Stability of Liposomes on Storage: Freeze Dried, Frozen or as an Aqueous Dispersion

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Received: October 28, 1983; accepted: December 12, 1983.

Abstract: For various types of liposomes carboxyfluorescein (CF) latency and physical stability on storage were investigated. Three regimens were compared: storage at 4–6°C in an iso-osmotic aqueous buffer, freezing of the dispersions at −5 or −30°C, or freeze drying of the dispersions. Reverse phase evaporation vesicles (REV) were used with mean diameters between 0.2 and 0.3 μm. Liposomes consisted of egg phosphatidylcholine (PC) and phophatidylserine (PS) without or with cholesterol (chol) (9/1 and 10/1/4, respectively) or of distearoylphosphatidylcholine (DSPC) and dipalmitoylphosphatidylglycerol (DPPG) without or with chol (10/1/[5]). PC/PS liposome dispersions lost 25 % of the entrapped CF within 10 days. The leakage rate decreased with the inclusion of cholesterol in the bilayer. Both after a freezing/thawing cycle and after freeze-drying no acceptable CF latency could be obtained. The cryoprotectants that were tested failed to raise CF latency significantly. However, the physical integrity of the liposomes could be maintained by proper choice of the cryoprotectant. When stored at 4–6°C, DSPC/DPPG/(chol) dispersions were stable for at least 6 months. Upon freezing/thawing less than 10 % CF was lost. Freeze drying without cryoprotectants reduced CF latency dramatically on rehydration. The physical structure was maintained and maximum latencies of 70 % could be obtained with the use of lactose as a cryoprotectant.

A prerequisite for the successful introduction of liposomes in therapy is a guaranteed quality of the product. Size, charge and composition are critical parameters controling their behavior *in vivo*. Moreover, the encapsulated drug should not leak out of the liposomes in the time interval between preparation and actual administration. Particle size, chemical stability and leakage have proven to present major problems during long term storage (1). The final overall stability strongly depends on the composition of the aqueous medium and the bilayer, the bilayer-drug interactions and the storage conditions.

The objective of this study was to increase the stability of liposomes compared to their stability at 4–6°C in aqueous media by freeze drying or freezing. Both size and drug leakage from the bilayer were monitored.

As there is a tendency to choose negatively charged liposomes for *in vivo* experiments this study also focussed on negatively charged liposomes. The effect of cholesterol addition to the bilayer and the physical state of the bilayer (gel- or fluid-like) were variables under investigation. 5,6-Carboxyfluorescein (CF) was used as a model compound for drugs that do not interact with the bilayer (2). Reverse phase evaporation vesicles (REV) have a high efficiency of aqueous phase encapsulation relative to multilamellar vesicles (MLV) (3). This is an advantage in particular for non-interacting compounds like CF. Therefore REV were used for CF encapsulation.

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Materials and Methods

Phospatidylcholine (PC) from egg yolk type V-E, phosphatidylserine (PS), distearoylphosphatidylcholine (DSPC), dipalmitoylphosphatidylglycerol (DPPG) and cholesterol (chol) were supplied by Sigma Chemicals (St. Louis, MO.). 5,6-Carboxyfluorescein was obtained from Eastman Kodak Co. (Rochester, N.Y.). Lactose met the requirements of the Ph. Eur., polyvinylpyrrolidone (PVP) of the U.S.P. (Plasdone K29/32) and mannitol of the B.P. Albumin was supplied by Organon Technika (Oss, The Netherlands).

CF was purified according to the procedure described by Ralston et al. (4). The lyso-phospholipid content was examined as reported before (5). PC, DPPG and DSPC contained less than 2 % and PS less than 5 % of the lyso-product.

REV were prepared as described by Wilschut (6). For PC/PS/(chol) diethyl ether and DSPC/DPPG/(chol), isopropyl ether was used to emulsify the CF-solutions, which contained 30–60 mmol/l CF in 0.01 mol/l tris buffer, pH 7.4. The compositions of the bilayers were (on a molar basis): PC/PS 9:1; PC/PS/chol 10:1:4; DSPC/DPPG 10:1; DSPC/DPPG/chol 10:1:5. During the initial stage of evaporation of the organic phase in a rotary evaporator, the pressure was kept at approximately 50 kPa. In the final stage it was decreased to 10 kPa. The pressure was controled by nitrogen addition to the system. After formation of a homogeneous dispersion the system was kept under low pressure (<10 kPa) for at least 30 minutes to remove traces of the organic phase. During preparation the temperature was kept at 30°C for PC/PS/(chol) vesicles and at 55–60°C for DSPC/DPPG/(chol) vesicles. The initial phospholipid concentration was 30 μmol/ml in all dispersions. The dispersions were sequentially extruded through membrane filters (Uni-pore, Bio-Rad, Richmond, CA) with pore diameters of 0.6 and 0.2 μm under nitrogen pressures up to 0.8 MPa. DSPC/DPPG/(chol) vesicles were extruded at temperatures between 55 and 60 °C. Free drug was removed by gel filtration over Sephadex G-50 fine (Pharmacia Fine Chemicals, Uppsala) packed columns. Iso-osmotic conditions were maintained unless otherwise stated by controlling the sodium chloride concentration of the extravesicular phase. In some cases solutions with additives (e.g. lactose) were mixed with these liposome dispersions that contained sodium chloride/tris after gel filtration.

CF latency (% CF in the vesicles) was determined by measuring the fluorescence (Perkin Elmer fluorescence spectrophotometer 204, Hitachi Ltd., Tokyo) of a diluted liposome dispersion at 515 nm before and after Triton X-100 (BDH chemicals Ltd., Poole, U.K.) addition. The excitation wavelength was 490 nm.

Particle size was measured by dynamic light scattering (Nanosizer, Coulter Electronics Ltd., Luton, U.K.). This apparatus provides a mean diameter in combination with a

polydispersity index ranging from 0 (monodisperse) to 9 (extremely polydisperse).

Freezing at −196°C was done by immersing the vials containing one ml of liposome dispersion into fluid, boiling nitrogen. The vials were stored at −30°C for at least 24 hours before thawing (room temperature). In case of freezing at −5 or −30°C glass spheres were added to the dispersions to induce ice formation. Otherwise supercooled systems could last for hours. The frozen material (−5 °C and −30°C) was kept at this temperature for at least 24 hours before thawing. For lyophilization a Leybold Heraeus GT-2 or a Minifast 470 freeze drier was used. Primary and secondary drying lasted 24 hours. All aqueous dispersions and freeze-dried materials were stored at 4–6 °C, protected from light.

Results

Storage of Aqueous Dispersions

CF latency in vesicles consisting of PC/PS 9/1, PC/PS/chol 10/1/4, DSPC/DPPG 10/1 and DSPC/DPPG/chol 10/1/5 as a function of time is shown in Figure 1. For the PC/PS liposomes cholesterol inclusion reduced the CF release rate substantially. For the DSPC/DPPG/(chol) liposomes less than 10 % CF loss was found over a period of one month. In a sequential study it was shown that for the DSPC/DPPG/(chol) vesicles CF latency still exceeded 90 % at the end of the experimental period (6 months). Mean particle size and polydispersity index did not change for any of the dispersions over the observed time interval. Decreasing the osmotic pressure of the outer aqueous phase by a 1000 times dilution of the dispersion with 0.01 mol/l tris buffer, increased the leakage rate of CF from PC/PS vesicles dramatically: 50 % was released in 2 hours. The permeability of DSPC/DPPG/(chol) vesicles was not affected by reduction of the osmotic pressure (not shown).

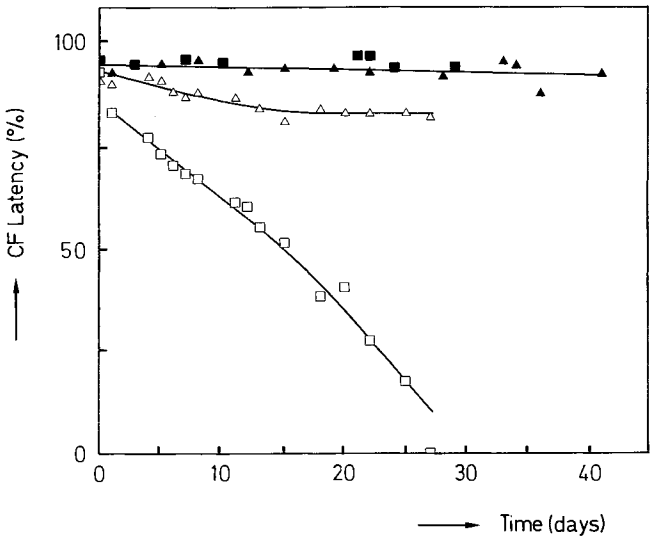

Fig. 1 CF latency in REV stored at 4–6 °C. Iso-osmotic sodium chloride/0.01 mol/l tris solutions, pH 7.4.

Composition	Mean particle diameter (μm)	(p.i.)[a]
□ PC/PS 9/1	0.21	(3)
△ PC/PS/chol 10/1/4	0.23	(3)
■ DSPC/DPPG 10/1	0.22	(2)
▲ DSPC/DPPG/chol 10/1/5	0.25	(3)

[a] p.i.= polydispersity index, ranging from 0 to 9

Freezing of CF Containing Liposomes

When a phospholipid bilayer structure changes from fluid- to gel-like upon cooling, an enhanced leakage has been observed in a narrow temperature range just around the transition temperature (7–9). The transition temperatures of PC and PS lie in the range from −15 to −7 °C and 6 to 8 °C, respectively. Because of the above mentioned reports it was expected that for PC/PS bilayers freezing at −5 °C, a temperature above the transition temperature of the main component, would result in a lower CF efflux after consecutive thawing than after freezing at −196 °C. For DSPC/DPPG vesicles (with transition temperatures above room temperature) no bilayer phase transition occurs on freezing, and therefore, no major bilayer rearrangement with concomitant excess of CF efflux is expected when the dispersions are cooled down to −196 °C instead of −5 °C. In addition to the freezing temperature the influence of lactose as cryoprotectant on liposome integrity was tested. Results are shown in Tables I and II.

Table I. CF latency (%) after a freezing/thawing cycle for various experimental conditions.
Experiments were carried out at least in duplicate; for n > 2 (n = number of experiments) the standard deviations are given. lac = 10 % m/v lactose solution added to iso-osmotic sodium chloride/0.01 mol/l tris buffer in 1:1 dilution.

Liposome composition	Freezing temperature (°C) −5	−5 +lac	−196	−196 +lac
PC/PS	42	46	18	19
PC/PS/chol	66	66	23	34
DSPC/DPPG	90±7	89±5	10±5	8±8
DSPC/DPPG/chol	94	88	51	49

Table II. Mean diameter (μm) and polydispersity indices (ranging from 0–9, given in parentheses) after a freezing/thawing cycle for various experimental conditions (see Table I).
lac = 10 % m/v lactose solution added to the iso-osmotic sodium chloride/0.01 mol/l tris buffer in 1:1 dilution.
ref = mean diameter of REV dispersions without freezing/thawing. Duplicate experiments.

Liposome composition	Freezing temperature (°C) −5	−5 +lac	−196	−196 +lac	ref
PC/PS	0.16(3)	0.17(2)	0.20(3)	0.17(2)	0.20(3)
PC/PS/chol	0.19(1)	0.20(1)	0.20(4)	0.18(3)	0.20(3)
DSPC/DPPG	0.21(2)	0.22 (1–2)	0.26(4)	0.25(4)	0.21(2)
DSPC/DPPG/chol	0.27(0–1)	0.29(2–3)	>3(9)	0.38(6)	0.26(3)

After freezing at −5 °C and thawing the PC/PS vesicles lost between 35 and 60 % of the encapsulated CF with or without cholesterol in the bilayer. Lactose did not decrease this loss. The CF efflux from the DSPC/DPPG liposomes was limited to approximately 10 %, independently of the inclusion of cholesterol or the addition of lactose. CF release after freezing at

−196 °C and thawing was more dramatic for all types of vesicles. Lactose did not significantly improve CF latency. But addition of cholesterol tended to decrease the efflux.

For PC/PS liposomes the mean particle size and size distribution was hardly affected by the freezing temperature, inclusion of cholesterol or addition of lactose. However, for DSPC/DPPG vesicles freezing at −196 °C destroyed the physical structure in particular for the cholesterol containing type without lactose.

Morris (10) reported liposome damage at low freezing rates because of osmotic dehydration. To minimize this phenomenon CF containing liposomes were dispersed in a hypotonic medium containing only 0.01 mol/l tris buffer (pH 7.4) after gel filtration. Here a low ionic strength and therefore increased zeta potentials (5) were combined with hypotonic aqueous extravesicular media. The results of freezing and thawing under these conditions are presented in Table III and IV. For convenience the CF latency and liposome dimensions for the corresponding iso-osmotic dispersions are included with the tables.

Table III. CF latency (%) after a freezing/thawing cycle. Effect of hypotonicity and ionic strength of the extravesicular aqueous medium.
a) Hypotonic; no sodium chloride in the aqueous extravesicular medium.
b) Iso-osmotic.
lac = 10 % m/v lactose solution added to the iso-osmotic or hypotonic aqueous medium in 1:1 dilution.
Measurements were carried out at least in duplicate; for n > 2 the standard deviations are given.

Composition		Freezing temperature (°C) −5	−5+lac	−196	−196+lac
PC/PS	a	48±1	51±5	27±7	23±6
	b	42	46	18	19
PC/PS/chol	a	48±11	51±3	25	23
	b	66	66	23	34
DSPC/DPPG	a	28±2	79±9	1	2
	b	90±7	89±5	10±5	8±8
DSPC/DPPG/chol	a	54	73	23	43
	b	94	88	51	49

Table IV. Mean diameter (μm) and polydispersity indices (ranging from 0–9, given in parentheses) after a freezing/thawing cycle. Effect of tonicity and ionic strength of the extravesicular aqueous medium.
a) Hypotonic; no sodium chloride in the aqueous extravesicular medium.
b) Iso-osmotic.
lac = 10 % m/v lactose solution added to the iso-osmotic or hypotonic aqueous medium in 1:1 dilution.
Measurements were carried out in duplicate.
ref = mean diameter of REV dispersions without freezing/thawing.

Composition		Freezing temperature (°C) −5	−5+lac	−196	−196+lac	ref
PC/PS	a	0.17(3)	0.17(3–4)	0.19(2)	0.18(3–4)	0.20(3)
	b	0.16(3)	0.17(2)	0.20(3)	0.17(2)	
PC/PS/chol	a	0.20(3–4)	0.19(2–3)	0.24(3)	0.17(2–3)	0.20(3)
	b	0.19(1)	0.20(1)	0.20(4)	0.18(3)	
DSPC/DPPG	a	0.23(2–3)	0.23(2–3)	0.21(5)	0.23(3)	0.21(2)
	b	0.21(2)	0.22(1–2)	0.26(4)	0.25(4)	
DSPC/DPPG/ chol	a	0.28(2)	0.29(1)	0.23(2)	0.27(1)	0.26(3)
	b	0.27(0–1)	0.29(2–3)	>3(9)	0.38(6)	

Addition of 10% m/v lactose to the hypotonic medium (1:1 dilution) always induced a considerable extravesicular osmotic pressure. But a low ionic strength (0.01 mol/l tris) was maintained, resulting in high zeta potentials. With PC/PS vesicles for both freezing temperatures (−5 and −196 °C) neither particle size nor CF latency was dramatically affected by the low ionic strength situation with or without lactose. However, with DSPC/DPPG vesicles in a hypotonic/low ionic strength aqueous medium an increase in CF efflux was found in comparison to an iso-osmotic/high ionic strength or low ionic strength (+ 5 % m/v lactose) situation. Tonicity appeared to be more critical than ionic strength to achieve a high CF latency for these vesicles. There is a tendency favoring the iso-osmotic/high ionic strength condition over the lactose containing aqueous medium with a low ionic strength for minimum leakage. It is remarkable that cooling of the DSPC/DPPG/chol vesicles to −196 °C in an hypotonic medium with low ionic strength stabilized the physical structure of the liposomes (mean diameter 0.23 μm) contrary to iso-osmotic conditions (> 3 μm), with a concomitantly reduced CF latency: 23 % vs 51 %, respectively. Under iso-osmotic conditions large aggregates of intact liposomes might be formed, whereas hypotonicity ensured the physical stability of the vesicles, but not of the barrier function of their bilayer. To discriminate between the osmotic contributions of lactose or specific lactose-bilayer interactions and its inhibitory effects on crystallization, a substance with crystallization inhibitory properties, but with negligible osmotic effect (PVP) was added to iso-osmotic DSPC/DPPG/(chol) dispersions. As can be seen in Tables V and VI, PVP in general did not act like lactose, and therefore, it is suggested that either osmotic effects or specific lactose-bilayer interactions control stability on freezing. The exclusiveness of lactose for size stabilization was not further investigated.

Table V. CF latency (%) after freezing/thawing cycle. Comparison between cryoprotective effect of lactose and PVP.
Measurements were carried out at least in duplicate. For n>2 standard deviations are given.
lac = 10 % m/v lactose solution added to an iso-osmotic aqueous medium in 1:1 dilution.
PVP = 10 % m/v PVP added to iso-osmotic medium in 1:1 dilution.

Composition	Freezing temperature (°C) −5	−5+lac	−5+PVP
DSPC/DPPG	90±7	89±5	40
DSPC/DPPG/chol	94	88	4

Table VI. Mean diameter (μm) and polydispersity indices (ranging from 0–9, given in parentheses) after a freezing/thawing cycle. Comparison of cryoprotective effects of lactose and PVP.
Measurements were carried out in duplicate.
lac = 10 % m/v lactose solution added to an iso-osmotic aqueous solution in 1:1 dilution.
PVP = 10 % m/v PVP solution added to an iso-osmotic aqueous solution in 1:1 dilution.
ref = mean diameter of REV dispersions without freezing/thawing.

Composition	Freezing temperature (°C) −5	−5+lac	−5+PVP	ref
DSPC/DPPG	0.21(2)	0.22(1–2)	0.33(3–5)	0.21(2)
DSPC/DPPG/chol	0.27(0–1)	0.29(2–3)	0.25(4)	0.26(3)

Freeze Drying of Carboxyfluorescein (CF) Containing Liposomes

The integrity of the liposomes after reconstitution of the freeze dried product was generally poor as far as CF latency was concerned (Tables VII and IX). The only exception were DSPC/DPPG vesicles, frozen at −15 °C, with lactose added to the iso-osmotic aqueous phase, with a latency of 62 %. Cholesterol destabilized the bilayer under these experimental conditions.

A cryoprotectant like lactose tended to increase the integrity of the liposomal physical structure (Table VIII). Without lactose no acceptable particle size distribution was obtained after rehydration. In addition to lactose other cryoprotectants were also tested. Tables IX and X show that the best results both in terms of bilayer permeability and physical integrity were obtained with a combination of DSPC/DPPG vesicles and lactose.

Table VII. CF latency (%) after rehydration of freeze-dried REV dispersions (Leybold Heraeus).
a) Freezing temperature −5 °C; iso-osmotic sodium chloride/0.01 mol/l tris buffer.
b) As a) but diluted (1:1) with 10 % m/v lactose solution.
c) Freezing temperature −196°C; iso-osmotic sodium chloride solution/0.01 mol/l tris buffer.
d) As c) but diluted (1:1) with 10 % m/v lactose solution.
Measurements were carried out at least in duplicate; for n > 2 the standard deviations are given.

Composition	Freezing temperature (°C) −5[a]	−5+lac[b]	−196[c]	−196+lac[d]
PC/PS	4±3	17±8	2±2	13±8
PC/PS/chol	4±3	5±11	3±5	17±13
DSPC/DPPG	0	66±10	1±1	4±4
DSPC/DPPG/chol	25±17	23	28±10	14

Table VIII. Mean diameter (μm) and polydispersity indices (ranging from 0–9, given in parentheses) after rehydration of freeze-dried REV dispersions (Leybold Heraeus).
For explanation of a), b), c) and d): see Table VII. Measurements were carried out at least in duplicate.
ref = mean diameter of REV dispersions without freeze drying.

Composition	Freezing temperature (°C) −5[a]	−5+lac[b]	−196[c]	−196 +lac[d]	ref
PC/PS	0.36(6–7)	0.17(3–6)	0.30(6–7)	0.15(2–3)	0.20(3)
PC/PS/chol	0.52(6–8)	0.29(4–7)	0.48(4–7)	0.19(3–4)	0.20(3)
DSPC/DPPG	>3(9)	0.23(2–3)	>3(9)	0.38(6)	0.21(2)
DSPC/DPPG/chol	>3(9)	0.29(3)	>3(9)	>3(9)	0.26(3)

Table IX. CF latency (%) after rehydration of freeze dried REV dispersions. Comparison between the effects of different additives (Minifast 470). Freezing temperature −15°C. Plate temperature in secondary drying phase +20°C. Sublimation phase lasted approximately 5 hours.
lac = 10 % m/v lactose solution added to an iso-osmotic aqueous medium in 1:1 dilution.
mann = 5 % m/v mannitol solution added to an iso-osmotic aqueous medium in 1:1 dilution.
alb = 0.1 % m/v albumin solution (in iso-osmotic aqueous medium) added to an iso-osmotic aqueous medium in 1:1 dilution.
lac/alb = solution containing 5 % m/v lactose and 0.05 % m/v albumin (in iso-osmotic aqueous medium) added to an iso-osmotic aqueous medium in 1:1 dilution.
mann/alb = solution containing 2.5 % m/v mannitol and 0.05 % m/v albumin (in iso-osmotic aqueous medium) added to an iso-osmotic aqueous medium in 1:1 dilution.
Measurements were carried out at least in duplicate; for n > 2 standard deviations are given.

Composition	Additive lac	mann	alb	lac/alb	mann/alb
DSPC/DPPG	62±6	5	0	43±25	1
DSPC/DPPG/chol	21±8	0	0	18±5	0

Discussion

The objective of this study was to explore ways to optimize liposome stability during long term storage. Freezing and freeze drying of the dispersions were tested as alternative techniques for storage of liposomes in aqueous media at 4–6 °C. Liposomes consisting of PC and PS with or without cholesterol have proven to be relatively successful in animal studies concerning the improvement of the therapeutic index of cytostatics (11–14). In this study PC/PS liposomes were used as representatives of the "fluid" bilayer type. It was expected that the "fluid" type would be sensitive to marker loss on storage. Lower permeabilities were predicted for bilayers in the gel-phase like DSPC/DPPG (1).

As a rule the physical structure of the liposomes both after freezing/thawing and freeze drying/rehydration could be maintained best in the presence of lactose. Other protective agents that were tested were by far less effective.

With PC/PS vesicles no acceptable solution was found to prevent CF leakage during long term storage. Both freezing and freeze drying under the conditions used in this study resulted in at least 35 % CF loss. A reduced permeability was found with cholesterol containing vesicles stored at 4–6 °C. Ten per cent loss was observed after approximately 10 days. Without cholesterol the leakage rate was much higher. As predicted, DSPC/DPPG vesicles with or without cholesterol were more stable during storage in an aqueous medium at 4–6 °C. Freezing the vesicles at −5 °C under iso-osmotic condi-

Table X. Mean diameter (μm) and polydispersity indices (ranging from 0–9, given in parentheses) after rehydration of freeze-dried REV dispersions. Comparison between the effects of different additives.
For experimental conditions and explanation of lac, mann, alb, lac/alb and mann/alb: see Table IX.
ref= mean diameter of REV dispersions without freeze-drying.

Composition	Additive lac	mann	alb	lac/alb	mann/alb	ref
DSPC/DPPG	0.20(1–4)	1.6(9)	0.79(8–9)	0.19(2–3)	0.21(5–6)	0.21(2)
DSPC/DPPG/chol	0.21(0–3)	0.24(5–8)	2.0(4–7)	0.21(0–3)	0.17(3–4)	0.26(3)

tions in the extravesicular aqueous medium resulted in a CF loss of 10 %. Addition of lactose or cholesterol inclusion did not significantly affect either CF loss or the physical stability. Cooling to −196 °C induced a substantial CF loss. Recrystallization of the frozen aqueous phase during rewarming might cause this degradation (15).

Freeze drying tended to destroy the membrane barrier function in both fluid- and gel-like bilayers. The only exceptions were the DSPC/DPPG vesicles in a lactose containing solution. Here the CF loss was limited to about 35 % and the physical structure was maintained after reconstitution. It is not yet clear what specific properties of lactose cause this protective effect. Neither a mere osmotic effect (mannitol), nor crystallization inhibition in general (PVP) provide a satisfactory explanation.

In this study neither freezing nor freeze drying could improve CF latency for the PC/PS/(chol) vesicles sufficiently. PC/PS vesicles containing drugs that do not interact with the bilayer like CF must be freshly prepared prior to use. With cholesterol in the bilayer these vesicles can be stored at 4–6 °C for a longer period of time if 85–90 % drug latencies are acceptable.

Under certain experimental conditions freezing and consecutive thawing of DSPC/DPPG/(chol) liposomes caused a CF loss of approximately 10 % and no significant change in mean particle size and size distribution. However, as these liposomes have a very high CF latency and good physical stability in aqueous dispersions at 4–6 °C, there is no direct need for freezing. Only if the low temperature is required to reduce the degradation of bilayer components or encapsulated drug, or if conditions unfavorable for bacterial growth are requested, freezing might offer an alternative for storage of the aqueous dispersions of DSPC/DPPG/(chol) vesicles at 4–6 °C.

After rehydration of the REV similar mean diameters and polydispersity indices were obtained as before freeze-drying. If the main objective is the recovery of the initial size distribution of liposome dispersions on rehydration, then freeze drying is a potential alternative for conventional techniques of storage. It is expected that markers interacting with the bilayer, like doxorubicin, show higher latencies than CF.

Preliminary results of the PC/PS/chol liposomes of the multilamellar type with doxorubicin showed drug-latencies of as much as 70 % and an excellent preservation of the physical structure upon freezing and freeze-drying after the addition of cryoprotectants.

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