but peak 1 was more pronounced than in the first scan. The heights of peak 3 in the first scan, and peak 1 in the second scan showed a substantial variability. The ratio of these peaks heights with the height of peak 2 varied with circa ±20% between batches. However, in each sample clear differences

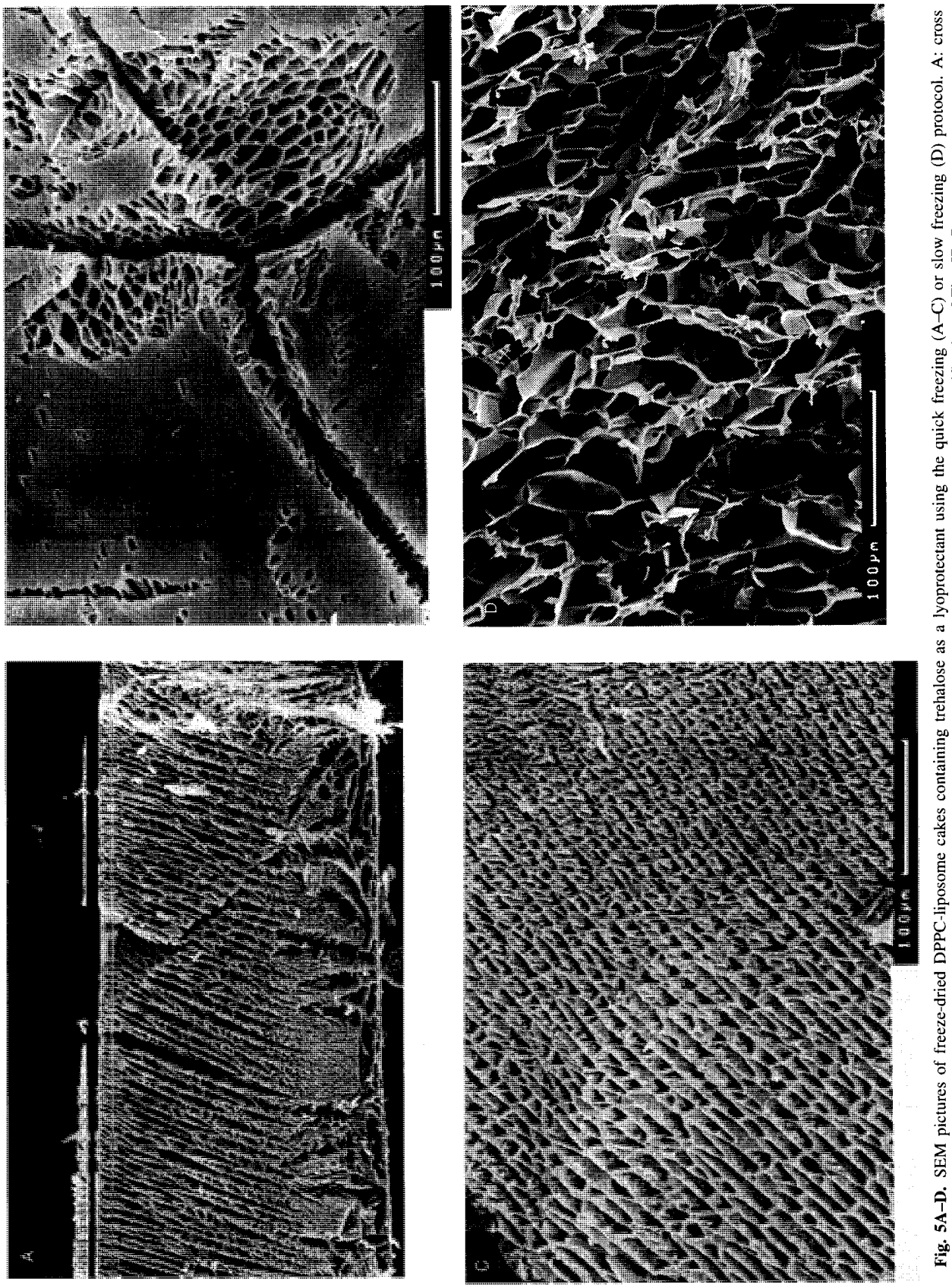

Fig. 5A–D. SEM pictures of freeze-dried DPPC-liposome cakes containing trehalose as a lyoprotectant using the quick freezing (A–C) or slow freezing (D) protocol. A: cross section, B: top view, C and D: cross section. The magnification of Fig. 5A was 1/5 of the magnification of Fig. 5B–D.

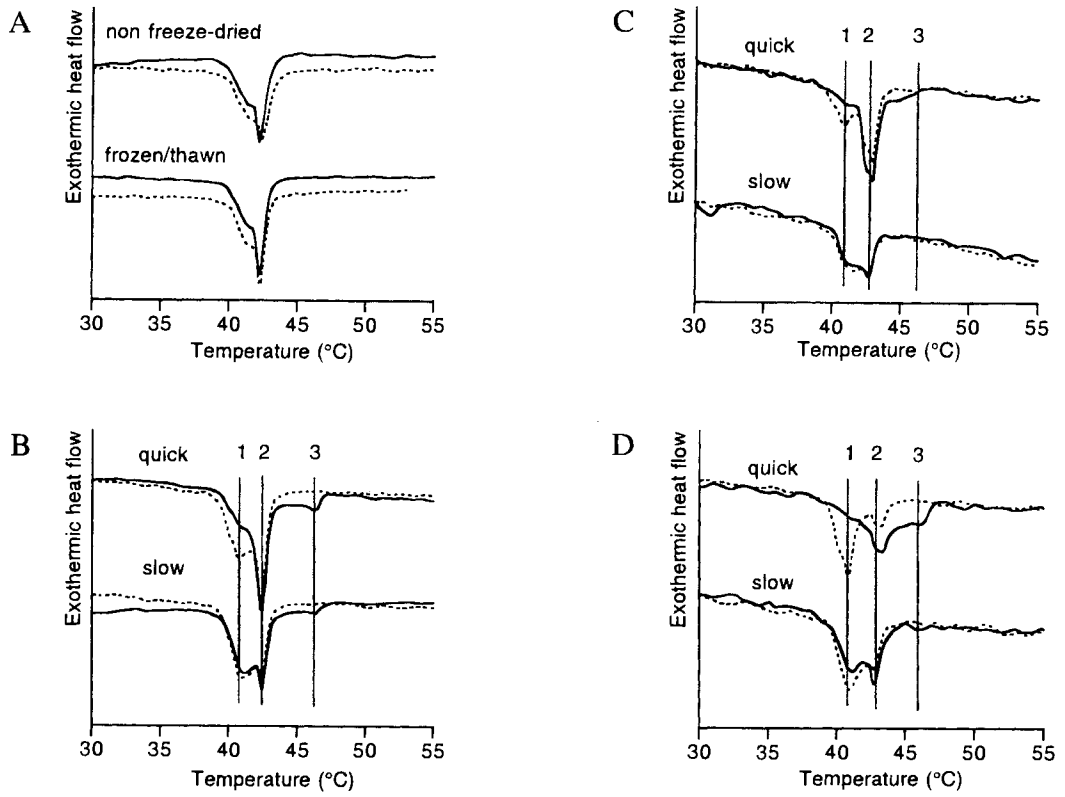

Fig. 6A–D. DSC heating profiles of (A:) untreated (non-freeze-dried) and quickly frozen/thawn, or (B–D:) freeze-dried and rehydrated liposomes. Freezing was performed in boiling nitrogen (quick) or with 0.5°C/min (slow). Lipid composition: DPPC:DPPG = 10:1, lyoprotectant: sucrose (A and B), trehalose (C) and glucose (D). Scan 1: (▬), scan 2: (▬). Heating rate: 2°C/min. For meaning of the numbers: see text.

were found between the first and second scan of the freeze-dried and rehydrated samples and between results obtained with freeze-dried and non freeze-dried liposomes.

The changes in the DSC profiles of the same liposomes after slow freezing, drying and rehydration (Fig. 6B) were different again, with a strong presence of the type 1 peak. Comparable, but different patterns were obtained with other sugars as a lyoprotectant. Figures 6C and D show the DSC profiles for DPPC:DPPG = 10:1 liposomes freeze-dried with trehalose and glucose, respectively. The most pronounced changes were observed with the monosaccharide glucose. Peak 1 was in the second scan even more dominant than peak 2 (untreated liposomes). In both cases, slow freezing resulted in less pronounced changes between the profiles of scan 1 and 2 of the rehydrated liposomes than quick freezing. For the disaccharides lactose and maltose and the trisaccharide raffinose profiles similar to those of trehalose were obtained. None of the investigated sugars exhibited changes between scan 1 and 2 of the untreated or frozen and thawn dispersion.

The changes after scan 1 suggested that the liposome bilayers were metastable after rehydration. We further investigated whether changes in this metastable state occurred during incubation in the rehydrated state below T_m (melting of bilayer in the rehydrated state) and whether the bilayer properties after the first scan further changed upon repeated scans. The results demonstrated that without heating through T_m, no change in melting behaviour occurred during a storage period of 2 weeks in the rehydrated state (data not shown). In addition, the melting behaviour after the first heating scan remained unchanged during 3 additional scans. Apparently, heating through T_m induced a transition of the bilayers from a metastable to a thermodynamically more stable state.

Until now, only rehydrated small (0.1 μm) liposomes made of DPPC:DPPG = 10:1 were examined. Theoretically, freeze-drying and rehydration may have induced a phase separation of DPPC and DPPG in the bilayer, resulting in distinct bilayer domains with different melting properties. However, these phospholipids generally mix well because of the equal acyl chain length. Moreover, assuming the main peak [2] of untreated liposomes represents the melting of DPPC, the large side peak [1] in e.g. the liposomes with glucose (Fig. 6D) can hardly be accounted for by the small fraction of DPPG in these liposomes.

An alternative explanation was based on a recent study by Viera *et al.* (28). These authors observed an increased bilayer permeability of dried and rehydrated multilamellar DPPC liposomes with trehalose which was maintained for at least 24 h. This was ascribed to the existence of 2 distinct phases in the liposomal bilayers, one unchanged phospholipid phase, and one phospholipid phase with trehalose molecules intercalated between the phospholipid headgroups. The existence of separate domains with different phases may result in an increased permeability, because of packing defects which may exist at the boundary of such domains.

To investigate whether intercalation of sugars could be involved in the complex melting behaviour of the liposomes in the present study, we examined DPPC liposomes (without

DPPG, vesicle size 0.1 μm) freeze-dried and rehydrated in the presence of trehalose. In such a sample, the occurrence of a phase separation between DPPC and DPPG can be excluded. The results, presented in Figure 7, indeed showed a (small) change in melting behaviour induced by freeze-drying and rehydration, and a metastable (scan 2) behaviour similar to observed for DPPC:DPPG = 10:1 liposomes. Thus, intercalation of trehalose after rehydration may explain the change in melting behaviour. Interestingly, our results differ from the findings by Viera *et al.* (28) who used DPPC MLV's and did not find a change in the melting behaviour of these liposomes.

In another study by our group (12) we observed an increased bilayer permeability of DPPC:DPPG = 10:1 MLV's after freeze-drying and rehydration, which is in line with the observations by Viera *et al.* (28) for DPPC MLV's. For small liposomes of the same lipid composition the permeability increase was less pronounced. Our conclusion was that this permeability enhancement was caused by reorganisation/reformation processes of the bilayer after rehydration. In order to compare the results of DSC analysis of rehydrated liposomes with these two studies (12,28), liposomes with different vesicle sizes and lamellarity (containing sucrose and prepared as described in our previous study (12)), were examined.

From the results it became clear that only vesicles with an average size of circa 0.4 μm and smaller exhibited a changed melting behaviour after freeze-drying and rehydration (data not shown). In contrast, the melting behaviour of multi- and plurilamellar vesicles of 1 μm were not affected by the freeze-drying and rehydration cycle, whereas these vesicles showed the highest bilayer permeability upon rehydration (12). Therefore, no correlation was found between these two phenomena: permeability enhancement and melting behaviour upon freeze-drying.

The role of sugars on the melting behaviour was also investigated by heating small freeze-dried DPPC liposomes with trehalose to temperatures between 80 and 130°C. This heating/cooling cycle resulted in an enhanced interaction between the carbohydrate and the phospholipid molecules in the freeze-dried state (13). Such an increased interaction may lead to a more pronounced (possible) intercalation of the sugar molecules between the phospholipid headgroups after rehydration. However, no consistent effect of this heating protocol on the bilayer transition of the rehydrated liposomes was found (data not shown).

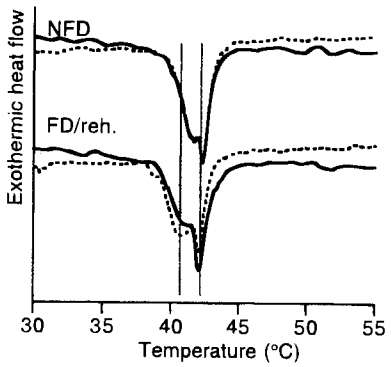

Fig. 7. DSC heating profiles of freeze-dried and rehydrated DPPC liposomes. Freezing was performed in boiling nitrogen (quick) or with 0.5°C/min (slow). Lyoprotectant: trehalose. Scan 1: (▬), scan 2: (▬▬). Heating rate: 2°C/min. NFD: non freeze-dried, FD/reh.: freeze-dried and rehydrated.

DISCUSSION

In this study, major effects of the freezing rate on the stability of liposomes after freeze-drying and rehydration were observed. These effects depend on the lipid composition (Fig. 1A and B). For example, CF retention in DPPC liposomes with sucrose or trehalose as a lyoprotectant was doubled from circa 40 to 80% when freezing slowly at 0.5°C/min instead of freezing in boiling nitrogen. The same change in freezing protocol resulted in an increase in CF retention from 40 to 90% in DPPC liposomes with glucose as a lyoprotectant, whereas poor lyoprotective properties have been reported for this monosaccharide previously (3,29). The freezing rate also affected the melting characteristics of the freeze-dried and rehydrated liposomes. Quick freezing resulted in the appearance of multiple side peaks in the DSC heating profiles, and a metastable behaviour of the rehydrated bilayers. Both effects were reduced by using the slow freezing protocol.

Interestingly, the retention in quickly frozen samples was slightly increased when the frozen sample was kept at −20°C for 3 h before drying (Fig. 3). Thus, not only the freezing rate, but also the temperature program after freezing determines the liposome stability during the freeze-drying process. Below we will discuss possible mechanisms involved in these phenomena.

A possible cause of CF leakage is a transition of the bilayer from the rigid gel to fluid liquid phase, or vice versa, at some point during the freeze-drying process. This may depend on the T_m of the bilayer, which is 41.5°C for fully hydrated DPPC liposomes (strong effect of freezing rate) and -10 ± 5°C for EPC liposomes (30) (no effect of freezing rate). The presence of CHOL in the bilayer abolishes this transition. It makes the fluid phase more ordered, and the gel phase more disordered leading to some kind of intermediate state. The stability of liposomes during quick freezing, drying and rehydration was better, and the improvement by slow freezing was less for DPPC/DPPG liposomes containing CHOL than for liposomes consisting of DPPC/DPPG only. However, during freezing, drying and rehydration, the sample temperature was always kept below T_m, excluding the occurrence of a bilayer transition during these processes. In addition, no difference between the T_m in the dried state of slowly and quickly frozen samples was observed. Therefore, we conclude that no phase transitions in the bilayer were involved in the effect of freezing rate.

The changed melting behaviour of the rehydrated liposomes correlated well with the effect of freezing rate on CF retention. Slow freezing reduced the changes in the bilayer transition characteristics after freeze-drying and rehydration and increased the CF retention. The presence of CHOL in the liposome bilayers abolished the bilayer transition in the hydrated state (data not shown), increased CF retention and reduced the difference between slowly and quickly frozen samples.

However, the changed melting behaviour did not correlate with the increased permeability of liposome bilayers after freeze-drying and rehydration, as observed in an earlier study (12). MLV's showed the strongest permeability increase, but no change in melting behaviour. In addition, the presence of

CHOL did not affect the low bilayer permeability in small liposomes, whereas it abolishes the melting endotherm.

Therefore, it is not clear how the changed melting behaviour is related to the CF leakage after freeze-drying and rehydration.

Our data suggest that the bilayer rigidity is an important factor in the occurrence of freeze-drying damage. The rank order of rigidity for the bilayer compositions used in this study is given in Table I (a). This rank order has similarities with the one for the improvement in CF retention after freeze-drying and rehydration when lowering the freezing rate (b). In contrast, the rank orders in Table I for the CF retention obtained with both freezing protocols did not match the sequence for bilayer rigidity. This indicates that at least two distinct stress vectors exist that induce CF leakage from lyoprotected liposomes during the freeze-drying and rehydration process. One stress vector that particularly affects rigid bilayers, is reduced by slow freezing instead of quick freezing. Freezing rate effects are correlated with bilayer rigidity.

A second mechanism of damage is not correlated with the bilayer rigidity and affects in particular the EPC containing liposomes. This agrees with the observation that the leakage in EPC liposomes could not be prevented by altering the freezing rate. This second stress vector could be the occurrence of a bilayer transition during rehydration. The T_m of EPC liposomes in the freeze-dried state is indeed above the rehydration temperature, as was reported in a previous study (13). The bilayer melting process in freeze-dried EPC:BPS = 10:1 liposomes was observed above 40°C, despite the presence of trehalose. However, it is unclear how EPC liposomes containing CHOL may leak CF via this mechanism, since these levels of CHOL (4 mol/11 mol phospholipid) abolish the bilayer transition.

The exact nature of the stress vector which is reduced by a slow cooling rate is not clear. Based on the data in this study, different hypotheses can be proposed.

The MTDSC thermograms (Fig. 4) suggest that a more pronounced devitrification may occur in samples frozen in boiling nitrogen and subsequently dried at −40°C, than in samples slowly frozen to −40°C and kept at that temperature. Whether devitrification damages the liposomes remains to be investigated. The formation of ice crystals in the glass during devitrification could rupture the bilayers in such a way that leakage and bilayer reassembling processes occur after drying and rehydration. This could explain the small size increase observed for the DPPC containing liposomes, since reformation of bilayers may lead to fusion of vesicles. This theory seems to be in conflict with the finding that ice formation and freeze-concentration did not induce leakage in a freezing/thawing cycle. However, the result of ice formation in the glassy or rubbery matrix (devitrification) where liposomes are located may differ in this respect from the effect of crystal growth with the less viscous freeze-concentrate in between the crystals.

A second hypothesis is that slow freezing allowed the vesicles more time to relax from deformations by the mechanical forces and high pressure induced by the ice formation before the freeze-concentrate was solidified above T_s. Deformed vesicles may be more leaky upon drying and rehydration than non-stressed liposomes. This would also explain the increase in CF retention (Fig. 3) when quickly frozen samples were incubated at −20°C, above T_s (Fig. 4) before the second, slow cooling step to −40°C.

A final hypothesis is that leakage is specifically induced for liposomes in the freeze-concentrate that are located at the glass—ice boundary. The incomplete encapsulation of liposomes by the glassy matrix may damage the liposomes in the dry state. The chance that liposomes are positioned at this glass-ice interface becomes smaller when the surface/mass-ratio of the sugar matrix decreases. This is obviously the case for the slowly frozen dispersions. Figures 5C and D show that the cake pores were much larger after slow freezing than after quick freezing. This implies a smaller glass surface, since the sugar concentration was the same in all samples.

In summary, marked effects of the freezing-rate on the CF retention and bilayer melting behaviour of freeze-dried and rehydrated liposomes were observed. These effects depended on the bilayer composition of the liposomes. At least two stress vectors which damage lyoprotected liposomes during the freeze-drying and rehydration process were hypothesised. One, correlating with bilayer rigidity, and a second one which damaged particularly the EPC containing liposomes. The results indicate that a proper design of the freezing protocol is essential for the optimal stability of liposomes during a freeze-drying and rehydration cycle.

Table I. Comparison of Liposomes with Different Lipid Compositions

(a) bilayer rigidity	DPPC > DPPC + CHOL > EPC + CHOL > EPC
(b) %Rslow–%Rquick	DPPC > DPPC + CHOL > EPC + CHOL, EPC
(c) %Rquick	DPPC + CHOL > EPC + CHOL, EPC > DPPC
(d) %Rslow	DPPC + CHOL > DPPC > EPC + CHOL, EPC

Note: Rank order of (a) bilayer rigidity, (b) improvement in retention after freeze-drying and rehydration by slow freezing instead of freezing in boiling nitrogen, % CF retention after freeze-drying and rehydration, with quick (c) or slow (d) freezing. The presence of charged phospholipids in the liposomal bilayers did not affect the rank order of the listed parameters.

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