

## Research paper

## Long term stability of freeze-dried, lyoprotected doxorubicin liposomes

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**Abstract**

The aim of the present study was to determine which parameters influence the long term stability of freeze-dried doxorubicin (DXR) liposomes. The DXR content, DXR retention and average vesicle size of the rehydrated liposomes were examined as a function of storage temperature, lyoprotectant (sucrose, maltose, lactose and trehalose), residual water content and onset temperature of the glass transition ( $T_{g,onset}$ ) of the freeze-dried cake. No significant physical instability or chemical degradation was observed in cakes containing less than 1% residual water after storage for 6 months at temperatures up to 30°C. However, a 25–50% decrease in the DXR content after rehydration was observed in samples stored at 50°C, which was accompanied by leakage of the encapsulated drug from the liposomes. All disaccharides selected for this study followed a similar pattern in this respect. Over the period of storage, no increases in average vesicle size (initial size around 0.1  $\mu\text{m}$ ) over 0.02  $\mu\text{m}$  were observed upon rehydration of these cakes, except for DXR-liposome samples containing sucrose and stored at 50°C. These effects all occurred below the  $T_{g,onset}$  of the freeze-dried cakes. The residual water content clearly affected the stability of the freeze-dried liposomes. In contrast, sucrose cakes containing circa 3.5% residual water showed a size increase, DXR degradation and leakage of encapsulated DXR already after storage at 30°C. Thermal analysis of the dry cakes showed clear differences between the intraliposomal phase and the extraliposomal matrix. Stability of the encapsulated DXR was primarily dependent on the physical states of the solids inside the liposomes. In conclusion, freeze-drying of DXR-liposomes resulted in formulations that are stable at 30°C for 6 months. In addition, the results suggest that the stability of liposome encapsulated drugs is largely determined by the characteristics of the intraliposomal solid, and only to a limited extent by the  $T_{g,onset}$  of the extraliposomal matrix. © 1997 Elsevier Science B.V.

**Keywords:** Liposomes; Doxorubicin; Freeze-drying; Stability; Modulated differential scanning calorimetry (MDSC); Lyoprotection

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**1. Introduction**

Liposomes have been introduced on the market as drug carriers formulated both as an aqueous dispersion (Dox-SL<sup>®</sup>) [1] and a freeze-dried product (Ambisome<sup>™</sup>) [2]. Phospholipids, contain ester bonds that are subject to hydrolysis. Degradation kinetics depend

on, e.g. pH temperature, and the presence of water [3]. Stability problems for aqueous liposomal drug formulations can be anticipated especially in those cases where the pH-values for optimal stability of the drug and the phospholipids (pH 6.5) differ. This is the case for the cytostatic doxorubicin, which has an optimal stability around pH 4 [4].

Freeze-drying was proposed as a tool to improve the long term stability of liposomes in 1978 [5]. However, until now, most studies have focused on the protection of liposomes against damage caused by the freeze-drying process itself (lyoprotection), as reviewed elsewhere [6]. Preservation of the physical integrity during freeze-

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drying and rehydration can be achieved by the presence of lyoprotectants such as disaccharides in and outside the liposomes. These agents can prevent fusion, aggregation and leakage of encapsulated compounds by the formation of an amorphous glass [7,8] and/or interaction with the phospholipid headgroups (e.g. [8,9]). However, the long term stability of liposomes in the freeze-dried state has not yet been systematically investigated.

As reported for other amorphous products [10,11], the stability of the freeze-dried, lyoprotected liposomes may depend on the glass transition temperature ( $T_g$ ) of the sugars. Below  $T_g$ , the low molecular mobility is generally considered to inhibit chemical and physical degradation. However, storage at temperatures below  $T_g$  does not always guarantee sufficient stability in the glass, as was described recently for a pharmaceutical protein freeze-dried in sucrose [12,13]. The chemical degradation observed in that study was ascribed to the formation of the reducing monosaccharide glucose, which can bind to the protein via a Maillard reaction.

Recently, the role of  $T_g$  for the short term stability of air-dried liposomes was described [14]. It was found that exposure of the dry samples to temperatures around  $T_g$  resulted into leakage of the encapsulated marker carboxyfluorescein and an increased vesicle size in the rehydrated dispersion. Below  $T_g$  no such phenomena were observed. However, results leading to different conclusions were obtained in our group with freeze-dried liposomes. It was concluded that the bilayer transition temperature ( $T_m$ ) of the dried liposomes is a better indicator for the short term stability of such formulations than  $T_g$  [15].

Exact prediction of the shelf life stability of amorphous products on the basis of accelerated, short term stability studies at elevated temperatures is difficult, as reaction kinetics above and below  $T_g$  are different. Moreover, the long term influence of slow relaxation processes may remain unnoticed in the short experimental time frame [16]. Therefore, long term studies should be performed to provide information on the shelf life stability of freeze-dried liposomes.

A few long term stability studies on freeze-dried liposomes have appeared in the literature, but limited insight was gained into the influence of excipients,  $T_g$  or residual water content. Isele et al. [17] reported good stability of liposomes containing the hydrophobic drug monomeric zinc phthalocyanine freeze-dried in lactose (residual water content: 1.6%) when stored for 6 months at 8, 25 and 40°C. Chong-Kook and Jeong described acceptable preservation of immunogenicity and vesicle size of freeze-dried liposomes with trehalose after 12 months storage at 4°C. However, in a study by Vermuri and Rhodes leakage of the water soluble drug [18] orciprenaline sulphate from liposomes and a vesicle size increase was observed upon storage at 2–8°C (no

residual water content given). As the experimental conditions in the above mentioned studies varied widely (or are not disclosed), it is not possible to fully explain these discrepancies. However, it is clear that the physicochemical nature of the compound is an important feature. For instance, physical instability of the liposomes may only result into leakage of the entrapped compound when this agent does not fully interact with the liposomal bilayer.

The aim of this study was to investigate parameters influencing the physical (particle size, retention upon rehydration) and chemical (encapsulated drug) stability of freeze-dried, lyoprotected liposomes loaded with doxorubicin, and to test the results for consistency with existing hypotheses on lyoprotection.

Doxorubicin (DXR) was chosen as a model drug because of the clinical relevance of DXR liposomes [19] and to serve as an example of a drug with a poor stability at pH 6.5, the optimal pH for phospholipid dispersions, as described above. Although DXR is known to interact with the bilayer [20] a major part of the compound is present in the free form, allowing leakage of encapsulated DXR when an increase in the bilayer permeability occurs [21]. The optimal vesicle size (0.1  $\mu\text{m}$ ), bilayer composition (DPPC:DPPG:CHOL = 10:1:4, Section 2) and freezing protocol (in liquid nitrogen) were used to minimise leakage of DXR during the freeze-drying and rehydration process, to fully concentrate on the 'aging' process [22].

We selected both reducing (maltose, lactose) and non-reducing disaccharides (sucrose, trehalose) as lyoprotectants, and examined in a wide temperature range (–20 up to 50°C) the influence of the residual water content and  $T_g$  of the freeze-dried cakes on stability. For packaging of the freeze-dried product, glass vials and rubber caps were used which are also customary in an industrial setting. Changes in residual water content and  $T_g$  of the cakes by the possible release of water from the caps were monitored throughout this study. Modulated Differential Scanning Calorimetry (MDSC), a novel extension of DSC [23], was applied to determine  $T_g$  in the freeze-dried samples. This technique enables detection of changes in heat capacity (indicator for  $T_g$ ) in samples which exhibit other overlapping thermal events. MDSC was shown to be very useful in the characterisation of freeze-dried lipid/sugar mixtures [24].

## 2. Experimental

### 2.1. Materials

Dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylglycerol (DPPG) were gifts from

Lipoid GmbH (Ludwigshafen, Germany). Cholesterol (CHOL) was obtained from Sigma Chemical Company (St. Louis, MO, USA). Doxorubicin (DXR) was a gift from Pharmachemie B.V. (Haarlem, The Netherlands). All other chemicals were of analytical grade and were used without further purification. All aqueous solutions were prepared with water purified by reverse osmosis.

## 2.2. Preparation of liposomes

A solution of DPPC, DPPG and CHOL (molar ratio = 10:1:4) in a mixture of chloroform/methanol was dried in a round bottom flask with a rotary evaporator. Nitrogen was blown over the dried lipid film for about 0.5 h in order to remove traces of organic solvents. The lipid film was hydrated at 60°C with a buffer solution containing 5% carbohydrate (w/w) and 120 mM  $(\text{NH}_4)_2\text{SO}_4$ , resulting in a phospholipid concentration of circa 60 mM. The dispersions were subsequently extruded several times at elevated temperatures through polycarbonate membranes with pore sizes of 0.6, 0.1 and 0.05  $\mu\text{m}$  (Uni-pore, Bio-Rad, Richmond, CA) until an average vesicle size of approximately 0.1  $\mu\text{m}$  was obtained. Doxorubicin was loaded into the vesicles by the remote loading method [25] with some modifications. In our group, aggregation of DXR liposomes was observed during loading which depended on DXR/phospholipid (PL) ratio, lipid composition, vesicle size and temperature [26]. In this study a special protocol was followed to circumvent the aggregation induced by DXR which remained in the extraliposomal phase after the loading procedure. An  $(\text{NH}_4)_2\text{SO}_4$  – gradient was created by replacement of the extraliposomal medium by a solution containing 15% (w/v) carbohydrate and 10 mM Hepes (pH 7.4) using gel permeation chromatography over a column with Sephadex GF-50 fine (Pharmacia, Uppsala, Sweden). A solution containing circa 7 mg/ml DXR (up to a DXR/PL ratio of 50 g/mol) and 15% carbohydrate was added to the extraliposomal medium at 60°C. After 30 min of incubation the dispersion was cooled down in ice water under stirring. In order to remove the non-encapsulated drug, an excess of the cation exchange resin Dowex 50WX-4 (0.5 g/mg DXR) was added when the dispersion reached a temperature of 30°C [27]. Subsequently, the resin was removed from the dispersion by filtration. The dispersion was diluted with the extraliposomal buffer to a PL-concentration of circa 20 mM.

## 2.3. Freeze-drying/storage

Liposomes were freeze-dried in aliquots of 0.4 ml in 13.5 ml freeze-dry vials to two different residual water contents (3.5 and 0.2–0.5%). The rubber freeze-dry caps (type V 9172 DZ, FM 257/5 C, Helvoet Pharma, Alken, Belgium) were dried at 100°C for 1 day prior to

use. The vials were frozen in liquid nitrogen for 10 min and placed on the freeze-dryer plate at a temperature of  $-40^\circ\text{C}$ . The low water content cakes were freeze-dried in a Leybold GT4 pilot-production freeze-dryer. In the first step of the freeze-drying process, the plate temperature was maintained at  $-40^\circ\text{C}$  and the chamber pressure at 10–13 Pa for 30 h, followed by additional drying steps at plate temperatures of  $-30^\circ\text{C}$ ,  $-16^\circ\text{C}$  and  $+20^\circ\text{C}$ , each for 5 h at a pressure of circa 1 Pa. The condenser temperature ranged between  $-55$  and  $60^\circ\text{C}$ . At the end of the freeze-drying process the chamber was filled with nitrogen gas and the vials were immediately closed with rubber caps by hand. The high water content cakes were dried in a Virtis Genesis 25 LL freeze-dryer with the same freezing method, but with a modified drying protocol. The plate temperature was maintained at  $-40^\circ\text{C}$  for 1 h, at  $-30^\circ\text{C}$  for 34 h and at  $-18^\circ\text{C}$  for 4 h at a pressure of circa 12 Pa. The vacuum was released by allowing nitrogen gas into the chamber up to atmospheric pressure. The vials were closed simultaneously by a hydraulic closing device before opening the freeze-dryer and exposing the cakes to room temperature. The vials were sealed with aluminum caps and stored at the indicated temperatures until analysis. The amount of water removed from the samples during freeze-drying was determined by weighing each vial before and after freeze-drying. For rehydration, the corresponding amount of water was added under ambient conditions, immediately followed by vortexing for 2 s.

## 2.4. Residual water content

The residual water content was determined with the Karl-Fisher method using a Mitsubishi moisture meter model CA-05 (Tokyo, Japan). To minimise exposure of the hygroscopic cakes to the environment, the rubber caps remained on the vials throughout the sampling procedure. The pressure inside/outside the vials was equalised via a needle through the rubber caps before weighing the closed vials containing the cakes. Cakes were dissolved in 1 ml of Hydranal Coulomat A (Riedel de Haen, Seelze, Germany) which was injected through the rubber caps, allowing the release of overpressure through a second needle. The water content of 100  $\mu\text{l}$  aliquots of the solvent was measured in duplicate. The weight of each empty vial was determined before usage, as was the weight of each used cap after cleaning and drying. Thus, the weight percentage of water in the freeze-dried cakes could be calculated.

## 2.5. MDSC analysis

A detailed description of the application of MDSC for the analysis of freeze-dried cakes is given in [24]. In order to minimise attraction of water by the hygro-

scopic samples, sampling was performed in a dry nitrogen gas environment, and the exposure time of the cakes to these conditions was less than 2 min. Samples (2.5–5 mg) of the freeze-dried cakes were punched out and transferred into aluminum pans, which were closed tightly. All scans were recorded with a DSC 2920 (TA Instruments, 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. Scans were recorded under 'heating only' conditions with 1 modulation/°C using the following settings: average heating rate ( $q$ ) = 1°C/min, period ( $p$ ) = 60 s, temperature amplitude ( $T_a$ ) = 0.159°C. The Thermal Analyst software versus 8.6 was used for data evaluation. The total, reversing and non-reversing heat flow curves are plotted on the same y-scale, but were repositioned for clarity reasons. The onset temperature values of glass transitions or peaks were determined as the point of intersection between the baseline and the tangent at the shift in baseline or peak slope.

#### 2.6. Phosphate determination

Lipid phosphate was determined by the colorimetric method of Rouser et al. [28].

#### 2.7. Vesicle size determination

Samples of the dispersion obtained after extrusion were diluted for the size measurements with the extraliposomal medium, but using in all cases sucrose as a carbohydrate. The Z-average size and polydispersity were determined at 25°C by dynamic light scattering with a Malvern 4700 system, using the automeasure versus 3.2 software (Malvern, UK). The values for the refractive index and viscosity used in the calculation of the particle size of the light scattering data were 1.3401 and 0.9906 g/m.s, respectively, for liposomes diluted in 5% (w/v) sucrose and 120 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, or 1.3549 and 1.3786 g/m.s, respectively, for liposomes diluted in 15% (w/v) sucrose, 10 mM Hepes pH 7.4. Average size measurements of 100 nm standard particles (Polymer Laboratories, Shropshire, UK) deviated less than 5% from the size indicated by the manufacturer.

#### 2.8. Doxorubicin retention

For DXR retention analysis a previously described method (27) was modified as follows. From each vial, samples of 60 µl were pipetted on Poly-Prep Chromatography Columns (Biorad, Veenendaal, The Netherlands) filled with circa 1 ml of the cation exchange resin Dowex 50WX-4. The sample was eluted from the column by washing twice with 0.5 ml of a

solution containing 1.35% NaCl and 10 mM Hepes (pH 7.4), and collected in 11 ml polypropylene tubes (liposomal fraction). DXR standards and total DXR-liposome samples were pipetted in the same polypropylene tubes, and identical amounts of dispersion and eluent were added. To all samples, 1 ml 2 propanol and 5 ml of a mixture of 2 propanol and H<sub>2</sub>O (1/1) were added, followed by vortexing. The fluorescence was measured in a Perkin Elmer LS 50B fluorescence spectrophotometer equipped with a well plate reader (Perkin Elmer, UK) ( $\lambda_{\text{excitation}}$  = 493 nm and  $\lambda_{\text{emission}}$  = 585 nm, slit width for excitation and emission: 15/15, integration time: 1 s).

#### 2.9. HPLC analysis of doxorubicin

Aliquots of 30 µl liposome dispersion in 1 ml-ependorf cups were diluted and solubilised by the subsequent addition of 200 µl 30 mM citric acid (pH 4.0) and 200 µl 2 propanol. pH 4.0 was chosen to ensure good solubility of DXR. After vortexing, the samples were centrifuged for 5 min; a dark red pellet was obtained in case of some degraded samples. Attempts to solubilise this precipitate with different organic solvents were unsuccessful. A 40 µl sample of the supernatant was analysed with a HPLC system consisting of a type 400 solvent delivery system (Kratos, Ramsey, NJ, USA), a Waters sampler (Waters Associates, Milford, MA, USA) and a type 783A absorption detector (Applied Bio-Systems, Ramsey, NJ USA). Chromatograms were collected and analysed with a computerised data system (WOW, Thermo Separation Products, Riviera Beach, Florida, USA). The separation of doxorubicin from degradation products was carried out on a Supelcosil LC-18-DB column (25 cm × 4.6 mm, Supelco, Bellefonte, PA, USA) with a C18 guard column. The mobile phase was a mixture of a solution containing 0.01 M sodium dodecylsulphate and 0.02 M H<sub>3</sub>PO<sub>4</sub> with acetonitrile (57/43 (v/v)). The absorption wavelength was 254 nm and the flow rate was 1.5 ml/min.

### 3. Results

The characteristics of the freeze-dried liposomes at the beginning of the stability study before and after rehydration are given in Table 1.

The average vesicle size after preparation of the liposomes ranged between 0.09–0.11 µm for the different batches. It tended to increase between 0.01–0.03 µm after exposure to a freeze-drying and rehydration cycle. No differences in lyoprotective capacity were found between the four disaccharides selected for this study. In all cases, the retention of doxorubicin in the liposomes after rehydration was > circa 90%.

Table 1

Characteristics of freeze-dried, lyoprotected DXR liposomes in the dried and rehydrated state

Lyoprotectant	Dried state		Rehydrated state			
	%H <sub>2</sub> O	T <sub>g</sub> °C	Mean size μm, {pd}	% Ret.	[DXR] mg/ml	[PL] mM
Sucrose	3.5 ± 0.2	38 ± 4	0.108 ± 0.002	91 ± 5	0.51 ± 0.02	19.3 ± 0.2
	[3.7–3.2]	[23–41]	{0.15 ± 0.01}	[83–103]		
	(10)	(14)	(9)	(10)	(9)	(10)
	0.4 ± 0.2	69 ± 4	0.107 ± 0.009	93 ± 1	0.51 ± 0.02	19.3 ± 0.2
Maltose	[0.2–0.7]	[62–74]	{0.2 ± 0.05}	[83–103]		
	(10)	(19)	(10)	(10)	(9)	(10)
	0.23 ± 0.03	101 ± 7	0.119 ± 0.002	91 ± 2	0.41 ± 0.01	18.0 ± 0.5
	[0.20–0.29]	[89–113]	{0.11 ± 0.02}	[89–94]		
Trehalose	(10)	(15)	(10)	(10)	(10)	(10)
	0.14 ± 0.05	114 ± 6	0.123 ± 0.001	89 ± 3	0.33 ± 0.01	18.9 ± 0.4
	[0.07–0.22]	[95–119]	{0.18 ± 0.02}	[84–93]		
	(10)	(17)	(10)	(10)	(10)	(10)
Lactose	0.29 ± 0.05	103 ± 8	0.110 ± 0.006	88 ± 4	0.39 ± 0.01	19.9 ± 0.1
	[0.2–0.4]	[93–114]	{0.19 ± 0.04}	[80–97]		
	(10)	(16)	(10)	(10)	(10)	(10)

All values are presented as mean ± S.D. (variability within one batch for sucrose, maltose and trehalose cakes), or pooled average and S.D. of two independent batches (lactose), [range of observed values], and (no. of determinations from different vials). The lipid composition was DPPC:DPPG:CHOL = 10:1:4.

Further experimental details are given under Section 2. %H<sub>2</sub>O, residual water content; T<sub>g</sub>, glass transition temperature determined from the reversing heat flow from the first MDSC scan, mean size with the polydispersity index {pd}, %Ret.: %doxorubicin retention, [DXR]: doxorubicin concentration, [PL]: phospholipid concentration.

### 3.1. Thermal analysis of frozen and freeze-dried dxr-liposomes

MDSC was used to study the liposome dispersions, as well as the intraliposomal medium, after freezing and freeze-drying. A critical parameter for the freeze-drying process which can be obtained from the thermograms is the softening temperature (T<sub>s</sub>) (see ref. [24,29]). Only when the product temperature is maintained below this value will collapse be prevented while removing the ice by sublimation. Drying at temperatures above T<sub>s</sub> results in softening and collapse of the material, and a reduced surface area. On the other hand, increasing the product temperature during primary drying is advantageous, because it shortens the required freeze-drying time. Insight into the T<sub>s</sub>-values of the formulations contributes to rational optimisation of the liposome formulation. Examples of MDSC curves of frozen DXR liposome dispersions with different lyoprotectants are displayed in Fig. 1, and the full array of key parameters is given in Table 2.

The extraliposomal media with and without liposomes were compared, and it was found that the presence of DXR-liposomes (4.4 g carbohydrate/g PL) did not significantly influence the thermal properties of these frozen solutions (data not shown). However, the freezing protocol clearly affected values found for T<sub>g</sub> and the onset temperature of the devitrification (T<sub>dev.</sub>). Fast freezing of 25 μl samples of a solution containing 15% sucrose in boiling nitrogen resulted in T<sub>g</sub> and

T<sub>dev,onset</sub> values 3–6°C lower than after freezing in the MDSC furnace with circa 17°C/min. Values for T<sub>s</sub> were only slightly (1.1 ± 0.2°C) decreased (*p* < 0.05). For practical reasons, we froze samples on the MDSC furnace in order to compare T<sub>s</sub> values of the DXR solutions with the different lyoprotectants. From Table 2 it is clear that the liposome dispersions containing maltose, lactose and trehalose have a similar T<sub>s</sub> (circa –30°C), whereas sucrose had a T<sub>s</sub> of –33.6 ± 0.2°C. This difference is small and in the present study the same freeze-drying protocol was applied for all dispersions when a low water content cake was requested. However, the solution containing sucrose, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and DXR (without phospholipids) at approximately the same concentrations as in the intraliposomal medium, had a much lower T<sub>s</sub> of –45°C, which is below the primary drying temperature in this study. It is unknown whether this has consequences for the drying process of the liposome contents. Effects of collapse of the liposome contents on actual freeze-drying rates may be limited because of the small particle size of the vesicles, which guarantees a high surface/mass ratio of the liposome contents. Apparently, the liposome stability was not affected, when considering the high retention of DXR in the liposomes after freeze-drying and rehydration (circa 90%).

The heat flow curves (scan 1) of the dry cakes are displayed in Fig. 2A and B. The glass transition was sometimes difficult to identify in the total heat flow curve, which is identical to the heat flow as obtained

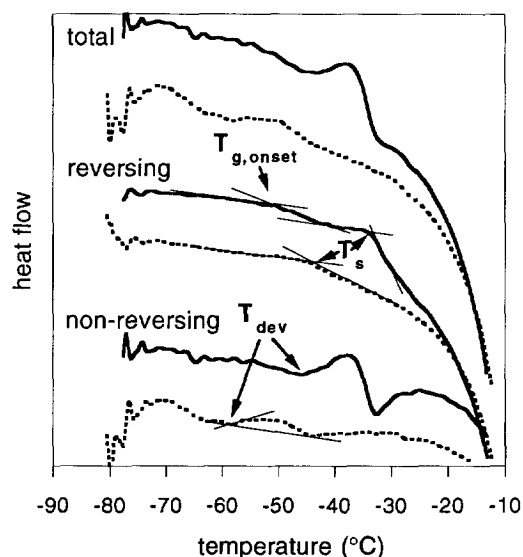


Fig. 1. MDSC profiles of (1) a DXR liposome dispersion (—) and (2) the intraliposomal medium of these liposomes (---), after freezing. Sample composition: (1) 15% sucrose (w/v), 10 mM Hepes pH 7.4 (extraliposomal), 20 mM PL, 0.5 mg/ml DXR, circa 8 mM  $(\text{NH}_4)_2\text{SO}_4$  (total dispersion), (2) 120 mM  $(\text{NH}_4)_2\text{SO}_4$ , 8 mg/ml DXR, 5% sucrose (w/v). Samples (25  $\mu\text{l}$ ) were frozen in sealed aluminum pans with circa 17°C/min, and kept at  $-80^\circ\text{C}$  for 5 min. Measurement settings:  $q = 1^\circ\text{C}/\text{min}$ ,  $T_a = 0.159^\circ\text{C}$ ,  $p = 60$  s.  $T_{g,\text{onset}}$ : onset temperature glass transition,  $T_s$ : softening temperature,  $T_{\text{dev}}$ : onset temperature of devitrification.

with standard DSC [23,24]. The total heat flow in scan 1 of freeze-dried samples often showed a strong endothermic signal; its origin is unclear to us. Second scans exhibited a less disturbed baseline, which suggests that relaxation processes are involved (not shown) [24]. In the reversing heat flow the glass transitions were clearly visible. In Fig. 2A the difference in  $T_g$  between sucrose/DXR liposome cakes with a low and high water content is visualised. In the same Fig. the MDSC curves of the freeze-dried intraliposomal medium containing DXR,  $(\text{NH}_4)_2\text{SO}_4$  and sucrose are displayed. This sample was collapsed after freeze-drying, and had a residual water content of circa 3%. The sample was

further desiccated under vacuum over dry  $\text{P}_2\text{O}_5$ , which lowered the water content to 0.6%. The  $T_{g,\text{onset}}$  values for the freeze-dried 'intraliposomal' sample and total dispersion were similar (see Table 2) but the total heat flow profiles were different. The DXR/ $(\text{NH}_4)_2\text{SO}_4$ /sucrose sample showed an endothermic process starting at  $59^\circ\text{C}$  which is  $12 \pm 2^\circ\text{C}$  below  $T_{g,\text{onset}}$  in the reversing heat flow (paired differences). For the total DXR liposome/sucrose cake, the onset temperature of the relaxation peak associated with the glass transition (in the total heat flow) was only  $3 \pm 1^\circ\text{C}$  lower than  $T_{g,\text{onset}}$  in the reversing heat flow. This comparison indicates that the endotherm in the 'intraliposomal' solid phase was different from the relaxation peak observed in sugar glasses without DXR and  $(\text{NH}_4)_2\text{SO}_4$ . The possible consequences of this observation will be discussed below.

### 3.2. Characteristics of the freeze-dried cakes upon storage

The stability of the freeze-dried liposomes was studied as a function of storage temperature, lyoprotectant and residual water content. In pilot studies, changes in water content of the cakes were observed due to the release and uptake of water by the rubber freeze-dry caps, which depended on the initial water content of the cakes, the storage temperature and the type of rubber caps. On the basis of these pilot studies, conditions were chosen where water exchange was minimised. In addition, both the residual water content and  $T_g$  were monitored throughout the study.

The variation in water content during storage is presented in Fig. 3 for sucrose cakes, as an example. For the cakes with a high water content a decrease was observed from  $3.5 \pm 0.2\%$  after preparation to  $3.0 \pm 0.3\%$  and  $2.3 \pm 0.2\%$  in cakes after storage for 6 months at  $-20$  and  $40^\circ\text{C}$ , respectively. For cakes with a low water content, no or (in absolute terms) a small increase in residual water was observed (from  $0.4 \pm 0.2\%$ – $0.4 \pm 0.2$  and  $0.8 \pm 0.1$  after 6 months at  $-20$  or

Table 2

Comparison of thermal parameters in freeze-concentrates of (1) the intraliposomal medium of sucrose containing DXR liposomes and of (2) DXR liposomes dispersions containing different lyoprotectants

Frozen sample	Reversing heat flow		Non-reversing heat flow
	$T_s$ ( $^\circ\text{C}$ )	$T_{g,\text{onset}}$ ( $^\circ\text{C}$ )	$T_{\text{dev}}$ ( $^\circ\text{C}$ )
(1) 'Intraliposomal medium' (sucrose)	$-45 \pm 1$	Not observed	$-55$
(2) Sucrose	$-33.6 \pm 0.2$	$-52 \pm 1$	$-46 \pm 2$
(2) Maltose	$-30.7 \pm 0.3$	$-49 \pm 1$	$-42 \pm 1$
(2) Trehalose	$-30.6 \pm 0.2$	$-48 \pm 1$	$-42 \pm 1$
(2) Lactose	$-29.7 \pm 0.2$	$-49 \pm 1$	$-42 \pm 1$

Softening temperature, ( $T_s$ ); onset glass transition, ( $T_{g,\text{onset}}$ ); onset devitrification temperature, ( $T_{\text{dev}}$ ) in MDSC total and reversing heat flow curves. Composition mimicking the intraliposomal medium before freeze-drying: 120 mM M  $(\text{NH}_4)_2\text{SO}_4$ , 8 mg/ml DXR, 5% sucrose (w/v).

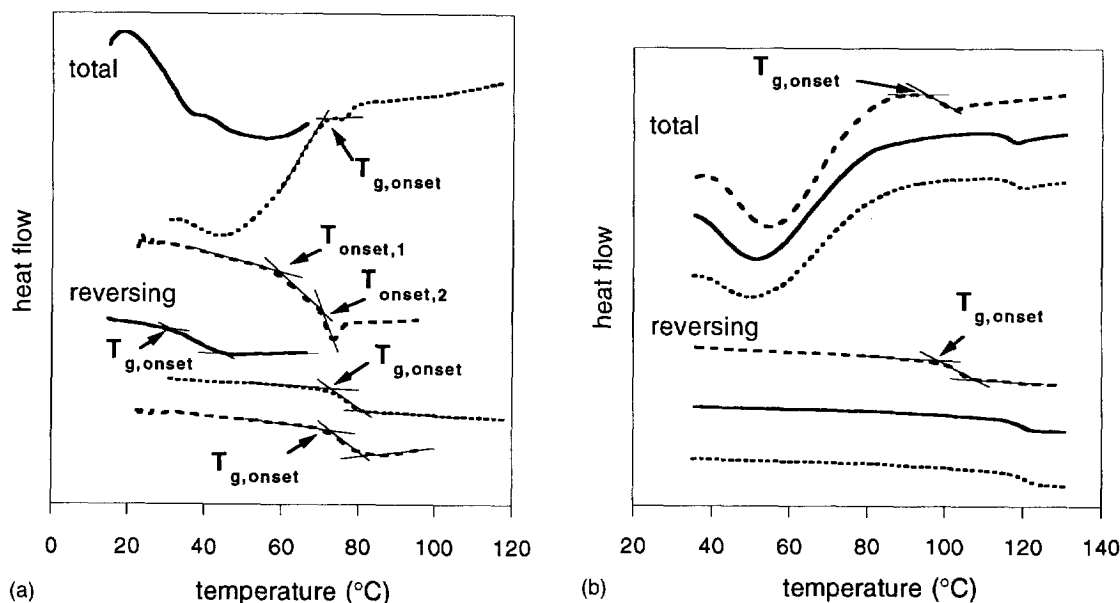


Fig. 2. Total and reversing heat flow of freeze-dried DXR-liposomes and the freeze-dried intraliposomal medium. Measurement settings:  $q = 1^\circ\text{C}/\text{min}$ ,  $T_a = 0.159^\circ\text{C}$ ,  $p = 60$  s. (A) Freeze-dried DXR liposomes (lyoprotectant: sucrose) with a residual water content of circa 3.5% (—) and 0.4% (---), and freeze-dried intraliposomal medium (---) (120 mM  $(\text{NH}_4)_2\text{SO}_4$ , 8 mg/ml DXR and 5% sucrose (w/v)) with a residual water content of circa 0.6%. (B) Freeze-dried DXR liposomes containing maltose (---), trehalose (—) and lactose (---) as a lyoprotectant.

50°C, respectively). Other batches with a low water content after freeze-drying showed similar patterns as found for sucrose.

The  $T_g$  values of the cakes during storage are plotted in the Fig. 4A and B.  $T_g$  values in sucrose cakes with a high water content increased from circa 40°C to similar values as for cakes with low water contents (circa 70°C). Apparently, the decrease in water content resulted in higher  $T_g$  values within 3 months, in spite of

the measures taken to reduce changes in residual water content of the cakes. It is unknown to us why the same values for  $T_g$  were found in these two sucrose batches, that contained different water contents (around 2.5% versus 0.5%). A possible explanation might be inhomogeneity of the water distribution over the cake. The glassy sucrose matrix of both batches might contain the same amount of plasticising water, whereas different amounts of water were associated with the phospholipids of the liposome bilayer, or the DXR- $(\text{NH}_4)_2\text{SO}_4$ -complex inside the liposomes. In Fig. 4A and B it can be seen that the  $T_g$  values of the cakes containing maltose, trehalose and lactose with a low water content remained relatively constant, the only exception being the trehalose cakes stored at 40 and 50°C.

After 6 months, the appearance of sucrose cakes containing 3.5% water could be described as displaying 'no shrinkage' (stored at  $-20^\circ\text{C}$ ), 'slight shrinkage' (20 and 30°C) and 'collapse' (40°C). The cakes with a low water content exhibited no collapse or shrinkage on visual inspection. Especially in the cakes containing less than 50% of the original DXR contents (see below) a change in colour was observed from red/white to purple/red.

### 3.3. Characteristics of rehydrated dxr-liposomes upon storage in the freeze-dried state

In Fig. 5A–B the DXR show contents of the sucrose cakes are plotted that were stored at different temperatures and over different periods of time. The curves

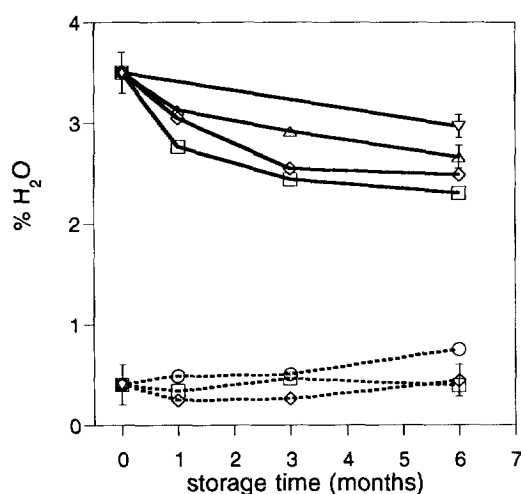


Fig. 3. Residual water content of freeze-dried DXR liposomes during storage. Lyoprotectant: sucrose, initial residual water content: 3.5% (—) and 0.4% (---). Storage temperature:  $-20^\circ\text{C}$  ( $\nabla$ ),  $20^\circ\text{C}$  ( $\Delta$ ),  $30^\circ\text{C}$  ( $\diamond$ ),  $40^\circ\text{C}$  ( $\square$ ), and  $50^\circ\text{C}$  ( $\circ$ ). Each data point represents the average values obtained from 2 (no error bars) or 6–10 (error bars: S.D.) vials.

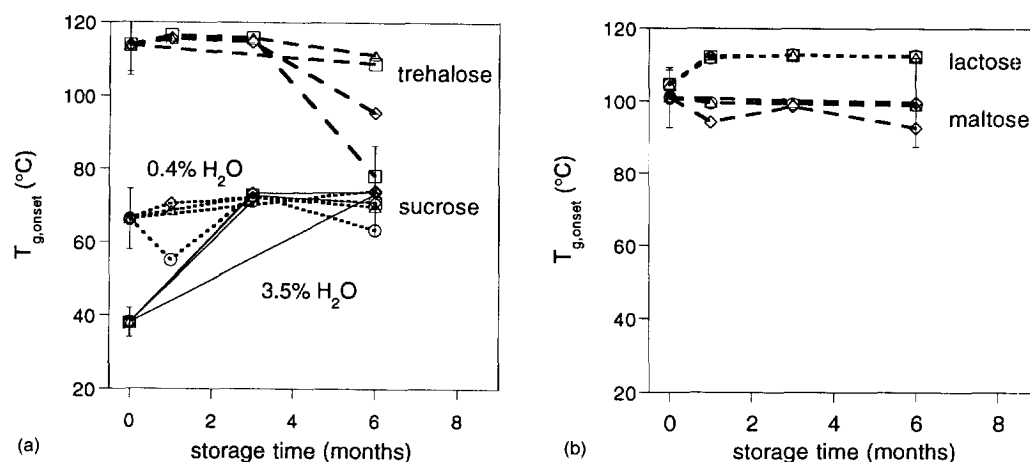


Fig. 4. A and B: variations in glass transition temperatures of freeze-dried DXR-liposomes during storage. Lyoprotectant: sucrose with an initial residual water content of (4A) 3.5% (—) and 0.4% (---), trehalose (---), and (4B) maltose (—) and lactose (---). Storage temperature:  $-20^{\circ}\text{C}$  ( $\nabla$ ),  $20^{\circ}\text{C}$  ( $\Delta$ ),  $30^{\circ}\text{C}$  ( $\diamond$ ),  $40^{\circ}\text{C}$  ( $\square$ ), and  $50^{\circ}\text{C}$  ( $\circ$ ). Each point represents the average of two samples taken from different vials. Error bars indicated the S.D. from 3–6 vials.

obtained for lactose, maltose and trehalose cakes with a low residual water content (0.1 to 0.4%) were similar to the curves observed for the sucrose cakes containing 0.4% water. None of the disaccharide cakes with a low residual water content stored at  $-20$  and  $30^{\circ}\text{C}$  showed significant degradation of DXR after 6 months. However, at  $40$  and  $50^{\circ}\text{C}$ , the DXR content (after rehydration) in all these batches was decreased, with no clear difference between the lyoprotectants selected for this study. The effect of temperature on DXR degradation is especially clear from Fig. 6. A major effect of the initial residual water content on degradation kinetics was observed in the sucrose cakes. The DXR content in the cakes with  $3.5 \pm 0.2\%$  residual water after freeze-drying decreased by circa 50% after 1 month of storage at  $40^{\circ}\text{C}$ , whereas the cakes with  $0.4 \pm 0.2\%$  water still contained circa 93% of the initial DXR content. The sample preparation of the rehydrated liposomes for HPLC analysis consisted of dilution and solubilisation of the liposomes with 30 mM citric acid (pH 4.0) and 2-propanol. In the samples of cakes with decreased DXR contents a red precipitate was observed after centrifugation (see methods). Attempts to solubilise this pellet in many different solvents were unsuccessful. It can not be excluded that these precipitates contained some undegraded DXR. Therefore, the observed DXR degradation could be an overestimation of the real values.

The determination of the DXR retention in liposomes was based on non-specific fluorescence measurements of the total and liposomal 'DXR' in the samples. In order to validate this assay, the DXR content of some of these samples were also measured with HPLC. The retention data obtained by HPLC

and fluorescence measurements were the same for samples containing 60% or more of their original DXR content. For samples that exhibited a more pronounced DXR degradation the retention data obtained with HPLC and fluorescence differed. Therefore, the retention assay only provides useful data when DXR degradation is less than 40%. Fig. 7 displays DXR retention after rehydration for all batches after 6 months of storage at different temperatures. DXR retention was dependent on the residual water content of the cakes, which is clear from the comparison of the two solid lines in Fig. 7, representing the sucrose cakes with two different water contents. No 'aging' effect on the DXR retention was observed for sucrose cakes with high (3.5%) water content stored up to  $20^{\circ}\text{C}$ , and for sucrose cakes with low (0.4%) water content stored up to  $30^{\circ}\text{C}$ . From these data, we concluded that all samples which remained chemically stable during storage (Fig. 6) also maintained their high initial values of DXR retention (circa 90%). DXR retention for maltose, lactose and trehalose cakes with a low water content showed a similar temperature dependency to the sucrose cakes containing 0.4–0.7% residual water. In samples stored at higher temperatures, the main retention decrease had already occurred after 1 month of storage (data not shown). This finding was qualitatively in line with the DXR degradation pattern of these samples.

For cakes with lactose, maltose and trehalose as a lyoprotectant, increases in mean vesicle size of the rehydrated liposomes remained below  $0.02 \mu\text{m}$  for all storage temperatures (Fig. 8A and B). In contrast, the sucrose cakes showed a pronounced size increase which was dependent on time and residual water content (Fig. 9A and B).



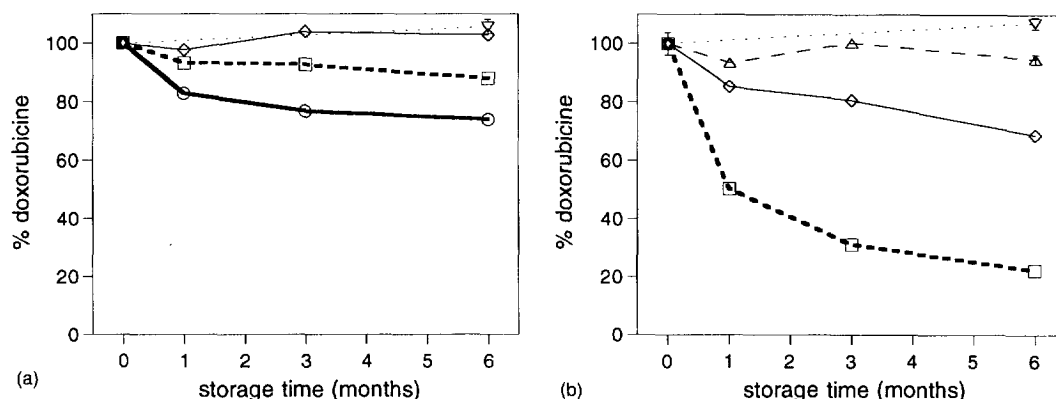


Fig. 5. DXR content of freeze-dried liposomes containing different lyoprotectants. Storage temperature:  $-20^{\circ}\text{C}$  ( $\nabla$ ),  $20^{\circ}\text{C}$  ( $\triangle$ ),  $30^{\circ}\text{C}$  ( $\diamond$ ),  $40^{\circ}\text{C}$  ( $\square$ ), and  $50^{\circ}\text{C}$  ( $\circ$ ). Each point represents the average values from 2–10 vials. Error bars indicate the S.D. of 6–10 samples. The DXR content measured at time point zero was set at 100%. The DXR contents are given in Table 1.  $\% \text{H}_2\text{O}$ : initial residual water content. (A) Lyoprotectant: sucrose,  $\% \text{H}_2\text{O}$ : 3.5%. (B) Lyoprotectant: sucrose,  $\% \text{H}_2\text{O}$ : 0.4%.

## 4. Discussion

### 4.1 Degradation below $T_g$

In the present study, we investigated the long term stability of freeze-dried DXR-liposomes as a function of temperature for different lyoprotectants and residual water contents. Our aim was to define parameters which controlled the solid state stability in order to rationally optimise the stability of freeze-dried liposomes.

Freeze-drying of DXR-liposomes with lactose, trehalose or maltose as a lyoprotectant to water contents below 0.5% resulted in an easily reconstitutable formulation with no signs of physical and chemical degradation after 6 months of storage at temperatures up to

$30^{\circ}\text{C}$ . For these lyoprotectants, DXR degraded up to 20 and 50% when stored at 40 and  $50^{\circ}\text{C}$ , respectively (Fig. 6). These data may represent an overestimation of the actual DXR degradation, as discussed under results, but they certainly reflect chemical instability under the specified conditions. In the same time period, DXR retention (based on a simple fluorescence analysis) decreased maximally by circa 10 and 20% after 6 months of storage at 40 and  $50^{\circ}\text{C}$ , respectively (Fig. 7). The average vesicle size in these samples (low residual water content) remained almost stable under all conditions described above. Only a slight trend ( $< 0.02 \mu\text{m}$ ) to size increase could be observed.

The onset temperatures of the glass transition (total heat flow) of these cakes were above  $90^{\circ}\text{C}$  throughout the study (Fig. 4A and B), except for some trehalose

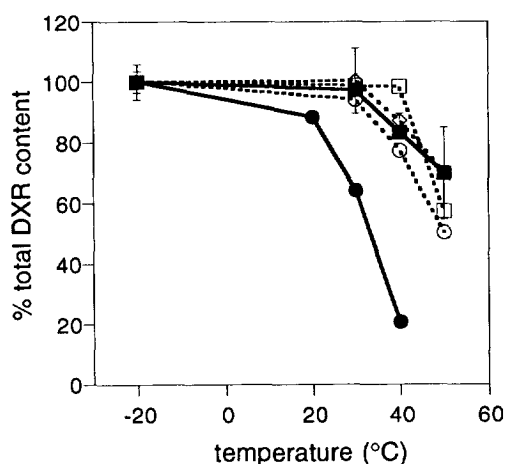


Fig. 6. DXR content in freeze-dried liposomes after 6 months of storage.  $\% \text{H}_2\text{O}$ , initial residual water content. Lyoprotectants, sucrose, (3.5%) ( $\bullet$ ) and (0.4%) ( $\blacksquare$ ); maltose (0.2%) ( $\diamond$ ); trehalose (0.1%) ( $\square$ ); lactose 0.3% ( $\circ$ ). Each point represents the average of two samples taken from different vials. Error bars indicated the S.D. from 3–6 vials.

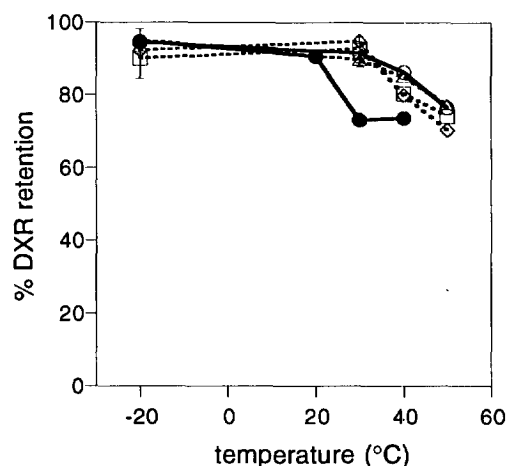


Fig. 7. DXR retention after 6 months storage in the dried state.  $\% \text{H}_2\text{O}$ , initial residual water content. Lyoprotectants, sucrose, (3.5%) ( $\bullet$ ) and (0.4%) ( $\blacksquare$ ); maltose (0.2%) ( $\diamond$ ); trehalose (0.1%) ( $\square$ ); lactose 0.3% ( $\circ$ ). Each point represents the average values from 2–10 vials. Error bars indicate the S.D. from 6–10 samples. The DXR retention before freeze-drying was 100%.

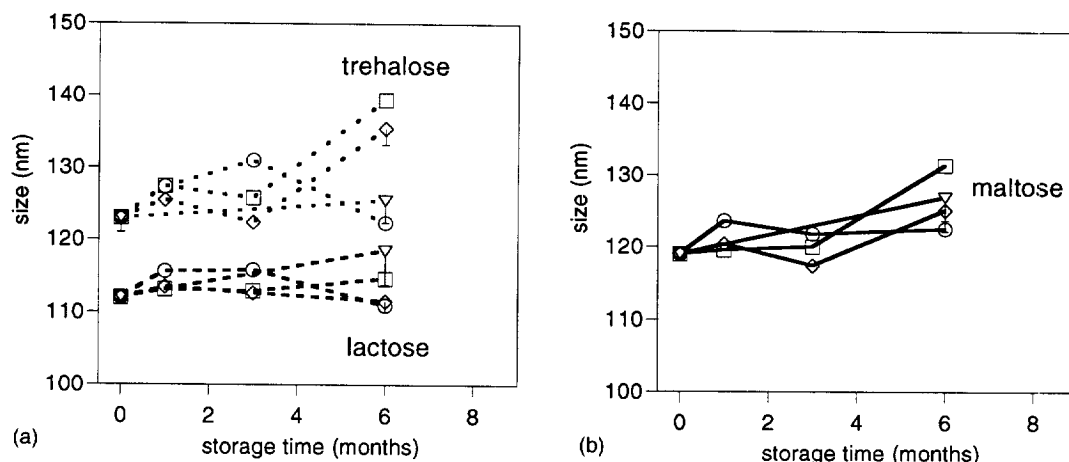


Fig. 8. Average vesicle size of DXR liposomes after rehydration as a function of storage time in the freeze-dried state. Lyoprotectant, (8A) lactose (---), trehalose (- - -), and (8B) maltose (—), all containing 0.2 to 0.7% residual water. Storage temperature,  $-20^{\circ}\text{C}$  (▽),  $20^{\circ}\text{C}$  (△),  $30^{\circ}\text{C}$  (◇),  $40^{\circ}\text{C}$  (□), and  $50^{\circ}\text{C}$  (○).

cakes at the last time point. This deviation from the general trend was not reflected in the other results, and is considered an artefact. Therefore, we conclude that all aging phenomena described above (DXR degradation, DXR leakage, size increase) occurred at  $40\text{--}60^{\circ}\text{C}$  below the  $T_{g,\text{onset}}$  of the sugar matrix.

#### 4.1. Effect of lyoprotectant and residual water content

The stability of DXR-liposomes freeze-dried in sucrose with a residual water content between  $0.4\text{--}0.7\%$  and a  $T_g$  between  $60\text{ and }70^{\circ}\text{C}$  was comparable with the stability of cakes containing lactose, trehalose or maltose with similar residual water contents, except for the average vesicle size which showed a pronounced increase after storage at  $50^{\circ}\text{C}$  for 6 months (Fig. 9B).

The residual water content had a pronounced effect on the solid state stability of freeze-dried liposomes. Sucrose cakes with a water content between  $3.5\text{ and }2.3\%$ , showed marked changes in DXR retention, DXR content and vesicle size when stored at temperatures of  $30^{\circ}\text{C}$  and higher. In contrast, such aging phenomena occurred only at a temperature of  $40^{\circ}\text{C}$  and higher in cakes with identical constituents but a residual water content of  $0.4\text{--}0.7\%$ .

#### 4.2. Mechanism of degradation

An important finding of this study was the observation of the physical and chemical instability of freeze-dried DXR liposomes below the  $T_g$  of the sugar matrices. These observations seem to contradict the general assumption that below  $T_g$  the low molecular mobility guarantees a high stability. However, two explanations may (partly) account for the observed degradation processes. First, DXR degradation will depend

on the direct local physical and chemical environment of these molecules. The thermal properties of a freeze-dried solution containing DXR,  $(\text{NH}_4)_2\text{SO}_4$  and sucrose, with a composition similar to the intraliposomal medium, differed from those of the freeze-dried extraliposomal medium, despite a residual water content below  $0.7\%$  in both samples (Fig. 2). The first deviation from the baseline of the total heat flow in the intraliposomal solid phase was observed circa  $12^{\circ}\text{C}$  lower than for the dried, full dispersion. This difference is ascribed to the presence of DXR and  $(\text{NH}_4)_2\text{SO}_4$  in this cake. Such an endothermal process may be indicative of decreased stability in the intraliposomal solid phase. It is not surprising that this endotherm is not observed in the total heat flow of liposomal cakes, since the estimated mass fraction of encapsulated solutes is only about  $3\%$ , which would give rise to only a small heat flow signal in the total sample. In conclusion, the data in this study suggest that the stability of encapsulated drugs is primarily dependent on the physical state of the solid inside the liposomes. Large amounts of DXR and  $(\text{NH}_4)_2\text{SO}_4$  inside the liposomes may decrease the stability of the solid phase in a temperature dependent fashion. The overall water content of the cakes does not provide information on the exact position of water at a molecular level. A factor that could not be addressed in the present study, is the possibility of heterogeneous distribution of residual water in the cakes. A relatively high water content inside the liposomes could provide an additional explanation for the enhanced physical and chemical activity in the intraliposomal solid. A second mechanism which may account for the physical degradation observed below  $T_g$  was described in a previous study [15] in which the short term stability of freeze-dried liposomes at elevated temperatures was examined. Liposomes freeze-dried with trehalose as a

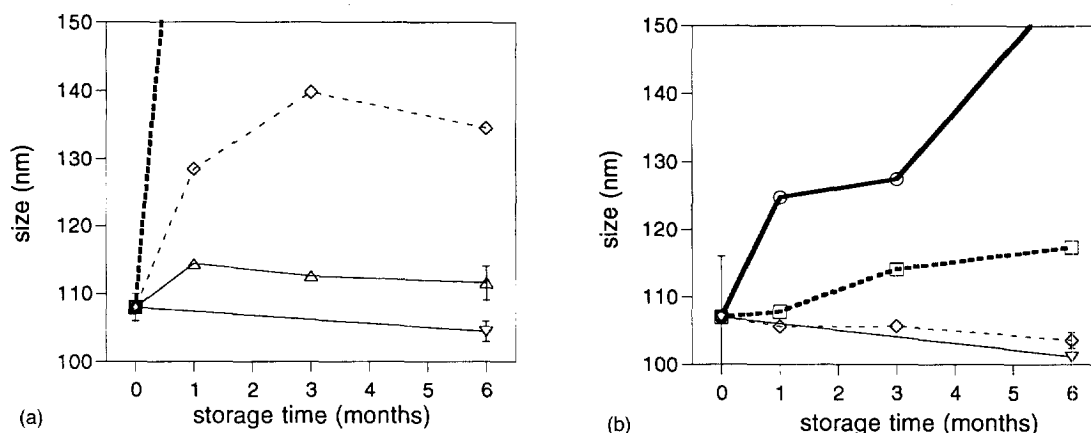


Fig. 9. Average liposome size after rehydration as a function of storage time. Storage temperature:  $-20^{\circ}\text{C}$  ( $\nabla$ ),  $20^{\circ}\text{C}$  ( $\triangle$ ),  $30^{\circ}\text{C}$  ( $\diamond$ ),  $40^{\circ}\text{C}$  ( $\square$ ), and  $50^{\circ}\text{C}$  ( $\circ$ ). Each point represents the average values from 2–10 vials. Error bars indicate the S.D. from 6–10 samples. (A): Lyoprotectant: sucrose, initial water content: 3.5%. (B): Lyoprotectant: sucrose, initial water content: 0.4%.

lyoprotectant leaked their encapsulated watersoluble marker when heated to the transition temperature of the liposomal bilayers. This so-called ‘annealing process’ was accompanied by an enhanced interaction between the lyoprotectant and the phospholipid headgroups. Also for liposomes with the same bilayer composition as used in this study (DPPC:DPPG:CHOL = 10:1:4), leakage and a size increase on rehydration was observed after exposure of the dry cakes to temperatures above  $50^{\circ}\text{C}$  for 30 min. In samples of the present study similar mechanisms may have been active during storage, especially at the highest storage temperatures. The short time scale required for these phenomena to occur may (partly) explain the present observations that the most pronounced physical changes were observed within the first month of storage (e.g. Fig. 5A and B).

The increase in vesicle size after storage of the liposomes in a sugar glass below  $T_g$  is most likely the result of aggregation and/or fusion after rehydration. It is hard to imagine how fusion of vesicles could occur in a glassy phase with a low molecular mobility. A factor which could contribute to this aggregation and/or fusion process is the presence of DXR which leaked out of the liposomes during storage/rehydration. In earlier studies, extraliposomal DXR has been shown to induce growth of the average vesicle size [26].

#### 4.3. Choice of lyoprotectant

At low storage temperatures, no differences in lyoprotective properties were found between the disaccharides selected for this study. That means that no consistent differences between the solid state stability of liposomes with reducing (maltose, lactose) and non-reducing sugars (sucrose, trehalose) were observed. At

elevated temperatures, the solid state stability of sucrose cakes was inferior to lactose, maltose and trehalose cakes with the same residual water content, which can be ascribed to its lower  $T_g$  in the extraliposomal matrix. Similar effects of the lyoprotectants may have influenced the stability of the intraliposomal solid in the freeze-dried cakes. In addition, the higher ‘softening temperature’ of the freeze-concentrates of lactose, maltose and trehalose (circa  $-30^{\circ}\text{C}$ , see Table 3) as compared with sucrose ( $-34^{\circ}\text{C}$ ) allows a slightly higher product temperature during freeze-drying, which enhances the drying rate.

#### 5. Conclusion

In summary, freeze-drying of DXR liposomes resulted in a stable formulation upon storage up to  $30^{\circ}\text{C}$  for the liposome/lyoprotectant cakes with low residual water contents. At higher temperatures, pronounced DXR degradation, DXR leakage and increase in vesicle size were observed after rehydration. The degradation processes occurred below  $T_g$ , thus in the solid state. Explanations were proposed to explain these phenomena mechanistically. First, the different thermal/physical properties of the intraliposomal solid may have provided an unstable environment for the encapsulated drug. Second, annealing processes between the bilayer and the lyoprotectant may have occurred at temperatures below  $T_g$ . It is clear that the  $T_g$  of the sugar matrix is not necessarily an appropriate indicator for the maximal temperature at which long term stability of freeze-dried liposomes is preserved, especially when high concentrations of encapsulated compounds destabilise the solid phase inside the liposomes.

Table 3

Comparison of onset temperatures in MDSC total and reversing heat flow curves of (1) freeze-dried DXR-liposomes containing sucrose and (2) the freeze-dried medium inside these liposomes

Freeze-dried sample	Total heat flow		Reversing heat flow
	$T_{\text{onset},1}$ (°C)	$T_{\text{onset},2}/T_{\text{g,onset}}$ (°C)	$T_{\text{g,onset}}$ (°C)
(1) Total dispersion	Not observed	$70 \pm 1$	$72.4 \pm 0.5$
(2) Intraliposomal medium	$59 \pm 1$	$69 \pm 1$	$71 \pm 2$

Composition before freeze-drying: (1) 15% sucrose (w/v), 10 mM Hepes pH 7.4 (extraliposomal), 20 mM PL, 0.5 mg/ml DXR, circa 8 mM  $(\text{NH}_4)_2\text{SO}_4$  (total dispersion), (2) 120 mM  $(\text{NH}_4)_2\text{SO}_4$ , 8 mg/ml DXR, 5% sucrose (w/v).  $T_{\text{onset},1}$ ,  $T_{\text{onset},2}$  and  $T_{\text{g,onset}}$  correspond with the labels in Fig. 2A.

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