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Lyophilization and rehydration of iopromide-carrying liposomes

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Abstract

Iopromide-carrying liposomes prepared by the ethanol-evaporation method which encapsulated approximately 35% X-ray contrast agent at an average vesicle size of 300 nm were stabilized by lyophilization. Freezing behaviour of the suspension was studied by differential scanning calorimetry (DSC) and resistance/temperature measurement with respect to the maximum allowable temperature during primary drying. While melting of the suspension was observed at −21°C by DSC, conductivity changes down to −40°C could be detected by simultaneous resistance/temperature measurement. Furthermore, the influence of two different shelf-temperatures (−15°C and +5°C) and three different chamber pressures (0.03 mbar, 0.08 mbar and 0.2 mbar) during primary drying on the quality of the lyophilized and rehydrated liposomes were investigated. A chamber pressure of 0.08 mbar and a shelf-temperature of −15°C during primary drying led to optimal product quality indicated by high contrast agent encapsulation. A chamber pressure of 0.08 mbar also proved to be optimal with respect to an economic lyophilization process. The residual water content of the freeze dried material proved to be very low (about 0.1%) independent of process parameters. Rehydration of the lyophilized liposomes with mannitol solution resulted in higher encapsulation efficiency of the rehydrated vesicles compared to rehydration with water or 20 mM tromethamine buffer (pH 7.5). The encapsulation efficiency could be further improved by increasing the concentration of the mannitol solution. Rehydration with iso-osmotic solutions of sucrose and KCl proved not to be as effective as mannitol solution in increasing the encapsulation efficiency of the rehydrated vesicles. Freeze-fracture images of lyophilized drug-free liposomes in contrast to iopromide-carrying liposomes revealed the lyoprotective effect of iopromide.

Keywords: Iopromide; Liposome; CT tumor diagnostic; Freezing behaviour; Lyophilization; Rehydration; Residual moisture; Lyoprotective effect of iopromide

1. Introduction

Liposomes as a drug carrier system for iodinated contrast media as well as paramagnetic

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metal complexes for computed tomography (CT) and magnetic-resonance imaging (MRI) have been described by several authors (Havron et al., 1981; Zerbin et al., 1983; Ryan et al., 1983; Benita et al., 1984; Cheng et al., 1987; Payne and Whitehouse, 1987; Musu et al., 1988; Henze et al., 1989; Janoff et al., 1991; Schwendener, 1992). For liver CT, liposomes loaded with X-ray contrast agents can provide a helpful diagnostic tool. Accumulating passively in the healthy liver and spleen they improve the difference in radio-density between normal liver tissue and tumors (Krause et al., 1991; Krause et al., 1993; Sachse et al., 1993). The accuracy of MR imaging is also improved by selective localization of liposomes encapsulating MR contrast agents in liver (Unger et al., 1989a; Unger et al., 1989b).

The introduction of a liposomal contrast agent in CT or MRI diagnostics requires a sufficient long-term product stability. In an aqueous liposome suspension chemical and physical degradation such as autoxidation, hydrolysis, aggregation, fusion and leakage of the incorporated compound are likely to occur (Frøkjaer et al., 1984; Grit et al., 1989; Grit and Crommelin, 1992; Grit et al., 1993). As a result, pharmacokinetic as well as imaging properties of the original liposomes may change. For stabilization of aqueous liposome suspensions lyophilization is employed (Shulkin et al., 1984; Van Bommel and Crommelin, 1984; Madden et al., 1985; Handa et al., 1987; Özer and Talsma, 1989; Tanaka et al., 1991). Lyophilization is considered a three step process: freezing, sublimation (primary drying) and subsequent removal of unfrozen, adsorbed water (desorption or secondary drying). Each of these steps comprises detrimental conditions for the liposomes. Thus, the vesicles may undergo thermo- and lyotropic phase transitions resulting in loss of bilayer integrity and incorporated agent, fusion and membrane separation (Crowe and Crowe, 1992). Nevertheless, sufficient stabilization of liposomes during freeze drying can be achieved in the presence of so-called lyoprotective compounds (Crowe et al., 1985; Crowe et al., 1986; Strauss et al., 1986; Hauser and Strauss, 1988). This study focuses on the systematic optimization of the lyophilization of MLV entrapping the highly water-soluble, monomeric contrast agent iopromide (Ultravist®, Schering AG, Berlin, Germany). The freezing behaviour of these liposomes is studied by DSC and simultaneous temperature/resistance measurement (Nail and Gatlin, 1985; Pikal, 1991) for determination of the highest allowable product temperature during primary drying. Furthermore, the effect of two different shelf-temperatures and various pressures during primary drying on the quality of the rehydrated material and the course of the drying process is investigated. The residual moisture of the lyophilized substances is determined by Karl-Fischer titration. During rehydration various media and conditions are tested in order to obtain liposomes with optimized product quality with regard to encapsulation efficiency and size. Electron microscopic studies of freeze-fracture preparations of the lyophilized material are employed to investigate the structure of the lyophilized material and to possibly prove the lyoprotective effect of the X-ray contrast agent iopromide.

2. Materials and methods

2.1. Materials

Soy phosphatidylcholine (Lipoid S100 (PC)) was obtained from Lipoid KG, Ludwigshafen, Germany. Cholesterol (CH), tromethamine, sucrose and 96% ethanol were supplied by Merck, Darmstadt, Germany. Stearic acid (SA) was obtained from Fluka, Buchs, Switzerland and iopromide solution was prepared by diluting aliquots of Ultravist®-370, Schering AG, Berlin, Germany with 20 mM tromethamine buffer (adjusted to pH 7.5 with HCl). Mannitol and KCl were purchased from Riedel-de Haen, Seelze, Germany.

2.2. Preparation and physicochemical characterization of liposomes

The iopromide-containing liposomes composed of PC/CH/SA at a molar ratio of 4:5:1 were prepared by the ethanol-evaporation method as described earlier by Sachse et al. (1993).

Liposome size was measured by photon correlation spectroscopy (PCS) using a Nicomp 270 Submicron Particle Sizer (Nicomp Instruments Corp.,

Goleta, CA). For calculation of particle size from light scattering data, viscosity and refractive index of pure water were used.

The encapsulation efficiency of the liposomes was determined by equilibrium dialysis using a dianorm system (Dianorm, Munich, Germany) and the iopromide concentration was measured at 243 nm using a Model 550 UV photometer (Perkin Elmer, Überlingen, Germany).

Simultaneous temperature/resistance measurements were carried out using the Eutectic Monitor AW 2 (Leybold-Heraeus, Cologne, Germany). Basically, about 2 ml of the liposome suspension were filled into a 10 ml glass vial and transferred to the Eutectic Monitor AW 2. The suspension was cooled down to −75°C at a rate of 0.5°C/min. At −75°C the sample was kept for 30 min and subsequently heated to 20°C at a rate of 0.5°C/min. During cooling and heating, temperature as well as resistance were recorded simultaneously with the DES program version 1.4, provided by Leybold-Heraeus. The results were shown as a semi-logarithmic plot of resistance (R) over temperature (T). The diagram also shows the first derivative of the log R versus T curve. At the start of each experiment the Eutectic Monitor was calibrated with simple KCl or NaCl solutions.

Differential scanning calorimetry (DSC) measurements were performed using a Mettler TA 4000, Mettler Toledo, Giessen, Germany. About 30 mg of the liposome suspension were weighed into an aluminium pan displaying a small pinhole. The sample was cooled from 20°C down to −60°C at a rate of 1°C/min and kept at that temperature for 10 min. Afterwards the sample was heated to 20°C at the same rate. As a reference an empty aluminium pan was used and the measurement was carried out in nitrogen atmosphere.

Freeze drying was performed in a GT 4 Lyovac (Leybold-Heraeus, Cologne, Germany). For this purpose 20-ml aliquots of the liposome suspension were weighed into 50-ml glass vials. Next, the vials were transferred to the pre-cooled (−50°C) shelves of the freeze dryer. The freezing temperature of −50°C was kept for 4 h. Afterwards the chamber pressure was reduced to about 0.08 mbar (0.03 or 0.2 mbar) and the shelf-temperature raised to −15°C or +5°C at a rate of 0.5°C/min. At the end of primary drying the pressure was further lowered to approximately 0.01 mbar and the shelf-temperature raised to 20°C at a rate of 0.5°C/min. The Lyovac GT 4 is equipped with a hydraulic closing device to ultimately seat the stopper which is placed on top of the vial in the lyophilizer.

The residual moisture of the lyophilized substance was determined by titration using a Karl-Fischer-Titrator DL 18 (Mettler Toledo, Giessen, Germany). The pyridine-free Hydranal-Solvent and Hydranal-Titrant 5 were obtained from Riedel-de Haen, Seelze, Germany and were used as solvent and titration medium.

For freeze-fracture images the lyophilized material was flash-frozen in liquid nitrogen. Freeze-fracture, shadowing with platinum (45°C) and stabilization of the replica with carbon (90°C) was performed following standard procedures. The samples were examined with a Siemens EM 101 electron microscope (Siemens, Munich, Germany).

3. Results

3.1. Freezing behaviour

The relationship between electrical conductivity and temperature of the iopromide carrying liposome suspension is shown in the plot of log R versus T in Fig. 1. The suspension supercools

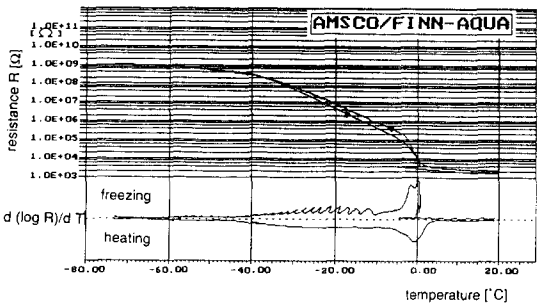

Fig. 1. Eutectic temperature of iopromide-carrying liposomes (PC/CH/SA 4:5:1, iodine/lipid 1:1) as determined with simultaneous temperature/resistance measurement, cooling and heating from +20°C to −75°C and vice versa.

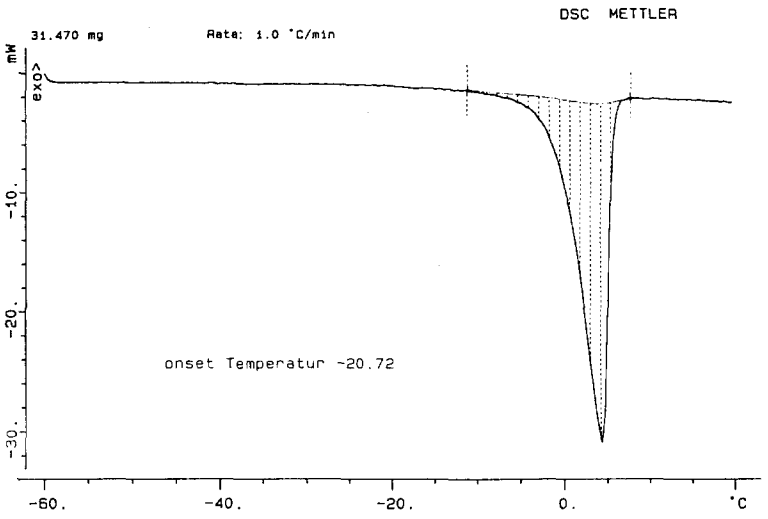

Fig. 2. Heat flow diagram of the iopromide-carrying liposomes determined by DSC measurement. Heat-flux versus temperature is shown.

until about −6°C. The heat of crystallization of the water causes the increase of the temperature of the suspension to 0°C. A sharp rise in product resistance over almost two orders of magnitude can be seen until approximately −6°C. Between −7°C and −40°C the slope of the log resistance versus temperature profile is nearly constant showing a continuous increase. At temperatures below −40°C no further changes on the resistance are observed. Upon heating, the conductivity of the suspension slightly increases from −40°C to approximately −7°C, evident as a broad, truncated region in the $d(\log R)/dT$ plot. From −7°C to 0°C the resistance drops dramatically due to melting of eutectic ice and the dissolution of eutectic solid. This transition can be identified in the $d(\log R)/dT$ plot as rather sharp peak.

Freezing and thawing behaviour was also studied by DSC. In Fig. 2 only the warming behaviour of the iopromide-carrying liposome suspension is shown as determined by DSC measurements. In the heating curve one endothermic heat-flow can be seen. This heat-flow is ascribed to the thawing of the suspension. The onset temperature of the thawing of the liposome suspension is determined to be −21°C.

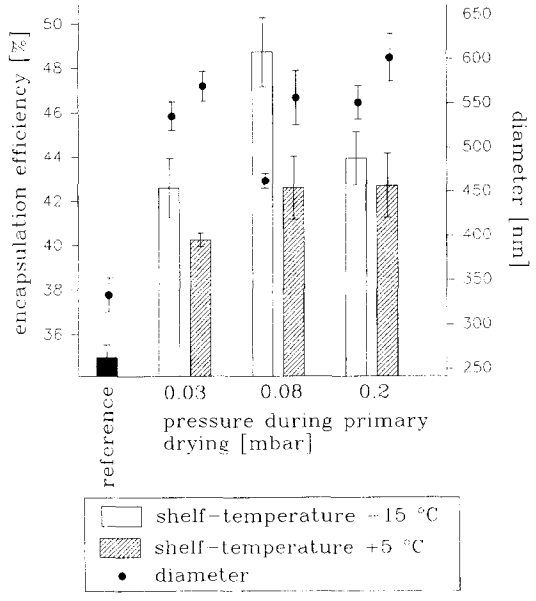

Fig. 3. Encapsulation efficiency and diameter of iopromide-carrying liposomes prior to lyophilization (reference) and after lyophilization and rehydration with 4 g of 135 mM mannitol solution per g lyophilized material. Each error bar represents six measurements.

Table 1
Residual water content of lyophilzed liposomes after primary drying using various freeze drying programs, the mean of triple measurement is given

Shelf-temperature (°C)	Pressure during primary drying (mbar)	Mean water content (%)
−15	0.03	0.12
	0.08	0.09
	0.2	0.16
+5	0.03	0.13
	0.08	0.08
	0.2	0.18

3.2. Variation of shelf-temperature and chamber pressure during primary drying

As shown in Fig. 3 the encapsulation efficiency and the diameter of the lyophilized and subsequently rehydrated vesicles increases compared to the freshly prepared liposomes (reference). The change of these two parameters which determine the quality of the rehydrated material varies with the shelf-temperature and the pressure used during primary drying of the liposome suspension. Irrespective of the employed pressures, a significant increase in encapsulation efficiency can be achieved by using a shelf-temperature of −15°C compared to +5°C during primary drying. The vesicles lyophilized at +5°C reveal larger diameters and lower encapsulation efficiencies after rehydration. Primary drying at 0.08 mbar not only results in optimum quality parameters of the resuspended vesicles such as high encapsulation of iopromide but also results in the fastest freeze drying process.

3.3. Effect of shelf-temperature and chamber pressure during primary drying on the residual moisture of the lyophilized liposome suspension

As shown in Table 1 the residual water content of the lyophilized iopromide-carrying liposomes as determined by Karl-Fischer titration is well below 0.2%. In these experiments the liposomes are lyophilized at different shelf-temperatures and pressures during primary drying with a secondary drying phase of 10 h. It can be demonstrated, that the temperature during primary drying does not significantly influence residual moisture of the lyophilized samples. In contrast, drying pressure shows a pronounced effect resulting in lowest residual water contents at 0.08 mbar.

3.4. Influence of duration of secondary drying time on the residual moisture of the lyophilized substance

Iopromide-carrying liposomes are lyophilized at a shelf-temperature of +5°C and a pressure of 0.08 mbar during primary drying. After 64 h of primary drying six vials were already stoppered in the freeze dryer. After 3 h, 6 h, 12 h or 18 h of secondary drying at 0.01 mbar and 20°C respectively six vials were closed and the residual water content measured. Increasing the time of secondary drying up to 12 h results in a decrease in residual water content of the freeze dried samples (Table 2). The residual moisture of the lyophilized liposomes is very low even without secondary drying (0.13%).

3.5. Relationship between various rehydration media (rm) and various amounts of rehydration media and the quality of the rehydrated vesicles

The influence of different rm on the quality of the rehydrated liposomes is investigated using either water, 20 mM tromethamine buffer (pH 7.5) or 135 mM mannitol solution. Furthermore, the influence of varying amounts of the respective rm (2 g, 4 g or 8 g(rm)/g lyophilized material) is

Table 2
Residual water content of lyophilized liposomes after various times of secondary drying, the mean of at least three measurements is given

Duration of secondary drying (h)	Mean residual water content (%)
0	0.13
3	0.11
6	0.10
12	0.08
18	0.08

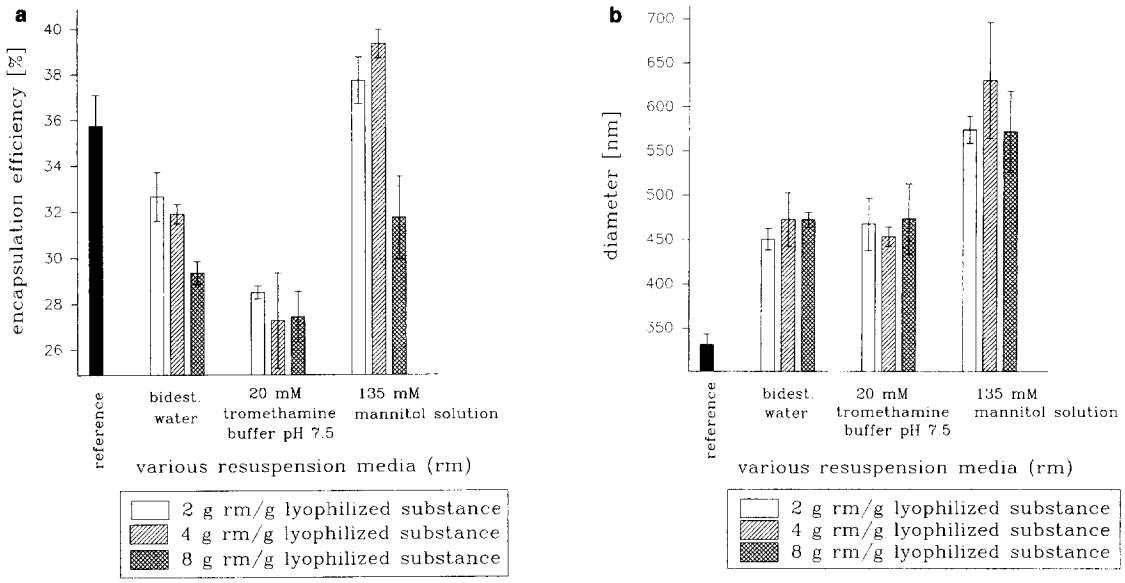

Fig. 4. Encapsulation efficiency (a) and diameter (b) of iopromide-carrying liposomes after preparation (reference) and after lyophilization and subsequent rehydration as a function of the rehydration media and their amount. Each measurement was performed sixfold.

tested. Application of 2 g(rm)/g freeze dried substance for rehydration results in a favourable, high total iodine concentration in the resulting liposomes. Unfortunately this suspension turns out to be very viscous, which is a major drawback for parenteral application. With 4 g(rm)/g lyophilized material the resulting liposomes display approximately the same iodine concentration as the freshly prepared liposomes. The iodine concentration of the rehydrated liposomes with 8 g(rm)/g lyophilized substance proves to be undesirable low.

In Fig. 4a,b the encapsulation efficiency and size of the rehydrated vesicles as a function of the amount and type of rehydration medium are shown. Rehydration with water and tromethamine buffer results in a significant decrease in encapsulation efficiency and increase of vesicle size compared to the non-lyophilized liposomes. In contrast, rehydration with 135 mM mannitol solution results in an increase in encapsulation efficiency and in size compared to the original liposomes. However, the size increase to approximately 600 nm is still acceptable for i.v. injection.

3.6. Relationship between temperature of the rm and the quality of the rehydrated material

Fig. 5a,b present the encapsulation efficiency and mean diameter of rehydrated vesicles as a function of the temperature of the rm. Again rehydration with 135 mM mannitol solution results in the highest encapsulation efficiencies and mean vesicle diameters. The amount of encapsulated contrast agent decreases with an increase of temperature of the mannitol solution. At room-temperature rehydration with water results in a higher encapsulation efficiency compared to rehydration with 20 mM tromethamine buffer. At 4°C, however, the encapsulation efficiency obtained when resuspended with water is even lower than after rehydration with tromethamine buffer. At 37°C no differences in encapsulation efficiency can be observed after rehydration with either water or tromethamine buffer. With regard to vesicle size, no difference can be observed between water and tromethamine buffer. Irrespective of the type of rm used, an increase in temperature is followed by an increase in vesicle size after rehydration.

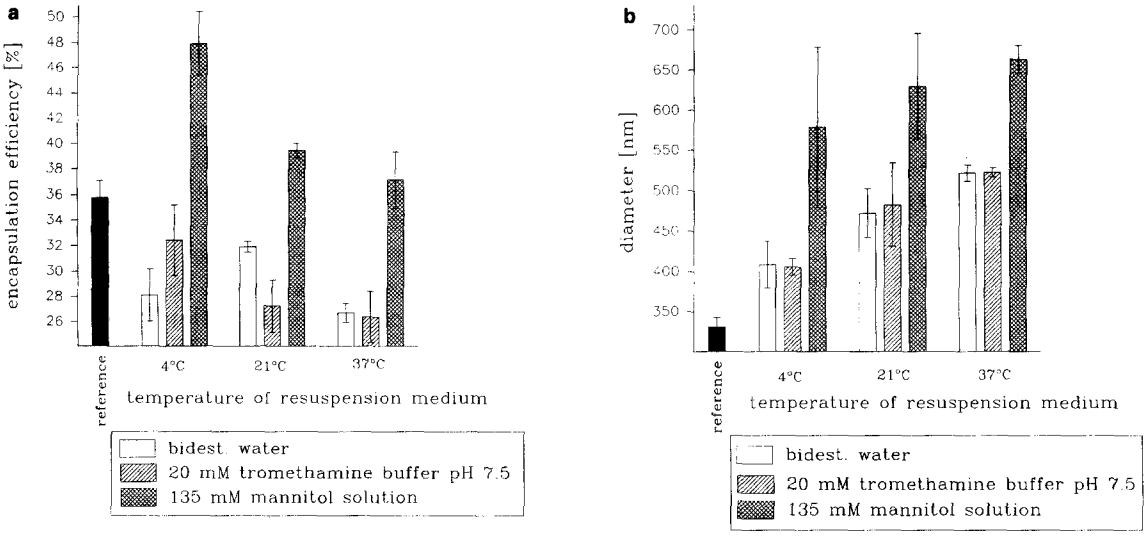

Fig. 5. Encapsulation efficiency (a) and diameter (b) of iopromide-carrying liposomes after preparation (reference) and after lyophilization and rehydration as a function of rehydration media and their temperature. Every experiment was performed sixfold.

3.7. Influence of the concentration of mannitol solution on the encapsulation efficiencies and vesicle sizes of the rehydrated vesicles

Rehydration of the lyophilized liposomes with mannitol solution results in an increase in encapsulation efficiency of the resulting liposomes compared to freshly prepared liposomes. The encapsulation efficiency and diameter of the rehydrated vesicles in dependence on the concentration of the mannitol solution is shown in Fig. 6. Increasing the concentration of the rm from 75 mM to 500 mM results in an increase in encapsulation efficiency and a corresponding increase in liposome diameter.

3.8. Rehydration with iso-osmolar rm

In the experiment shown in Fig. 7 different rm are employed which are iso-osmotic to 500 mM mannitol solution. The respective sucrose- or KCl-solutions show a lower encapsulation efficiency and vesicle size compared to the values obtained by rehydration with the mannitol solution.

3.9. Freeze-fracture images of the dry, lyophilized material

Electron microscopic studies of the freeze-fracture preparation of the lyophilized iopromide-carrying liposomes reveal that the vesicular structure

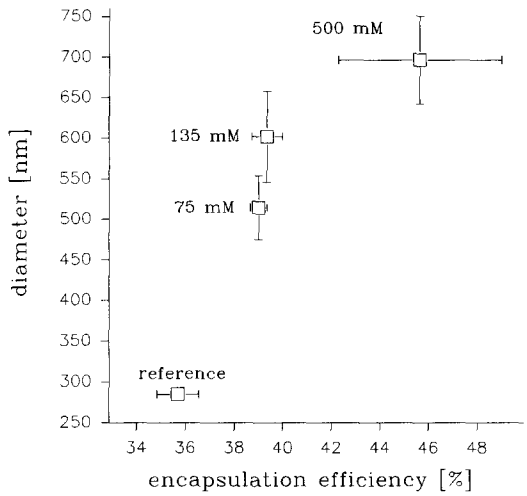

Fig. 6. Encapsulation efficiency and diameter of iopromide-carrying liposomes after preparation (reference) and after loyphilization and subsequent rehydration. For rehydration mannitol solution at various concentrations (75 mM, 135 mM, 500 mM) was used. Each experiment was performed sixfold.

of some of the liposomes is preserved, even in the dry, freeze-dried state as shown in Fig. 8. In contrast, the lyophilized, dry substance of liposomes prepared without the contrast-agent iopromide (drug-free liposomes) reveal the loss of vesicular structure (Fig. 9). The EM-image of these placebo-liposomes clearly shows lipid layers.

4. Discussion

The freezing behaviour of the iopromide-carrying liposomes determined either by DSC or by simultaneous resistance/temperature measurement varies considerably depending on the method. While only one heat-flux in the heating curve of the DSC reflecting the thawing of the suspension is seen at −21°C, constant changes of conductivity can be observed by resistance/temperature measurement between −40°C and −7°C. From −7°C to 0°C the conductivity of the liposome suspension increases sharply, indicating the eutectic melting of the suspension. One reason for differences between these two methods might be the differences in sample size or sensitivity of the methods. Thus the relatively small volumes (30 mg) used for DSC might not be sufficient to allow detection of smaller heat flows (Nail and Gatlin, 1985).

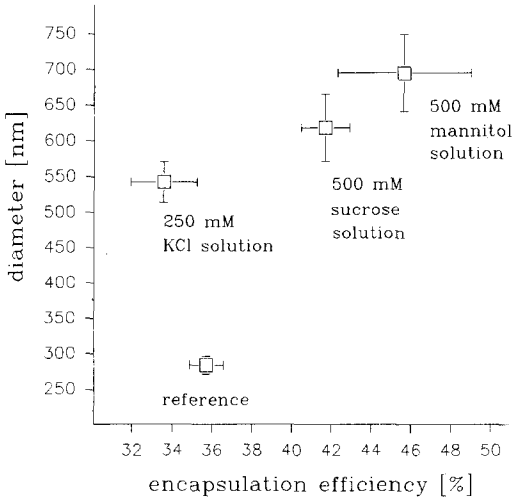

Fig. 7. Encapsulation efficiency and diameter of liposomes directly after preparation (= reference) and after lyophilization and subsequent rehydration with iso osmolar solutions of sucrose, mannitol or KCl. Each square represents the mean of six experiments.

The iopromide-carrying liposome suspension represents a complex system revealing several conductivity changes during cooling and warming between −40°C and −20°C, which are not observed by thermal analysis. The low temperature transitions, however, seem not to be relevant for the outcome of the lyophilization process of the iopromide-carrying liposomes. Thus product temperatures between −33°C and −20°C which were measured in our experiments during primary drying resulted in lyophilized materials with a homogeneous cake-like structure. Temperature and pressure during primary drying were shown to significantly influence the product quality. After rehydration the liposomes dried at a shelf-temperature of +5°C reveal larger vesicle diameters and smaller encapsulation efficiencies, possibly indicating a more multilamellar structure than vesicles dried at −15°C. The freeze drying process proceeds faster at a higher shelf-temperature meaning a more economic process design. A compromise has to be made between economic freeze drying and optimal product parameters. At a chamber pressure of 0.08 mbar the lyophilization course is faster than at the lower and higher pressures used.

Residual water content of lyophilized liposomes has been described to be about 0.5–4% by Henry-Michelland et al. (1985) and by Crowe et al. (1987). In our experiments the residual water content of the lyophilized vesicles is even below 0.2%. The shelf-temperature during primary drying has little if any effect on the residual water content of the lyophilized iopromide-carrying liposomes. Again, a chamber pressure of 0.08 mbar results in optimal product quality. As expected, prolongation of secondary drying time leads to a decrease of residual moisture of the lyophilized samples until the residual water level seems to approach a plateau after approximately 12 h. This has already been described by Pikal et al. (1990).

The contrast agent iopromide seems to protect the liposomes during lyophilization against dehydration damages. This is reflected by the existence of some intact liposome structures observed in the freeze-fracture image (Fig. 8). A possible lyopro-

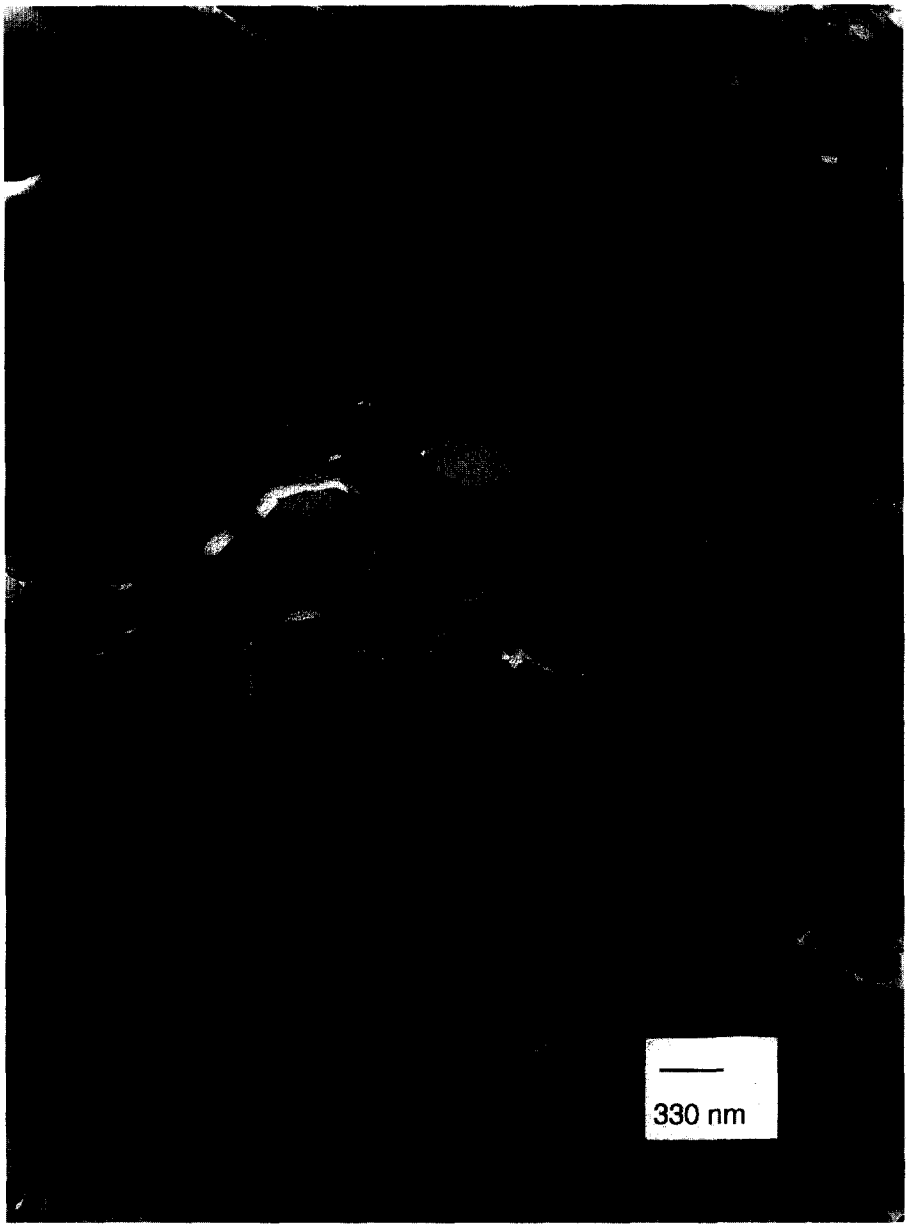

Fig. 8. Freeze-fracture image of lyophilized, dry iopromide-carrying liposomes.

tective effect of the X-ray contrast agent sodium ioxitalamate has been suggested in 1985 by Henry-Michelland et al. In the tested liposome preparation iopromide was present in the inner and outer aqueous phase prior to lyophilization. The presence of lyoprotective substances on both sides of the liposome membrane has been shown to be essential for the prevention of fusion and leakage during lyophilization (Crowe et al., 1985). In contrast, a liposome suspension without iopromide shows thermo- and lyotropic phase transitions during lyophilization which result in planar

Fig. 9. Freeze-fracture image of lyophilized, dry drug-free liposomes.

lipid layers as demonstrated by freeze-fracture EM (Fig. 9). Nevertheless, some of the iopromide-carrying liposomes also undergo changes during lyophilization due to limited protection. Upon rehydration new liposomes are formed, reflected by an increase in vesicle size and changes in

encapsulation efficiency. In 1984 Kirby and Gregoriadis employed a dehydration-rehydration method to produce liposomes with high encapsulation efficiencies (Kirby and Gregoriadis, 1984). Rehydration with water and tromethamine buffer results in vesicle size growth but a loss in encapsulation efficiency. This indicates that liposomes which are destroyed during freeze-drying reorganize to form more multilamellar structures. Due to their unfavourable lipid/water ratio MLV display lower encapsulation efficiencies compared to unilamellar vesicles. However, rehydration of the dry, lyophilized material with 135 mM mannitol solution results in higher encapsulation efficiencies and vesicle sizes of the rehydrated vesicles compared to the liposomes directly after preparation. A 300 mM mannitol solution has already been used for the rehydration of vesicles which were produce by a simplified dehydration-rehydration method (Cruz et al., 1993). By increasing the concentration of the mannitol solution we were able to further improve the encapsulation of iopromide after resuspension. Mannitol solutions prove to be more effective than isoosmolar sucrose- and KCl-solutions. Rehydration at low temperatures yields higher encapsulation efficiencies with mannitol solution and tro-methamine buffer as rm. Increasing the temperature of these two rm results in a decrease of encapsulation efficiency and an increase of vesicle size of the resuspended vesicles. With increasing temperature of the rm the microviscosity of the resulting liposomes decreases which might give rise to fusion and the formation of larger multilamellar liposomes which display a lower encapsulation efficiency.

In conclusion, iopromide-carrying liposomes could successfully be stabilized by lyophilization. By careful evaluation of the freeze-drying parameters and rehydration conditions an optimal lyophilization process as well as resuspension procedure was identified. Additionally a lyoprotective effect of the contrast agent iopromide could be demonstrated.

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