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Freeze drying of liposomes with free and membrane-bound cryoprotectants – the background of protection and damaging processes

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Abstract

Studies of the protective effects of different amounts of sucrose and glucose and a carbohydrate directly linked to the liposome surface on large unilamellar vesicles (LUV) built from soybean phosphatidylcholine (SPC) during lyophilization were carried out. Analyses of freeze-dried liposomes were conducted by particle size determination, retention of entrapped water-soluble marker and lipid mixing assay employing resonance energy transfer (RET). The extent of functionality of carbohydrates depends on their concentration and results from spacing mainly preventing fusion at low concentrations, membrane stabilization preventing leakage and the bulk sugar matrix mainly depressing aggregation at higher concentrations. By incorporating hexadecyl-β-D-galactopyranoside in SPC-LUV as membrane-bound cryoprotectant it could be shown that fixation of the sugar head of galactosides at the membrane surface only leads to prevention of fusion of liposomes. Although the galactoside does not exhibit a membrane stabilizing effect alone, it improves the protective effects of the free carbohydrates hyperadditively. However, this fact is discussed on the basis of sugar-sugar interactions by means of hydrogen bonding.

Key words: Liposome; Freeze-drying; Membrane fusion; Resonance energy transfer; Glycolipid

1. Introduction

Certain carbohydrates are capable of protecting membranes and vesicles from dehydration damage and of maintaining the integrity and barrier function of membranes. Experimental investigations on lyophilized and freeze-thawed liposomes have shown that carbohydrates can suppress vesicle fusion and phase separation of lipids (Strauss et al., 1986; Crowe et al., 1987).

The protective effects of sugars has been related to their ability to form hydrogen bonds between the polar region of the membrane lipids and the carbohydrate hydroxy groups (Crowe et al., 1984a). These interactions result in membrane fluidization of dehydrated lipids, which without sugars would undergo phase transitions

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and phase separations. Thus, damage of membranes consisting of fluid lipids can be prevented due to pseudohydration. In contrast, phase transition of rigid lipids which are in the gel phase is not affected (Crowe et al., 1988). There is only sparse knowledge of both the specific mechanisms of pseudohydration generation and the reasons for the different efficacy of various carbohydrates as cryoprotective agents (Crowe et al., 1984b; Madden et al., 1985). Relatively small amounts of carbohydrates are effective in preventing fusion, however, alone this is not sufficient to prevent leakage of vesicular contents. Only greater quantities of sugar can completely protect the membrane by fluidization, resulting in no phase transition or separation (Crowe et al., 1989).

By using fusion assays employing RET (Vanderwerf and Ullman, 1980; Struck et al., 1981) and by detecting the efflux of an entrapped marker out of the vesicles, the cryoprotective action of carbohydrates dissolved during the lyophilization process can be characterized (Crowe et al., 1986).

An approach to attaining a local, high concentration of carbohydrates on the membrane surface has been taken by incorporating synthetic glycolipids into the liposomal membrane. The galactose and maltose residues linked to cholesterol via polyoxyethylene spacers were effective cryoprotectants in the freeze-thaw process (Goodrich et al., 1988, 1991; Testoff and Rudolph, 1992). Recently, similar experiments have been performed using other glycolipids (Park and Huang, 1992).

We have undertaken this study in order to examine the effectiveness of the cryoprotection afforded by different amounts of glucose and sucrose to lyophilized LUV from SPC by detecting the fusion and aggregation events and by determinating 5(6)-carboxyfluorescein (6-CF) retention. For monitoring the effects of sugars on all possible liposome damage, we employed the fluid SPC lipids and the less effective cryoprotectants glucose and sucrose.

In addition to explaining the mechanism, we also wished to examine the possible cryoprotective effect of a carbohydrate directly attached to the polar region of SPC membranes. Therefore, taking hexadecyl-β-D-galactopyranoside as an example, a glycolipid was incorporated into SPC-LUV before lyophilization with different amounts of glucose. Comparison of these results should shed some light on the mechanisms of action of saccharide cryoprotectants and on the use of linked carbohydrates during lyophilization processes for mimicking the effects of bulk sugars.

2. Materials and methods

2.1. Chemicals

SPC was obtained from Lucas-Meyer (Germany), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), glucose and sucrose from Sigma (Germany) and 5(6)-carboxyfluorescein (6-CF) from Serva (Germany). 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) (NBD) and dipalmitoyl-*sn*-phosphatidylethanolamine-*N*-(Lissamine Rhodamine B sulfonyl) (Rh) were purchased from Avanti Polar Lipids (U.S.A.). All materials were used without further purification. All salts and buffer were of analytical grade. Hexadecyl-β-D-galactopyranoside was synthesized as described (Ogawa et al., 1981).

2.2. Vesicle preparation

LUV were prepared by extrusion of multilamellar vesicles (MLV). Briefly, a lipid film (30 μmol) was suspended either in 1 ml phosphate-buffered solution (PBS) (pH 7.4) or in 1 ml 0.2 M 6-CF containing the required amount of sugars. The resulting MLV dispersions were extruded (Extruder, Lipex Biomembrane, Vancouver) six times through a 200 nm polycarbonate membrane (Nucleopore, Heidelberg, Germany). Excess 6-CF was removed by dialysis against PBS.

2.3. Lyophilization and rehydration

Aliquots of 250 μl were rapidly frozen in a methanol bath of −45°C for 10 min and then freeze-dried for 20 h (Christ Beta 1–8K freeze

dryer, Osterode, Germany). The resulting lyophilization cake was rehydrated to its original volume at room temperature with distilled water.

2.4. Fluorescence measurements

Fluorescence emission was monitored with a Shimadzu model RF 5001 Spectrofluorometer.

2.5. Retention studies

Encapsulated and non-encapsulated 6-CF were determined before and after lyophilization/rehydration. Since 6-CF is encapsulated at self-quenching concentrations, non-encapsulated 6-CF was determined by direct measurement of the liposome dispersion. Encapsulated 6-CF was quantified by lysis of liposomes with 1% Triton X-100 (Serva, Germany).

2.6. Fusion / aggregation studies

The NBD/Rh lipid mixing assay (Struck et al., 1981) was used as dilution and mixing assay (Duezguenes et al., 1987). In the probe dilution assay (NBD and Rh in one vesicle population), the residual fluorescence of the labelled vesicle population containing 1 mol% of NBD and Rh was taken as 0% fusion. Aliquots of fluorescence labelled and unlabelled vesicles were mixed. 40 μl of this mixture was dispersed in 2 ml of 0.2 M PBS and measured before and after lyophilization. Complete intermixing could be initiated by addition of 1% Triton X-100 to this dispersion (= 100% fusion). NBD fluorescence was measured at 450 nm excitation and 530nm emission. Fusion was calculated from the increase in NBD fluorescence between 0 and 100%.

For the probe mixing assay, NBD (1 mol%) and Rh (1 mol%) were incorporated in two different liposome preparations. Both liposome populations were mixed in a 1 : 1 ratio and the resulting NBD fluorescence taken as 0% fusion/aggregation. After lyophilization/rehydration, the NBD fluorescence was followed as described above. Intermixing of lipids of these two vesicle populations leads to RET between NBD and Rh and a decrease in the NBD fluorescence signal.

2.7. Particle size

Vesicle size was determined by dynamic light scattering (DLS) using a Malvern IIc AutoSizer (Malvern, U.K.). The quoted values demonstrate

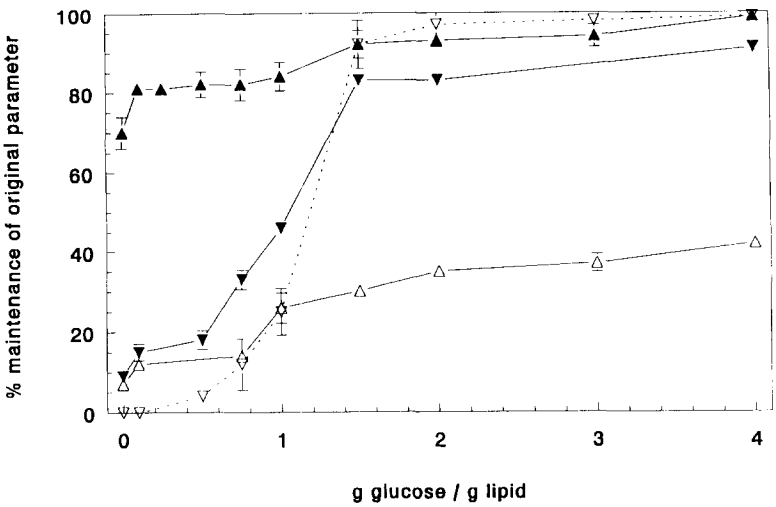

Fig. 1. Characterization of SPC-LUV after lyophilization with various amounts of glucose: (△) 6-CF retention; (▽) particle size; (▲) fusion; (▼) aggregation.

the maintenance of the original particle size in the mass distribution mode after lyophilization/rehydration.

2.8. Calorimetry

LUV for differential scanning calorimetry (DSC) were prepared as described above. For scans of hydrated probes, water instead of buffer for suspension of lipid film was used. Freeze-dried LUV were removed from the freeze dryer and stored at 55°C under pump vacuum. Samples were scanned from 250 to 370 K at a rate of 5 K per min (DSC 2C, Perkin Elmer).

3. Results

Stability of liposomes is an overall expression and includes different parameters. We investigated the retention parameters of fusion during the freeze-drying process. These parameters are compared in Fig. 1 and are expressed as maintenance of the original quality. For this, SPC liposomes were freeze-dried with different amounts of glucose and the parameters determined before and after the freeze-drying/rehydration process. The stability of freeze-dried SPC liposomes increases with increasing amounts of glucose. However, the influence of glucose on the single parameters is different. It is evident that fusion even without cryoprotectant is incomplete. The parameters of retention, aggregation and particle size were improved markedly, but to different extents, with increasing amounts of glucose. At a concentration of about 150% glucose (1.5 g glucose/g lipid) all parameters remained nearly constant. Glucose concentrations up to 400% only result in minor improvement of the curves. This indicates saturation of cryoprotective activity at about 150% glucose.

3.1. Aggregation and fusion

Size determination with DLS covers aggregation as well as fusion. The extents of aggregation and fusion processes were differentiated by an RET assay. The different curves for mixing and dilution assay in Fig. 2 demonstrate the different relevance of fusion and aggregation processes for liposome stability.

Fusion, which is selectively measured by dilution assay, only occurs to a minor extent. Also, Fig. 1 shows that the influence of fusion on the overall stability was very small, even if liposomes were dried without a cryoprotectant. In accor-

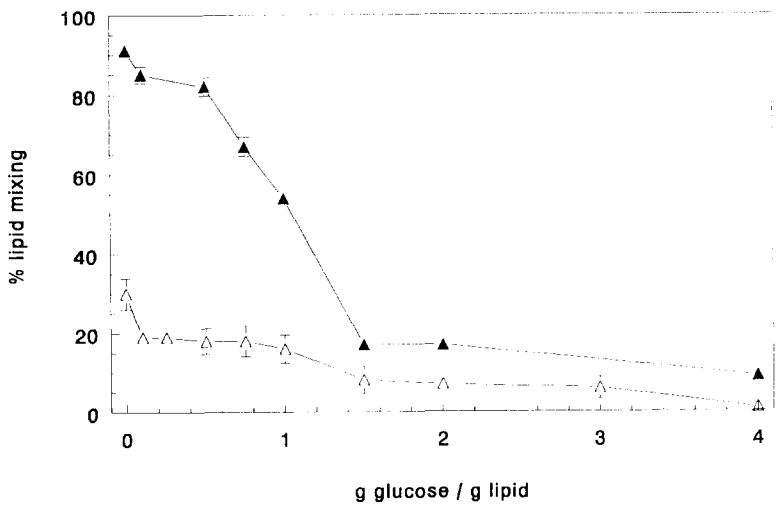

Fig. 2. Lipid mixing of lyophilized SPC-LUV as a function of glucose concentration: (△) dilution assay (fusion); (▲) mixing assay (aggregation/fusion).

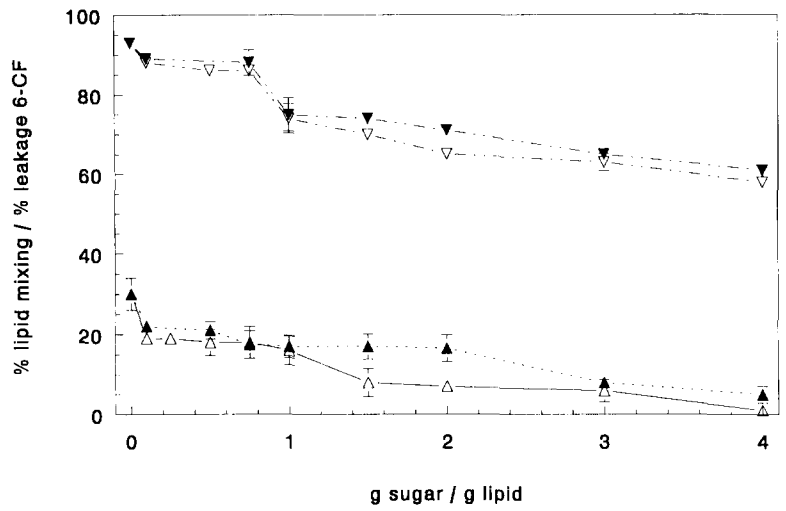

Fig. 3. Cryoprotective effect of glucose and sucrose as a function of sugar concentration (6-CF leakage and fusion): (▽) 6-CF leakage, glucose; (▼) 6-CF leakage, sucrose; (△) dilution assay, glucose (fusion); (▲) dilution assay, sucrose (fusion).

dance with literature data (Womersley et al., 1986), it was found that small amounts of glucose (< 10%) are sufficient to depress the fusion processes. This confirms the theory that small amounts of sugar molecules on the liposome surface may act as spacers between liposomes and thus protect against fusion during freeze-drying processes.

Aggregation, measured by mixing assay, also includes the RET signal of fusion processes. Because minor fusion was measured by dilution assay, conclusions regarding aggregation are pos-

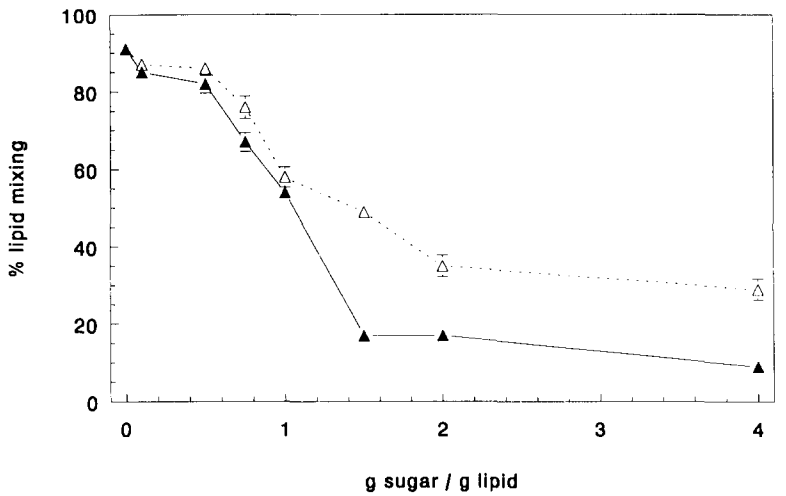

Fig. 4. Cryoprotective effect of glucose and sucrose as a function of sugar concentration (aggregation/fusion): (▲) mixing assay, glucose; (△) mixing assay, sucrose.

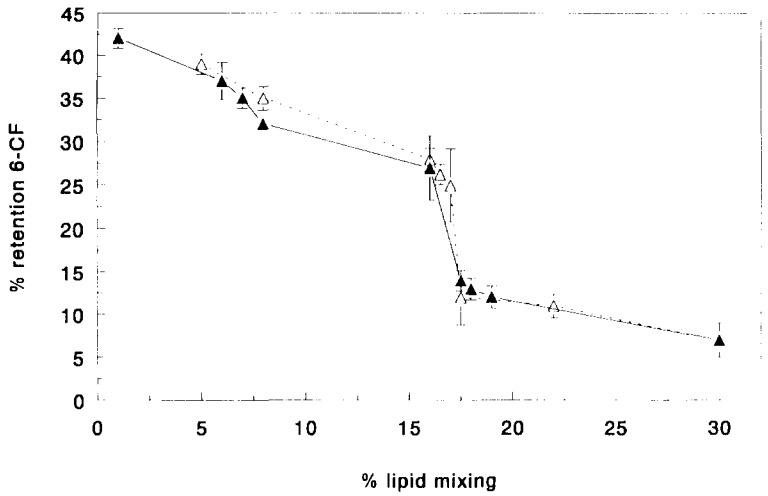

Fig. 5. Correlation between fusion (dilution assay) and 6-CF retention: (▲) glucose; (△) sucrose.

sible (Morgan et al., 1983). The mixing function, like the dilution function, shows a similar decrease between 0 and 10% glucose. This possibly can also be attributed to prevention of fusion due to the spacer function of sugar molecules between liposomes (Harrigan et al., 1990). Further increases in glucose concentrations result in considerably improved stability of the system. This is provides an indication for increasing amounts of cryoprotectant which are now available as bulk sugar and build up a matrix preventing aggregation between liposomes.

3.2. Comparison of cryoprotective activity of glucose and sucrose

In Fig. 3 and 4 the different parameters of retention, fusion (dilution assay) and aggregation (mixing assay) are compared for sucrose and glucose. The results confirm that stability improves

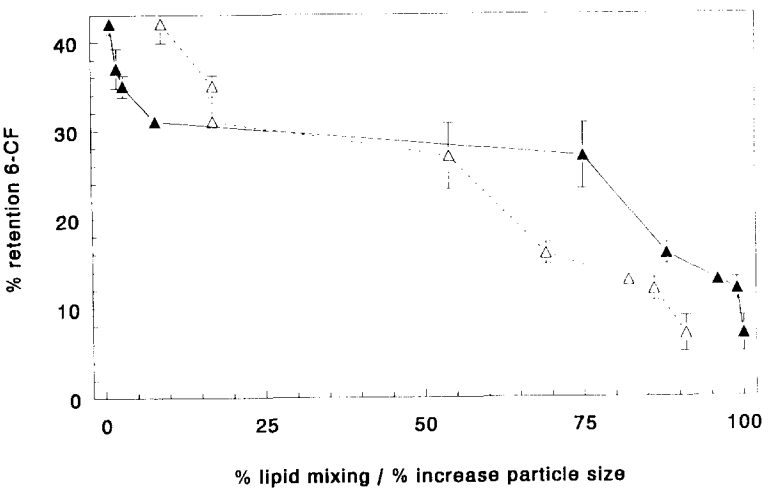

Fig. 6. Correlation between aggregation/fusion (mixing assay), particle size and 6-CF retention: (▲) particle size; (△) aggregation/fusion (mixing assay).

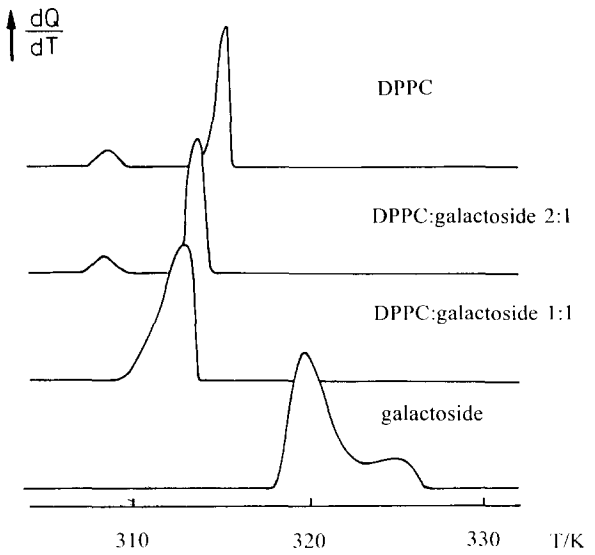

Fig. 7. DSC scans of lyophilized and rehydrated preparations of DPPC, galactoside and DPPC/galactoside mixtures.

with increasing amounts of cryoprotectant. It was interesting that the trends of the curves for glucose and sucrose are more or less the same. The effectiveness of the protective influence of sucrose is slightly lower than that of glucose. However, it should be noted that this is only the case for calculations in weight percent (M_w sucrose = 342.3 g/mol, M_w glucose = 180.2 g/mol). If calculations are carried out in mole percent, sucrose is about twice as active as glucose.

3.3. Interdependence of leakage, phase transition, fusion and aggregation

The functions for retention, fusion and aggregation in Fig. 3 and 4 are inconsistent and nonlinear. Different parts of the curves show drastically increasing protective effectiveness, for the single parameters in the same or different ranges of sugar concentration. This might indicate different qualities in the cryoprotective effect as a function of sugar concentration. The curves in Fig. 3 show that the increase in retention (expressed as a decrease in leakage) follows a more or less proportional prevention of fusion. This dependence is very obvious for cryoprotectant concentrations between 0 and 75%. The drastic increase in retention (less leakage) between 75 and 100% cryoprotectant (Fig. 3) is the result of complete membrane stabilization. This process is involved in the increasing formation of a sugar matrix at concentrations of 50 up to 150% cryoprotectant, preventing liposomes from aggregation and fusion, which results in a decrease in lipid mixing (Fig. 4).

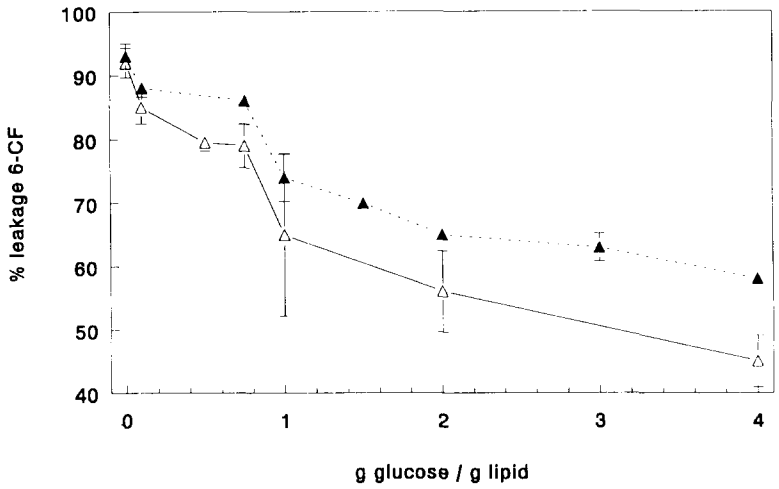

Fig. 8. 6-CF leakage of SPC/galactoside liposomes compared to SPC liposomes after lyophilisation with various amounts of glucose: (▲) SPC liposomes; (△) SPC/galactoside liposomes (2:1).

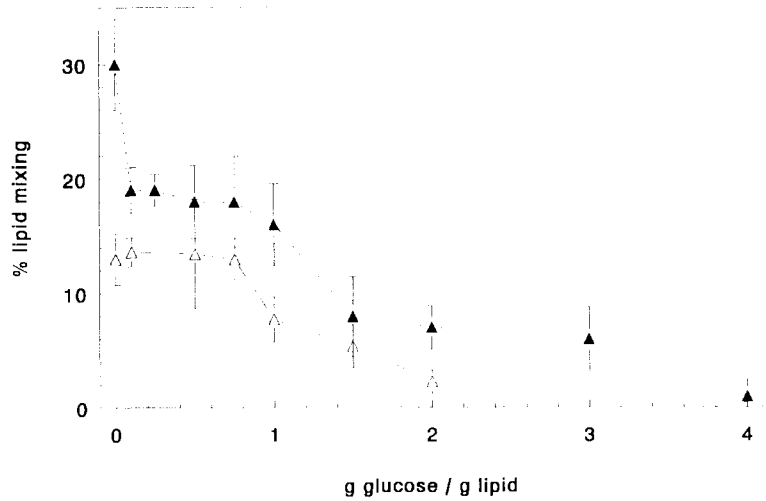

Fig. 9. Fusion (dilution assay) of SPC/galactoside liposomes compared to SPC liposomes after lyophilisation with various amounts of glucose: (▲) SPC liposomes; (△) SPC/galactoside liposomes (2:1).

Direct comparison of retention with fusion, aggregation and particle size illustrates qualitative differences in protection of liposomes at different sugar concentrations (Fig. 5 and 6). Improving stabilization of the liposomal preparation is indicated by decreased lipid mixing, maintenance of particle size and increased retention. However, the increase of the curves is not continuous. Retention of 6-CF improves linearly with suppression of fusion up to about 16% fusion. Between 16 and 17% fusion, retention increases by 16% and subsequently improves again linear with suppression of fusion (Fig. 5). Fig. 6 shows that after an initial improvement of retention up to 27% and depression of aggregation/fusion, the formation of a solid sugar matrix predomi-

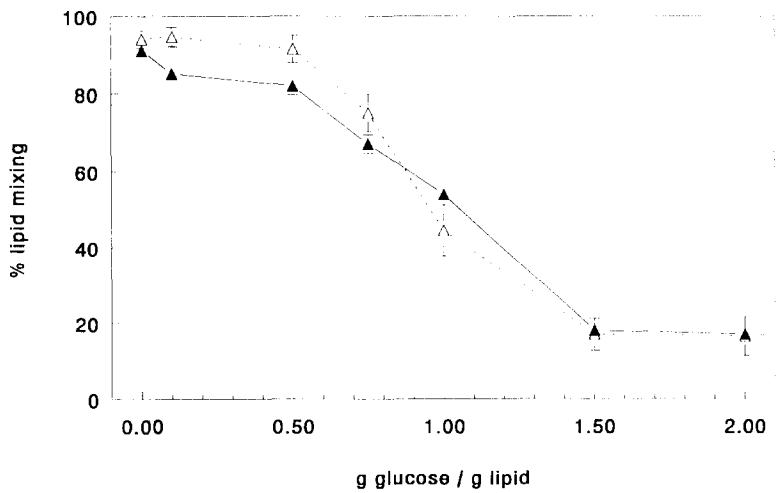

Fig. 10. Effect of glucose on inhibition of lyophilization induced aggregation/fusion (mixing assay) of SPC/galactoside liposomes compared to SPC liposomes after lyophilisation with various amounts of glucose: (▲) SPC liposomes; (△) SPC/galactoside liposomes (2:1).

nates between 27 and 31% retention, mainly depressing aggregation and fusion processes.

3.4. Cryoprotective activity of membrane-bound sugars

The previous investigations showed that sufficiently high concentrations of cryoprotectants on the membrane surface are essential for the efficient protection of liposomes during freeze-drying and rehydration. In addition to free cryoprotectants, incorporation of alkylglycosides into the phospholipid bilayer may also fulfill this requirement. To examine the cryoprotective activity of membrane-bound sugars, experiments for determination of retention, fusion and aggregation were repeated for SPC-LUV with incorporated glycolipid in a molar ratio of 2:1, as performed for free carbohydrates. The data from these experiments are shown for SPC-LUV containing hexadecyl-β-D-galactopyranoside, as an example. According to the DSC results (Fig. 7) and X-ray data (not shown), the integrity of the phospholipid bilayer is not affected by incorporation of galactoside. DSC scans of DPPC/galactoside liposomes (2:1) even demonstrated the typical DPPC pretransition, which is very unusal for lipid mixtures. Also, there was no indication of any phase separation.

Replacement of 33% (m/m) SPC against the glycolipid leads to direct fixation of 7.5% (w/w) sugar on the membrane surface, resulting in depression of the phase transition temperature (T_m), similar to free carbohydrates, in the dehydrated state (Table 1). It becomes evident, that fusion and leakage of SPC/galactoside vesicles are less compared to SPC vesicles (Fig. 8 and 9). It is very interesting that the curves for SPC and SPC/galactoside are qualitatively identical. The improvement of retention by galactoside cannot be equalled even with concentrations of free glucose up to 300%. Galactoside containing vesicles also show lower fusion rates up to 150% of free glucose. However, aggregation of galactoside containing vesicles is greater than for plain SPC liposomes up to 75% of free glucose and becomes nearly equal at higher concentrations of free cryoprotectant (Fig. 10).

Table 1
T_m of lyophilized DPPC, DPPC/glucose and DPPC/galactoside mixtures

Composition	T_m max (K)
DPPC monohydrate	346.5
DPPC + 10% glucose	335.2
DPPC + 25% glucose	322.6
DPPC + 100% glucose	319.6
DPPC + 400% glucose	317.9
DPPC + galactoside 2:1 (= 7.5% sugar)	332.4
DPPC + galactoside 1:1 (= 11.4% sugar)	329.8
DPPC + H_2O	314.4

4. Discussion

To design stable liposomal carriers, which survive the freeze-drying protocol, it is important to differentiate between the single damaging processes which may influence their stability. Here, we have investigated the parameters of the retention of entrapped water-soluble marker, particle size (DLS), aggregation and fusion (RET), to differentiate between these damaging processes.

Even if it is not possible simply to compare absolute values of the measured parameters, due to their different physical basis of measurement, comparison of the trends of the curves in Fig. 1 allows quantitative estimations of the overall liposome stability. It becomes clearly evident that liposome stability is a complex parameter. Determination of only a single parameter does not reflect the complexity of the problems and leads to erroneous conclusions (Burgess et al., 1991). Since liposome stability is very sensitive to dehydration this becomes very important for optimization of freeze-drying protocols. Conflicting results in the literature, especially for freeze-drying, illustrate this fact (Madden et al., 1985; Crowe et al., 1986; Womersley et al., 1986). It should be noted that the different methods for liposome preparation and characterization used by different groups are scarcely comparable with each other. This makes drawing of common conclusions for all systems very difficult if not impossible. Optimization of encapsulation and retention of water-soluble marker correspond to the ulti-

mate goal in designing stable liposomal carriers for water-soluble drugs. Retention of water-soluble marker is the most sensitive parameter and reflects all damaging processes which may occur during the freeze-drying process. Each membrane disturbance during freezing, drying and rehydration caused by phase transition, phase separation and fusion will result in loss of encapsulated marker (Crowe et al., 1988). However, retention of encapsulated marker does not allow one to differentiate between damaging processes. DLS provides overall information about processes which result in an increase of particle size which includes fusion and aggregation processes. Both can be differentiated with RET by use of the dilution and mixing assays. Fig. 2 shows that aggregation (mixing assay) compared to fusion (dilution assay) is the most predominant damaging process during freeze-drying. Fusion is especially relevant at low sugar concentrations (< 10%). Sugar concentrations between 50 and 150% result in drastic inhibition of aggregation. Higher concentrations of cryoprotectant have only minor effects on fusion and aggregation. Thus, the increase in particle size measured with DLS is predominantly caused by aggregation.

Fig. 3 compares the cryoprotective activity of glucose and sucrose for the parameters of retention and fusion. Surprisingly, it was found that the profiles for sucrose and glucose are nearly identical. This indicates that the curve characteristic for cryoprotection is independent of the cryoprotectant used. If sucrose and glucose concentrations (mass) are equal, the same cryoprotective activity is found. If, however, cryoprotective activity is expressed as mol sugar/mol lipid, only half of the amount of sucrose is necessary to achieve the same activity as glucose, indicating that only half as many sucrose molecules are necessary for the same protection. Better prevention of fusion by sucrose may be related to the larger molecular size of sucrose compared to glucose (Womersley et al., 1986). Also, the greater number of available OH groups of sucrose which can interact with the phospholipid head group and replace free water may be responsible for better cryoprotection. Differences between sucrose and glucose are observed for inhibition of aggregation (Fig. 4). If the masses of glucose and sucrose are compared, glucose shows better activities above 100% sugar.

Fig. 3–6 allow the following general conclusions to be drawn. Low sugar concentrations (up to 10%) result in clear suppression of fusion, probably by spacer effects of sugar molecules between the liposomes, which cover the liposome surface. Further increase of used sugar has only minor effects on fusion processes. Above 10% sugar (especially between 75 and 100%) liposome stability increases due to effective replacement of hydration water by pseudohydration with sugar OH groups. For this mechanism fewer sucrose molecules than glucose molecules are necessary. Beginning at about 50% sugar, an aggregation and fusion inhibiting matrix is also built up. At sugar concentrations above 150% all parameters only improve slowly. Depression of fusion and aggregation and improvement of retention are minor and increase to the same extent until they reach their maximal retention and minimal fusion/aggregation value (between 200 and 400% sugar). This differentiation of the freeze-drying process into different damaging and protecting mechanisms should allow a more rational evaluation and design of lyophilization processes.

Fixation of sugar molecules on the liposome surface appears to be a promising new means of improving liposome stability during freeze-drying. Calorimetric data of freeze-dried mixtures of DPPC/galactoside indicate interactions of sugar head groups with phosphate head groups. These interactions lead to depression of the gel-liquid phase transition in the dehydrated state. As seen in Table 1, addition of 400% glucose results in a transition which is close to that of hydrated DPPC. Because the amount of covalent bound sugar is limited to about 12%, depression of the phase transition is also insufficient and subsequently results in incomplete protection.

As shown in Fig. 8 and 9, incorporation of galactoside into the SPC membrane suppresses fusion and improves retention of 6-CF to a certain extent. Fig. 9 indicates that also without addition of free glucose fusion is suppressed to extent of about 17% by 7.5% covalently bound glucose (compared to SPC without glucose). Un-

fortunately, this protection is not reflected by increased 6-CF retention. If, however, free glucose is added, galactoside containing liposomes become less leaky than pure SPC liposomes. These differences become more obvious with higher glucose concentrations. Comparison of leakage for SPC and SPC/galactoside liposomes indicates that improved stability is not simply caused by the additional 7.5% sugar which is immobilized on the liposome surface. Moreover, retention of galactoside containing liposomes was improved hyperadditively and indicates an amplification of the cryoprotective activity of free sugar. The characteristic curve trend is maintained.

Preparations with covalently attached sugars show increased hydration. DSC investigations demonstrated strong interactions of glycolipid head groups, which are even stronger than those of phospholipids with equally hydrophobic regions (Iwamoto et al., 1982; Curatolo, 1987). It may be speculated that such interactions between free and membrane-bound sugar molecules may lead to enhanced localization of free sugar at the membrane surface and may be responsible for the hyperadditive cryoprotective effect of the galactoside. A high affinity of galactoside head groups to each other may also explain increased aggregation at low free sugar concentrations (Fig. 10). Glucose concentrations higher than 50% build up a sugar matrix between liposomes and equalize enhanced aggregation. There is no difference in aggregation between galactoside containing and plain SPC liposomes above 100% free glucose.

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References

Burgess, S.W., Massenburg, D., Yates, J. and Lentz, B.R., Poly(ethylene)-induced lipid mixing but not fusion between synthetic phosphatidylcholine large unilamellar vesicles. *Biochemistry*, 30 (1991) 4193–4200.

Crowe, J.H., Crowe, L.M. and Chapman, D., Infrared spectroscopic studies on interactions of water and carbohydrates with a biological membrane. *Arch. Biochem. Biophys.*, 232 (1984a) 400–407.

Crowe, J.H., Crowe, L.M. and Hoekstra, F.A., Phase transitions and permeability changes in dry membranes during rehydration. *J. Bioenerg. Biomembr.*, 21 (1989) 77–92.

Crowe, J.H., Crowe, L.M., Carpenter, J.F. and Aurell Wistrom, C., Stabilization of dry phospholipid bilayers and proteins by sugars. *Biochem. J.*, 242 (1987) 1–10.

Crowe, J.H., Crowe, L.M., Carpenter, J.F., Rudolph, A.S., Wistrom, C.A., Spargo, B.J. and Anchordoguy, T.J., Interactions of sugars with membranes. *Biochim. Biophys. Acta*, 947 (1988) 367–384.

Crowe, L.M., Crowe, J.H., Womersley, C., Reid, D., Appel, L. and Rudolph, A., Prevention of fusion and leakage in freeze-dried liposomes by carbohydrates. *Biochim. Biophys. Acta*, 861 (1986) 131–140.

Crowe, L.M., Mouradian, R., Crowe, J.H., Jackson, S.A. and Womersley, C., Effects of carbohydrates on membrane stability at low water activities. *Biochim. Biophys. Acta*, 769 (1984b) 141–150.

Curatolo, W., The physical properties of glycolipids. *Biochim. Biophys. Acta*, 906 (1987) 111–136.

Duezguenes, N., Allen, T.M., Fedor, J. and Papahadjopoulos, D., Lipid mixing during membrane aggregation and fusion: Why fusion assays disagree. *Biochemistry*, 26 (1987) 8435–8442.

Goodrich, R.P., Crowe, J.H., Crowe, L.M. and Baldeschwieler, J.D., Alterations in membrane surfaces induced by attachement of carbohydrates. *Biochemistry*, 30 (1991) 5313–5318.

Goodrich, R.P., Handel, T.M. and Baldeschwieler, J.D., Modification of lipid phase behavior with membrane-bound cryoprotectants. *Biochim. Biophys. Acta*, 938 (1988) 143–154.

Harrigan, P.R., Madden, T.D. and Cullis, P.R., Protection of liposomes during dehydration or freezing. *Chem. Phys. Lipids*, 52 (1990) 139–149.

Iwamoto, K., Sunamoto, J., Inoue, K., Endo, T. and Nojima, S., Importance of surface structure in liposomal membranes of glyceroglycolipids. *Biochim. Biophys. Acta*, 691 (1982) 44–51.

Madden, T.D., Bally, M.B., Hope, M.J., Cullis, P.R., Schieren, H.P. and Janoff, A.S., Protection of large unilamellar vesicles by trehalose during dehydration: retention of vesicle contents. *Biochim. Biophys. Acta*, 817 (1985) 67–74.

Morgan, C.G., Thomas, E.W. and Yiannakis, Y.P., The use of fluorescence energy transfer to distinguish between poly(ethylene glycol)-induced aggregation and fusion of phospholipid vesicles. *Biochim. Biophys. Acta*, 728 (1983) 356–362.

Ogawa, T., Beppu, K. and Nakabayashi, S., Trimethylsilyl trifluormethansulfonate as an effective catalyst for glycoside synthesis. *Carbohydr. Res.*, 93 (1981) C6–C9.

Park, Y.S. and Huang, L., Cryoprotective activity of synthetic glycophospholipids and their interactions with trehalose. *Biochim. Biophys. Acta*, 1124 (1992) 241–248.

Strauss, H., Schurtenberger, P. and Hauser, H., The interaction of saccharides with lipid bilayer vesicles: stabilization during freeze-thawing and freeze-drying. *Biochim. Biophys. Acta*, 858 (1986) 169–180.

Struck, D.K., Hoeckstra, D. and Pagano, E., Use of resonance energy transfer to monitor membrane fusion. *Biochemistry*, 20 (1981) 4093–4099.

Testoff, M.A. and Rudolph, A.S., Modification of dry 1,2-dipalmitoylphosphatidylcholine phase behavior with synthetic membrane-bound stabilizing carbohydrates. *Bioconjugate Chem.*, 3 (1992) 203–211.

Vanderwerf, P. and Ullman, E.F., Monitoring of phospholipid vesicle fusion by fluorescence energy transfer between membrane-bound dye labels. *Biochim. Biophys. Acta*, 596 (1980) 302–314.

Womersley, C., Uster, P.S., Rudolph, A.S. and Crowe, J.H., Inhibition of dehydration-induced fusion between liposomal membranes by carbohydrates as measured by fluorescence energy transfer. *Cryobiology*, 23 (1986) 245–255.