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Critical parameters in freezing of liposomes

G.J. Fransen [1], P.J.M. Salemink [2] and D.J.A. Crommelin [1]

[1] *Department of Pharmaceutics, Subfaculty of Pharmacy, State University of Utrecht, Utrecht (The Netherlands)* and [2] *Organon International B.V., P.O. Box 20, 5340 BH Oss (The Netherlands)*

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Summary

The effect of freezing rate, freezing temperature, freezing time and the cryoprotective capabilities of a number of additives on the carboxyfluorescein (CF) retention and physical stability (resistance against aggregation and fusion) of liposomes after a freezing/thawing cycle was investigated. Negatively charged multilamellar vesicles consisting of hydrogenated soybean lecithin and DCP (10/1) were used. No aggregation or fusion and over 90% CF retention was found after freezing at −25°C (freezing rate 7°C min^{-1}) for 20 min in the presence of various cryoprotectants; other freezing conditions proved to induce a substantial CF loss or aggregation and fusion.

Introduction

In the near future liposomes might be introduced as drug carriers in a number of therapeutic fields, e.g. cancer chemotherapy, antifungal and antiparasitic therapy. In general, the proposed types of liposomes do not meet the required standards for long term stability of pharmaceutical preparations if they are stored as aqueous dispersions. The encapsulated drug tends to leak out of the bilayer structure and the liposomes might aggregate or fuse on storage. These processes can cause a change in the pharmacokinetic profile of the encapsulated drug and therefore reduce the reproducibility of the therapeutic effect.

Correspondence: D.J.A. Crommelin, Department of Pharmaceutics, Subfaculty of Pharmacy, State University of Utrecht, Catharijnesingel 60, 3511 GH Utrecht, The Netherlands.

As an alternative for storing aqueous dispersions, freeze dried liposomes are proposed. A number of articles and patents have been published on this subject (Evans et al., 1978; Vanlerberghe and Handjani, 1979; Gordon et al., 1982; Shulkin et al., 1984; Crommelin and Van Bommel, 1984; Van Bommel and Crommelin, 1984; Henry-Michelland et al., 1985; Abu-Zaid et al., 1984). The results show that liposome integrity on freeze drying can be preserved in the presence of properly selected cryoprotectants as far as the stability against aggregation and fusion is concerned. Lipophilic, bilayer associated compounds show a high level of drug retention (% of liposome associated drug) on rehydration of the freeze dried product. However, for hydrophilic, non-bilayer interacting compounds drug retention on rehydration is low.

The objective of this study was to explore different routes that can lead to an improved quality

of liposomes containing hydrophilic compounds after a freeze drying/rehydration cycle. Freeze drying is a technique that consists of 3 separate steps. First the product is frozen, then sublimation (primary drying) starts, followed by the secondary drying process. For a maximum drug retention after rehydration optimization of process parameters in all 3 steps is required. Here we will focus on the optimization of freezing conditions. Existing concepts in the process of freezing/thawing of liposomes are based on insights gained in the past in the field of cryobiology (e.g. Ashwood-Smith and Farrant, 1980; Mazur, 1970). The effects of freezing rate, freezing temperature, freezing time (time that the sample is kept at the selected temperature) and of the presence of different types and concentrations of cryoprotectants were studied for multilamellar, negatively charged liposomes containing carboxyfluorescein (CF), a hydrophilic non-bilayer interacting compound, as a model drug. Drug retention and mean diameter were the parameters that were monitored.

Materials and Methods

Phospholipon 100H (hydrogenated soybean phosphatidylcholine: PC-H) was a gift of Nattermann (Köln, F.R.G.). This product contains at least 93% phosphatidylcholine, less than 2% lysophosphatidylcholine and about 2% water. The fatty acid composition is 10% $C_{16:0}$, 90% $C_{18:0}$ and traces of $C_{20:0}$. The transition temperature of PC-H in an aqueous dispersion measured by differential scanning calorimetry (Perkin Elmer DSC-2, Perkin Elmer Corp., U.S.A.) was 51°C. Dicetylphosphate (DCP) and cholesterol were supplied by Sigma Chemicals (St. Louis, MO). 5,6-Carboxyfluorescein was obtained from Eastman Kodak Co. (Rochester, NY). Lactose, glucose, sucrose, ascorbic acid, citric acid and glycerol met the requirements of the Ph. Eur., mannitol and PVP of the USP XXI, PEG 300 of NF 15. Methylcellulose was supplied by Hoechst (Frankfurt, F.R.G.). Apart from CF all chemicals were used as such. CF was purified according to the procedure described by Ralston et al. (1981).

Multilamellar vesicles were prepared with the classical "film method". PC-H and DCP (molar ratio 10 : 1) were dissolved in chloroform in a pear shaped vessel. The chloroform was evaporated in a rotary evaporator to yield a film. Traces of chloroform were removed in a vacuum desiccator for 2 h. Then glass beads were added and the film was hydrated in a 50 mmol/l CF and 10 mmol/l Tris containing aqueous solution (pH 7.4) at 70°C by hand shaking. The phospholipid concentration after hydration was around 30 mmol/l. Lipid phosphorus was determined according to the procedure of Fiske and Subbarow (1925).

The dispersion was extruded at 70°C twice through 600 nm polycarbonate membrane filters (Uni-pore, Bio-Rad, Richmond, CA) under nitrogen pressures up to 0.8 MPa. The external CF solution was replaced by a 100 mmol/l sodium chloride, 10 mmol/l Tris solution (pH 7.4) by eluting the dispersion over a Sephadex G-50 fine column (Pharmacia Fine Chemicals, Uppsala). The fraction of non-encapsulated CF after gelchromatography was 3–4%. The final liposome dispersion contained between 5 and 10 mmol/l PC-H. The dispersions were kept at 4–6°C. Aqueous solutions of cryoprotectants were added in a 1 : 1 volume ratio to the liposome dispersions. The concentrations mentioned are the actual cryoprotectant concentrations in the frozen/thawed dispersions. The principle and details of the determination of the CF retention were described elsewhere (Lelkes, 1984). The fluorescence signal (Perkin Elmer fluorescence spectrophotometer 204, Hitachi Ltd., Tokyo) of a diluted liposome dispersion before and after destruction of the liposomes by Triton X-100 (1%) (BDH chemicals Ltd, Poole, U.K.) was detected at 515 nm. The excitation wavelength was 490 nm. For complete release of CF from the liposomes the Triton X-100 containing dispersions were heated for 1 h at 100°C in sealed vials. Particle diameters were measured by dynamic light scattering (Nanosizer, Coulter Electronics Ltd., Luton, U.K.).

Glass vials containing 0.6 ml of the dispersion were placed in the cryostat (Julabo F40/HC). After freezing under the selected conditions the vials were taken out of the cryostat and thawed at 4°C. For measuring the conductivity as a function of the temperature a freezing analyzer (Edwards,

Marburg, F.R.G.) was used. A 5 ml sample of the solution or dispersion was transferred to the freezing analyzer. The sample was frozen down to −60°C at a rate of 7°C/min and subsequently slowly heated to +4 or +12°C. During this heating process the temperature and electrical conductivity (AC) were recorded simultaneously as a function of time. The calibration curve for the conductivity cell was obtained from the manufacturer (Edwards, Marburg, F.R.G.). For analysis of the experimental data, the analog curves were digitized with a Hewlett Packard 9872 plotter and a Hewlett Packard 9825 desk-top calculator.

Results

Freezing temperature

CF retention after a freezing/thawing cycle depended on the selected freezing temperature (Fig. 1a). A freezing rate of 0.12°C/min was selected. Between −6 and −8°C ice crystal formation occurred. Then a detectable leakage of CF from the liposomes was found. This loss increased when lower temperatures were reached; CF retention dropped to 30% in the range between −25 and −30°C. After thawing the particle diameter was found to be unchanged down to freezing temperatures of −18°C; at lower temperatures aggregation or fusion was observed (Fig. 1b).

Freezing conditions: freezing rate and temperature

The influence of freezing rate and temperature on the integrity of CF containing liposomes was investigated in more detail. CF retention and particle diameter after a freezing/thawing cycle are presented in Table 1.

Under the experimental conditions a freezing rate of 7°C min^{-1} has to be preferred for temperatures down to −32°C (Table 1, Figs. 1 and 2). Lowering the temperature to −196°C induced an almost complete loss of CF from the liposomes.

Freezing conditions: freezing temperature and freezing time

Liposome dispersions were not stable once the selected freezing temperature was reached. Aggregation or fusion and CF leakage continued as the liposome dispersions were kept at the selected temperature. Optimum conditions for CF retention were found at −30°C. Fig. 2 shows the increased loss of CF on prolonged storage under freezing conditions.

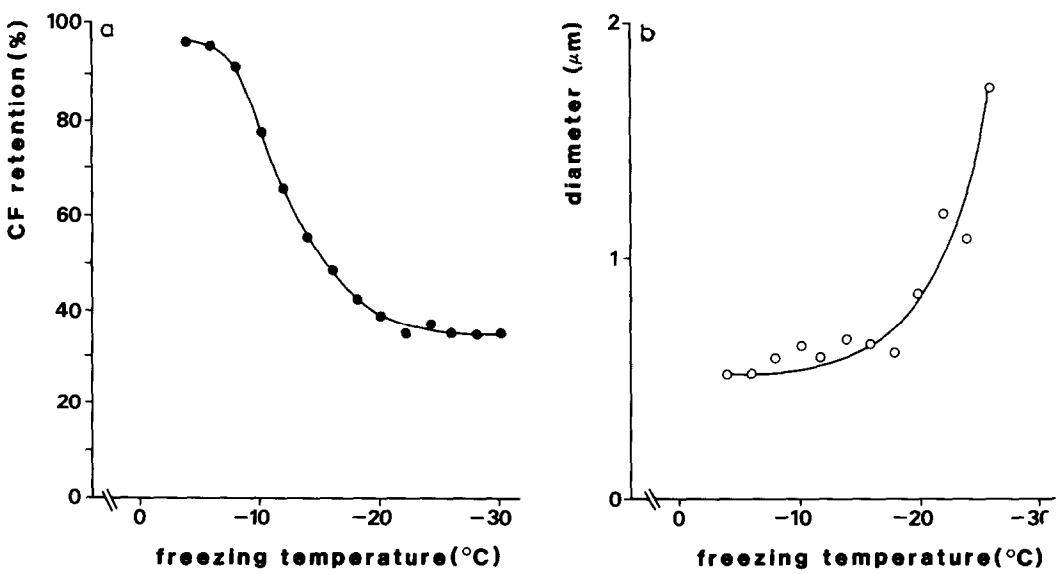

Fig. 1. The effect of freezing temperature on CF retention (a) and particle diameter (b) after a freezing/thawing cycle. Mean of two separate experiments. Freezing rate, 0.12°C/min. Time at the selected freezing temperature, 10 min.

TABLE 1

THE EFFECT OF FREEZING CONDITIONS (RATE AND TEMPERATURE) ON CF RETENTION AND PARTICLE DIAMETER AFTER A FREEZING/THAWING CYCLE

Mean of two separate experiments: reference, −4°C (supercooled for 10 min); CF retention, 97%; diameter, 0.51 μm.

Freezing temperature (°C)	Freezing rate (°C min^{-1})	CF retention (%)	Diameter (μm)
−20 [a]	7	87	0.49
−20 [a]	0.12	39	0.83
−32 [a]	7	85	0.56
−32 [a]	0.12	36	> 3
−32 [b]	7	83	1.7
−196	[c]	17	0.68
−196	[d]	6	> 3

[a] Time at selected temperature: 10–15 min.

[b] Time at selected temperature: 60 min.

[c] No exact freezing rate could be established. Glass vials were immersed into a boiling liquid nitrogen containing vessel; time at the selected temperature: 2 min.

[d] Vials were first cooled down to −32°C (rate 7°C min^{-1}) and subsequently cooled down to −196°C (cf. c).

Resistance against osmotic stress

If ice crystal formation occurs in the external phase of a liposome dispersion, then the salt concentration in the external phase will increase. In the literature there is substantial evidence that ice formation in the internal phase does not parallel external ice formation. As a result, at least temporarily an osmotic gradient over the bilayer structure will be present. To gain insight into the resistance of the liposomes against osmotic stress the osmotic pressure in the external phase of the liposome dispersions was increased 10- and 20-fold by mixing liposome dispersions with hyperosmotic aqueous media. CF retention was monitored over a period of 30 min at 22°C. The results and experimental conditions are presented in Fig. 3. A considerable loss of CF was observed as a result of the osmotic shock. Hardly any difference between 10- and 20-fold increase of the salt concentration could be detected.

Cryoprotectants

The influence of a number of potentially cryoprotective agents on liposome integrity after a freezing/thawing cycle was studied. CF retention data was presented mostly at two cryoprotectant concentrations. From Table 2 it can be derived that as a rule the addition of the cryoprotectants under investigation hardly influenced CF retention after 20 min. Only for methylcellulose (1.25 and 2.5%) and citric acid (5%) the retention on

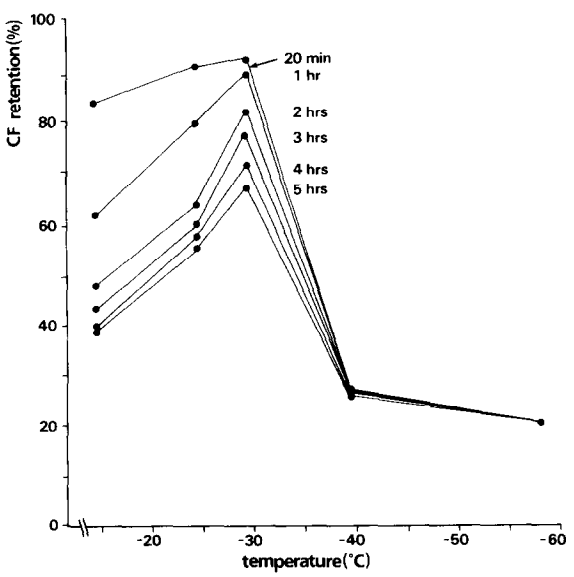

Fig. 2. The influence of freezing temperature and freezing time on the CF retention (%). Freezing rate, 7°C/min.

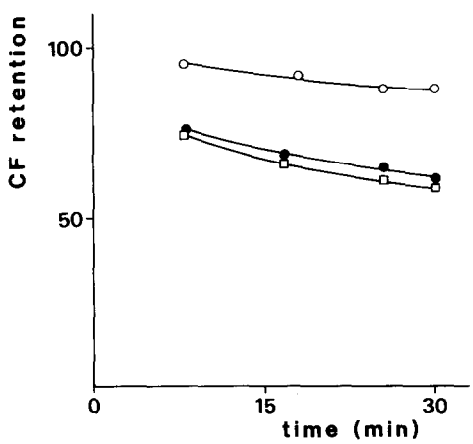

Fig. 3. CF retention (%) as a function of time in liposomes exposed to a hyperosmotic external phase. ○, iso-osmotic, 0.1 mol/l sodium chloride, 0.01 mol/l Tris, pH 7.4; ●, 1 mol/l sodium chloride, 0.1 mol/l Tris, pH 7.4; □, 2 mol/l sodium chloride, 0.2 mol/l Tris, pH 7.4. Temperature 22°C, results of duplicate experiments.

TABLE 2

THE INFLUENCE OF CRYOPROTECTANTS ON CF RETENTION (%) AND PARTICLE DIAMETER (μm) AFTER A FREEZING/THAWING CYCLE

Freezing rate, 7°C min^{-1}; freezing temperature, −25°C; n.d., not determined.

Cryoprotectant	Concentration [a]	CF retention	Freezing time	Particle diameter	Freezing time
		20 min	3 h	20 min	3 h
Glucose	2.5	90	54	0.63	0.50
	5	90	53	0.59	0.52
Lactose	2.5	85	57	0.53	0.52
	5	91	51	0.54	0.57
Saccharose	2.5	86	59	0.58	0.54
	5	90	49	0.51	0.50
Glycerol	2.5	89	47	0.57	0.53
	5	89	57	0.61	0.54
Mannitol	2.5	90	24	0.58	0.80
	5	73	n.d.	0.65	n.d.
PEG 300	2.5	88	47	0.63	0.53
	5	82	n.d.	0.61	n.d.
PVP	2.5	76	48	0.55	0.50
	5	89	41	0.61	0.48
Methylcellulose	1.25	65	n.d.	0.55	n.d.
	2.5	0	n.d.	0.87	n.d.
Ascorbic acid	2.5	n.d.	34	n.d.	0.48
	5	n.d.	27	n.d.	0.57
Citric acid	2.5	82	n.d.	0.61	n.d.
	5	68	n.d.	0.70	n.d.
Mannitol/glycerol	10/10	95	n.d.	0.54	n.d.
[b]	–	91	60	0.54	1.3
[c]	–	97	97	0.50	0.47

[a] %, m/v, except for glycerol v/v.

[b] No cryoprotectant added.

[c] Reference dispersion, stored under refrigerator conditions.

thawing dropped below 70%. After 3 h of freezing at −25°C CF retention in the thawed dispersions decreased to 40–60%; only for mannitol (2.5%) and ascorbic acid (2.5 and 5%) CF retention did not reach 40% anymore. After a freezing/thawing cycle the physical stability was preserved with all cryoprotectants except with methylcellulose (2.5%, 20 min) and mannitol (2.5%, 3 h). The combination of glycerol 10%/mannitol 10% was chosen in anticipation of the actual freeze drying process as on freeze drying a solid, porous structure was formed. For temperatures below −30°C the addition of a 10% (v/v) glycerol, 10% (m/v) mannitol mixture reduced CF leakage from the liposomes considerably compared to the situation without the cryoprotectants (Fig. 4).

Eutectic temperatures

As it was hypothesized that the eutectic temperature played a critical role in the preservation of liposome integrity, the eutectic temperatures were established by determining the conductivity of the various aqueous media as a function of the temperature. Figs. 5 and 6 show two examples of the conductivity profiles that were obtained. The eutectic temperatures were read from the intersection of the tangents to the curve in the low conductivity region as indicated in Figs. 5 and 6.

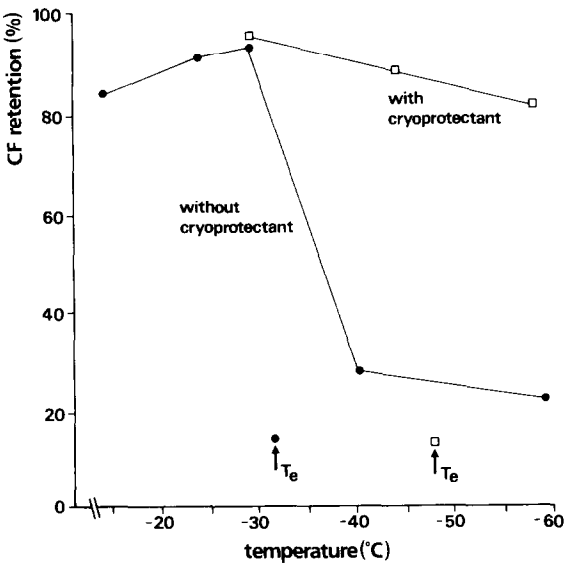

Fig. 4. The influence of freezing temperature on CF retention (%) after a freezing/thawing cycle with (□) and without (●) a cryoprotectant mixture (10% glycerol, 10% mannitol). The positions of T_e (eutectic temperature) are indicated. Freezing time, 20 min; freezing rate 7°C min^{-1}.

These curves were recorded for complete liposome dispersions. Internal and external phases were also recorded separately. An overview of the eutectic temperatures of a number of aqueous solutions and dispersions is presented in Table 3.

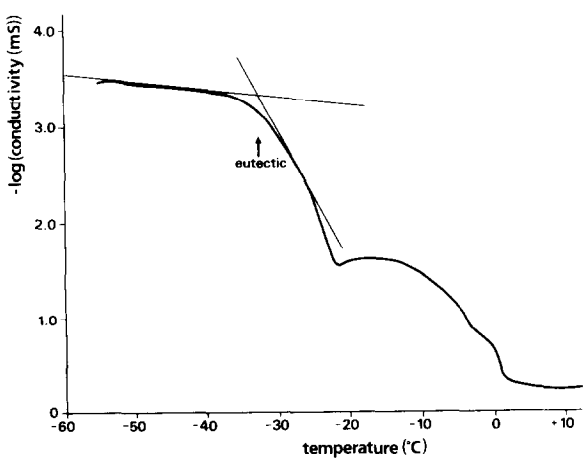

Fig. 5. The conductivity (mS) of the liposome dispersion as a function of temperature in a solution of 100 mmol/l sodium chloride and 10 mmol/l Tris, pH 7.4.

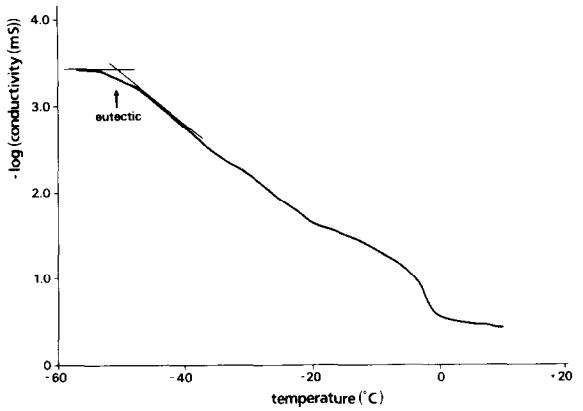

Fig. 6. The conductivity (mS) of the liposome dispersion as a function of temperature in an aqueous solution containing 10% (v/v) glycerol, 10% (m/v) mannitol, 100 mmol/l sodium chloride and 10 mmol/l Tris, pH 7.4.

TABLE 3

EUTECTIC TEMPERATURES (T_e) CALCULATED FROM CONDUCTIVITY MEASUREMENTS

Liposomes: PC-H/DCP (10/1), MLV; pH 7.4

	T_e (°C)
Liposome dispersion (100 mM NaCl, 10 mM Tris)	−33
Internal phase liposome (50 mM CF, 10 mM Tris)	−37
External phase (100 mM NaCl, 10 mM Tris)	−29
Liposome dispersion (100 mM NaCl, 10 mM Tris 10% glycerol–10% mannitol)	−48
Liposome dispersion (100 mM NaCl, 10 mM Tris 10% glycerol–10% lactose)	−48

Discussion

No cryoprotectants

For the selected low freezing rate (0.12°C/min) the liposomes tended to lose CF as soon as ice crystal formation occurred (between −6 and −8°C). Particle aggregation or fusion was only observed below −20°C (Fig. 1a, b). CF leakage therefore started while the particles had not irreversibly aggregated or fused. A similar phenomenon was described by Strauss (1984). A faster freezing rate (7°C/min) in combination with a short freezing time resulted in CF retention levels over 85% (Table 1). This high retention suggested

that under these conditions thawing is not the critical step in the freezing/thawing cycle. An increased osmotic stress over the bilayer due to ice formation in the external or internal phase might be responsible for the damage to the bilayer structure as indicated by the high loss of CF observed upon thawing when the low freezing rate was used or when the system was frozen at 7°C/min and kept in the frozen state for a longer period of time. As the initiation of ice nuclei is a random process the formation of ice crystals in the large external phase precedes the formation of ice crystals in the small internal volumes of the lipo-somes (Farrant, 1977) when the system is cooled down to temperatures in the range between −10 and −30°C. This ice crystallization process results in an increase of the salt concentration in the external phase. Therefore, an osmotic stress will exist as soon as freezing in the external phase occurs. When the liposomes were exposed to hyperosmotic conditions in the external phase at room temperature a significant increase in rate of CF leakage compared to the iso-osmotic situation was observed (Fig. 3). Morris and McGrath (1981) studied the effect of a hypertonic external phase on the release of glucose from liposomes and found results similar to ours. At these subzero temperatures the resistance against osmotic pressure differences over the bilayer is expected to be larger than at room temperature. But, the possibility that osmotic stress was responsible for the drop in the CF retention on thawing cannot be excluded.

In the literature the occurrence of internal ice in cells and liposomes has been reported (Siminovitch and Chapman, 1971; Mazur, 1977; Morris and McGrath, 1981; McGrath, 1984). It is difficult to predict under what conditions of freezing (rate, temperature or time) internal ice formation will occur in the temperature range between −10 and −30°C. Critical factors are the water permeability of the bilayer and the potential of the vesicles to shrink to compensate for the increased salt concentration in the external phase as a result of ice crystal formation.

Upon fast cooling to −196°C over 80% of CF was released after thawing. Under these conditions internal ice formation will occur. For cells it was reported that recrystallization of internal ice on thawing was responsible for membrane damage (Mazur, 1970, 1977; Farrant, 1977).

From Fig. 2 it can be derived that: (1) CF retention on thawing dropped dramatically when the freezing temperature was lowered from −30 to −40°C; and (2) keeping the dispersion at the selected temperature increased CF loss: at −30°C CF retention was 93% after a freezing time of 20 min, 67% after 5 h and 25% after 22 h. On the basis of these observations and the determined eutectic temperatures of the systems (Table 3) it was hypothesized that above −30°C there is a combination of ice and a sodium chloride/Tris aqueous solution. The liposomes could find a place in the liquid phase. When the temperature was lowered from −30 to −40°C, the liposomes became more concentrated in the decreasing liquid state volume and finally they became immobilized in the completely solid matrix. This solidification process damaged the liposomes.

With cryoprotectants

Cryoprotectants have been shown to reduce damage to cells and liposomes after a freezing/thawing cycle (Mazur, 1970; Huggins, 1975; Williams and Harris, 1977; Strauss and Ingenito, 1980; Morris, 1982; Franks, 1983; Strauss, 1984; Machy, 1984; Machy and Leserman, 1984; Fahy et al., 1984; Crommelin and Van Bommel, 1984; Van Bommel and Crommelin, 1984). The cryoprotectants tested stabilized the liposome dispersions as far as aggregation or fusion was concerned at −25°C with a few exceptions (Table 2). It was noted that after 20 min of freezing at −25°C also the dispersion without cryoprotectant was resistant against aggregation or fusion. At this temperature CF efflux rates were not affected or even increased in the presence of cryoprotectants (Table 2). Below −25°C cryoprotectants such as a combination of 10% (v/v) glycerol, 10% (m/v) mannitol reduced the efflux rate as compared to the situation without cryoprotectants (Fig. 4). Glycerol might protect the liposomes by one or a combination of the following mechanisms.

(1) A direct interaction of glycerol with the bilayer structure might diminish the damaging effects of bilayer dehydration during ice for-

mation (Crowe et al., 1983).

(2) A reduction of the ice crystallization rate because of an increased viscosity (Michelmore and Franks, 1982). The observation that at −25°C no significant improvement in CF retention could be detected by the addition of cryoprotectants (Table 2) after 20 min and 3 h indicated that under these conditions the cryoprotective effect was not the result of a reduced ice crystallization rate.

(3) The decreased efflux can be ascribed to the lower eutectic temperature of the aqueous system with the cryoprotectant compared to the situation without the cryoprotectant (Table 3). Glycerol was added to the external phase. As this compound is known to pass through bilayer structures (Rendi, 1967), it was also expected to be present at the inside of the liposomes. It can thereby influence internal ice formation. Even at temperatures below the eutectic temperature of −48°C (Fig. 4) still over 80% of CF is inside the liposomes after a 20 min freezing time. It was therefore concluded that the eutectic temperature can not be the only critical physical parameter in the process of preservation of the liposome integrity.

In the present study it was found that freezing temperature, freezing rate, freezing time and the presence of cryoprotectants were critical parameters to preserve liposome integrity on freezing/thawing. No aggregation or fusion occurred and over 90% CF retention was observed in the presence of a number of cryoprotectants when the liposomes were frozen at −25°C (freezing rate 7°C/min) for 20 min. This information can be used to select freeze drying conditions for optimum liposome quality after rehydration by rationale instead of by "trial and error".

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