Stability of Dry Liposomes in Sugar Glasses

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ABSTRACT Sugars, particularly trehalose and sucrose, are used to stabilize liposomes during dehydration (freeze-drying and air-drying). As a result, dry liposomes are trapped in a sugar glass, a supersaturated and thermodynamically unstable solid solution. We investigated the effects of the glassy state on liposome fusion and solute retention in the dry state. Solute leakage from dry liposomes was extremely slow at temperatures below the glass transition temperature (T_g); however, it increased exponentially as temperature increased to near or above the T_g, indicating that the glassy state had to be maintained for dry liposomes to retain trapped solutes. The leakage of solutes from dry liposomes followed the law of first-order kinetics and was correlated linearly with liposome fusion. The kinetics of solute leakage showed an excellent fit with the Arrhenius equation at temperatures both above and below the T_g, with a transitional break near the T_g. The activation energy of solute leakage was 1320 kJ/mol at temperatures above the T_g, but increased to 1991 kJ/mol at temperatures below the T_g. The stabilization effect of sugar glass on dry liposomes may be associated with the elevated energy barrier for liposome fusion and the physical separation of dry liposomes in the glassy state. The half-life of solute retention in dry liposomes may be prolonged by storing dry liposomes at temperatures below the T_g and by increasing the T_g of the dry liposome preparation.

INTRODUCTION

Preservation of dry liposomes has been achieved by the discovery that sugars are able to protect membranes during dehydration (air-drying or freeze-drying). The roles of sugars, particularly trehalose and sucrose, include the stabilization of membrane by lowering lipid phase transition temperature (T_m) and the prevention of liposome aggregation and fusion (Crowe et al., 1984, 1986, 1989; Hoekstra et al., 1992; McKersie et al., 1989; Anchordoguy et al., 1987; Womersley et al., 1986). It has been demonstrated that sugar molecules directly interact with membranes by forming hydrogen bonds with the polar headgroups of phospholipids (Crowe et al., 1984, 1994).

Because sugars are added at high concentrations before drying, dry liposomes inevitably exist in a sugar glass matrix. As water is removed during air-drying, sucrose solution is increasingly concentrated. The transition of a dilute solution to a stable glass is achieved by removing 99% water in the samples. Because sucrose does not precipitate along the solidus line, the concentrated sucrose solution becomes supersaturated when it is dried to a water content below the saturation point. As drying continues, the concentrated solution will transform into a syrup, then to a viscoelastic rubber, and finally to a stable glass. During freeze-drying, a similar conversion from a liquid to a glass is also achieved by freeze-induced dehydration (ice formation) and then vacuum sublimation.

It has been shown that the glassy state is essential for stabilizing liposomes during freeze-drying (Crowe et al., 1994). It is possible that the glass matrix prevents liposome fusion and protects liposomes from the mechanical damage of ice crystals during freeze-drying. Recent studies by the same group concluded that the glassy state was also required for liposome stabilization during air-drying at ambient temperatures. They found that the samples must be dried at temperatures below the glass transition temperatures (T_g) (Crowe et al., in press). However, these studies did not investigate the mechanism by which the glassy state stabilizes liposomes during dehydration, or the stabilization role of the glassy state during storage of dry liposomes.

The glassy state is a supersaturated, thermodynamically unstable solid state. Its real-time stability depends on kinetic stability, which is achieved by virtue of the extremely high viscosity of the glassy state (Franks, 1993). The kinetic stability of the glass could play an important role in dry liposome stabilization. However, as far as we know, there has been no study on the kinetic stability of dry liposomes in the glassy state, although products using dry liposomes as drug carriers are already commercially available. Studies on kinetic stability are essential for understanding the role of the glassy state in liposome stability, estimating shelf-life, and formulating the product for long-term storage.

In the present study, we investigated the effects of the glassy state on liposome fusion and solute retention inside air-dried liposomes. Here we report a direct relationship between the glass state transition and solute leakage of dry liposomes. The stabilization effect of sugar glass is associ-

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ated with the high energy barrier for liposome fusion in the glassy state and the physical separation of dry liposomes.

MATERIALS AND METHODS

Dry liposome preparation

Liposomal samples were prepared with sucrose (Fisher Scientific, Fair Lawn, NJ) and egg phosphatidylcholine (egg PC) (Avanti Polar Lipids, Birmingham, AL). To remove chloroform in which the lipid is delivered by the manufacturer, the lipid was dried under a stream of nitrogen, followed by vacuum for 2 to 3 h (<20 μm Hg). The dried lipid was mixed with an equal mass of sucrose and rehydrated (25 mg lipid/ml) in 0.2 M carboxyfluorescein (CF). The lipid suspension was extruded through membrane filters (pore size, 100 nm) 15 times, using a Lipofast extruder (Avestin, Ottawa, ON, Canada). CF outside the liposomes was removed by passage through a 16-cm Sephadex G-50 column. The liposomes were collected and diluted with sucrose solution to 5 mg lipid/ml and 55 mg sucrose/ml. The liposomes were air-dried in 20-μl aliquots on the lids of 0.5-ml Eppendorf tubes for 2 to 4 h in 0% relative humidity as described elsewhere (Crowe et al., in press). Liposomal samples prepared for fusion study contained 20 mg lipid/ml and 50 mg sucrose/ml. Residual water content in the liposomal sample was 0.02 to 0.04 g water/g dry sample (g/g dw), measured gravimatrically after heating overnight at 110°C. Dry liposomes were sealed and stored with C_aSO_4 as the desiccant. Size of the vesicles was measured with a Brookhaven particle size analyzer, using 2000 scans. CF retention inside the liposomes was determined by spectrofluorometry as described previously (Crowe et al., 1986).

Storage experiments

Two storage experiments were carried out to investigate the kinetics of liposome fusion and solute retention at temperatures above and below glass transition. In the first experiment, air-dried liposomes were stored at constant temperatures from 4°C to 90°C for 2 h, and then rehydrated with distilled water for CF retention measurements. The increase in liposome size, as a measure of fusion, was determined after 1 h of storage. In the second experiment, liposomes were stored for various periods at constant temperatures. CF retention was plotted against storage time, and the rate constants at various storage temperatures were calculated.

Effects of sorption of water vapor on stability

The stability of dry liposomes in the glassy state was also studied by altering the glass transition temperatures through sorption of water vapor in different relative humidities. Dry liposomes (with sucrose) were equilibrated over various saturated salts to provide known relative humidities (Rockland, 1960; Winston and Bates, 1960; Young, 1967), and CF leakage during rehydration was measured over time. Water contents were determined after drying the liposomal samples at 95 to 98°C under vacuum for 48 h. From the gravimetrical determinations we constructed the sorption isotherm (Fig. 1), which was subsequently used to estimate glass transition temperatures of samples incubated at the same relative humidities.

Determination of glass transition temperature

Sucrose was dissolved in distilled water (1 g sucrose/ml water). The lipid-sucrose suspension, rehydrated with 0.2 M carboxyfluorescein, was sonicated to clarity. Sucrose and liposomal samples were pipetted into 25-μl differential scanning calorimetry (DSC) aluminum pans, air-dried to various water contents, and mechanically sealed. Glass transitions of samples were determined with a Perkin-Elmer DSC-2 differential scanning calorimeter. Samples were cooled first at a rate of 20°C/min to −80°C and then scanned at a rate of 5°C/min from −80°C to 90°C. Glass transition temperatures were taken as the midpoint in the baseline shift that accompanies glass transitions. The samples were weighted both before and after DSC measurement. No detectable change in sample weight was recorded. After the DSC scanning, the sealed sample pans were punctured, and samples were dried in 98°C under vacuum for at least 48 h. Dry samples weighed approximately 13 to 16 mg. Water contents were determined gravimetrically.

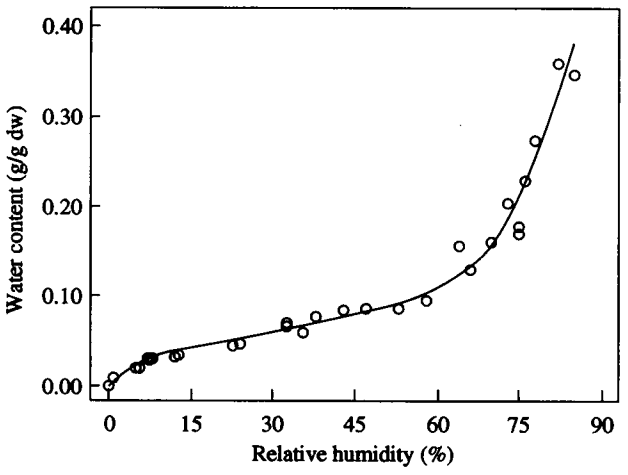

FIGURE 1 Water sorption isotherm of egg PC-sucrose liposomes at 23°C (room temperature). Dried samples were equilibrated for 2 days over various saturated salt solutions with known relative humidities (with excessive salts present). The data were fitted with the D'Arcy-Watt sorption model (*solid line*). Note that water activity in samples equals relative humidity divided by 100.

RESULTS

In the present study, a high mass ratio of sucrose:lipid (11:1) was used to prepare dry egg PC liposomes. The sucrose concentration before air-drying was 55 mg/ml. It was previously reported that this concentration was sufficient to stabilize liposomes during dehydration (Crowe et al., 1994; Mobley and Schreier, 1994). During air-drying, sucrose was expected to form the glassy state, and dried liposomes were preserved in such a sugar glass matrix. The mean diameter of liposomes was found to be 97 nm before air-drying, and 102 nm after air-drying and rehydration. CF retention inside liposomes was approximately 78% after drying. This result showed that the liposome aggregation and fusion during air-drying were effectively prevented.

To study the effect of the glassy state on the retention of trapped solutes in liposomes, we developed glass transition state diagrams of sucrose and egg PC-sucrose liposome preparation. The glass transition was determined with differential scanning calorimetry for samples at water contents from 0.02 to 0.40 g water/g dry sample (Fig. 2). The T_g of liposomal samples increased as sample water content was reduced. The glass transition state diagram of liposomal samples was essentially identical to that of the sucrose-water system, as we expected. These data of the glassy state transition showed that when the concentrated solution was dried to water content below 0.05–0.06 g/g dw, it transformed to a stable glass even at room temperatures (Fig. 2).

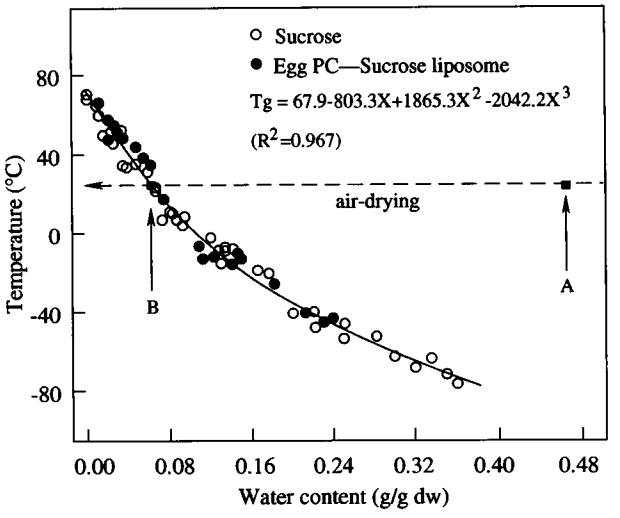

FIGURE 2 State diagrams of glass transition for sucrose and egg PC-sucrose liposomal samples. The formation of a stable glass from a dilute solution is achieved by air-drying to remove 99% water. (*A*) 0.46 g/g dw, saturation points of sucrose solutions at room temperature (25°C). (*B*) 0.05–0.06 g/g dw, water contents at which the glass transition occurs during air-drying. Liposomes were prepared with a lipid/sucrose mass ratio of 1:11 (see text for details). Glass transition was measured by DSC at a scan rate of 5°C/min. The solid line shows the equation of the best fit to the T_g data for both sucrose and egg PC-sucrose liposomes. All T_g data for liposomal samples are from the present study. Nineteen points of sucrose T_g data are from the present study, and 20 points are taken from Mackenzie (1977), Green and Angell (1989), and Roos and Karel (1990, 1991). Saturation points of sucrose solutions are calculated with the solubility data from Windholz (1983).

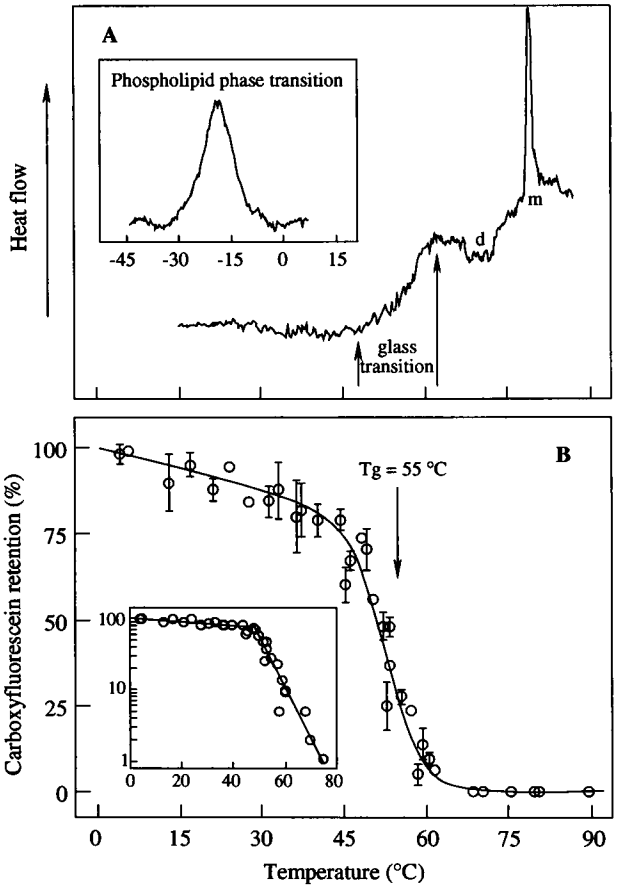

FIGURE 3 The effect of the glassy state on carboxyfluorescein (CF) retention inside egg PC-sucrose liposomes. (*A*) The DSC thermogram of air-dried egg PC-sucrose liposomes at a scan rate of 5°C/min. Sample water content was approximately 0.02–0.03 g water/g dry sample. The melting of phospholipids occurred at −20°C (*inset*), and the glass transition between 48 and 63°C. d, devitrification. The peak at 80°C was probably from the melting of crystallized carboxyfluorescein. (*B*) CF retention inside egg PC-sucrose liposomes after storage at different temperatures for 2 h. CF retention decreased dramatically at temperatures near the glass transition. Error bars are ± standard deviation. The same data set was plotted in logarithmic scale (*inset*).

The air-dried liposomal sample had a residual water content of 0.02–0.03 g/g dw. The sample was scanned with differential scanning calorimetry at 5°C/min, and the glass transition was observed between 48°C and 63°C (Fig. 3 *A*). The T_g of the sample, the midpoint temperature, was 55°C. The phase transition of egg PC occurred at approximately −20°C. The sharp peak at 80°C was not identified. This endothermic event could not be sucrose crystallization after devitrification or the melting peak of sucrose crystals, because crystallization should give an exothermic peak, whereas the melting temperature of sucrose crystals is approximately 186°C. This peak might be due to the melting of crystallized carboxyfluorescein in the dry sample.

To investigate the CF retention inside liposomes at temperatures above and below the glass transition, air-dried liposomal samples were stored at constant temperatures from 4°C to 90°C for 2 h. CF retention in liposomes remained quite high after storage at temperatures below the T_g of the sample. However, as the storage temperature was increased to the region of glass transition or above T_g, CF retention decreased remarkably (Fig. 3 *B*). After storage for 1 h, a set of samples was rehydrated and the size of liposomes (as a measure of fusion) was measured. The average liposome diameter increased dramatically when samples were stored at temperatures near T_g and above T_g, showing liposome fusions increased as the storage temperature was raised (Fig. 4). The data in Figs. 3 and 4 show a clear correlation between liposome fusion and solute leakage. No similar temperature-dependent effect was observed in several studies on solute leakage and liposome fusion of egg PC liposomes in solutions between 5 and 80°C (Anchordoguy et al., 1992; Torchilin et al., 1992; Kono et al., 1994).

The kinetics of CF leakage from dry liposomes followed first-order kinetics during storage. The plots of ln (CF retention) against storage time showed linear relationships at all temperatures tested from 4°C to 90°C (Fig. 5). The rate constants (k) of CF leakage, calculated from the first-order plots, were extremely small when samples were stored at temperatures below T_g, but increased exponentially at temperatures above T_g (Fig. 6 *A*). The kinetics of CF leakage showed an excellent fit with the Arrhenius equation at temperatures both below and above the T_g (*solid curve*).

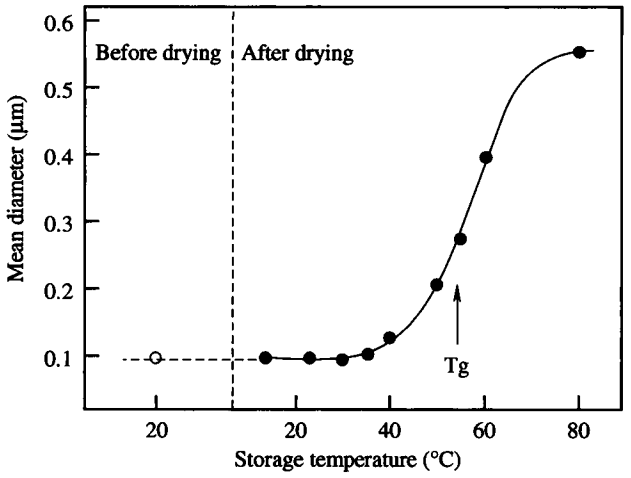

FIGURE 4 Average diameters of air-dried egg PC liposomal samples after 1-h storage at various temperatures. The glass transition was observed between 48°C and 63°C with differential scanning calorimetry. The arrow indicates the T_g, the mid-point temperature. The size of fully rehydrated liposomes was measured with a Brookhaven particle size analyzer using 2000 scans.

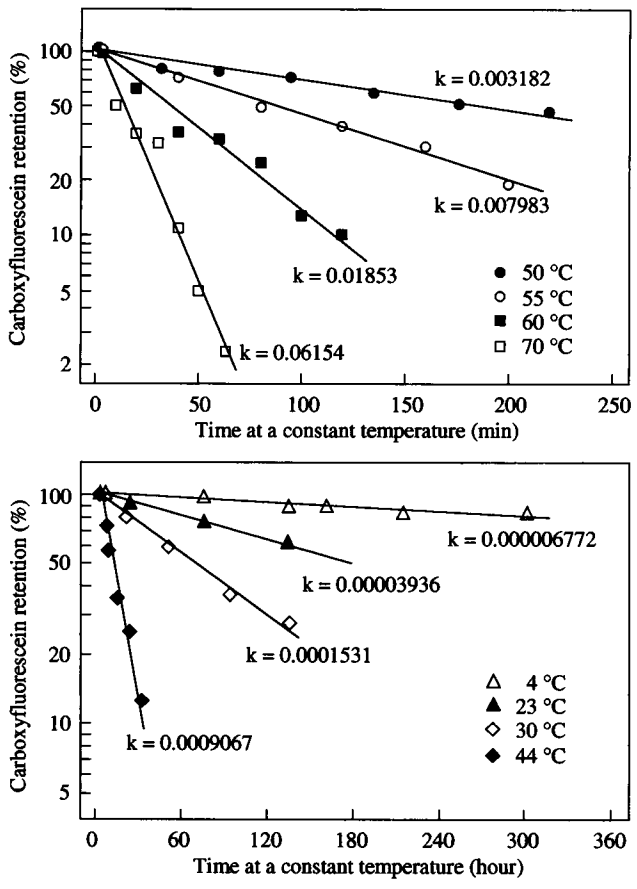

FIGURE 5 First-order plots of carboxyfluorescein retention inside dry egg PC-sucrose liposomes during storage at constant temperatures. The rate constants (k, the slopes) were calculated for various temperatures. For clarity, not all temperature experiments were plotted.

The effect of the glassy state on CF retention in air-dried samples was also investigated by altering the T_g of samples through sorption of water vapor in different relative humidities. The CF leakage during rehydration also followed the law of first-order reaction (Fig. 7). We estimated the T_g of liposomal samples rehydrated at different relative humidities and measured CF retention inside liposomes after 48 h (Fig. 8). Again, when the T_g of rehydrating samples was near or above the rehydration temperature, CF retention inside liposomes remained high. On the other hand, when the sample was not in the glassy state, CF retention decreased rapidly. The data of isothermal experiments (Figs. 7 and 8) were treated similarly with these techniques, which were used to analyze the data of storage experiments at various temperatures. The plot of rate constant (k) of CF leakage against $T - T_g$ (T = 23°C) (data not shown) resembled the relationship between the rate constant and temperature in Fig. 6.

DISCUSSION

Effects of the glassy state on liposome fusion and solute retention

We tested in the present study the stability of air-dried liposomes in the glassy state through two kinds of experiments. First, dry liposomal samples were stored at constant temperatures above or below T_g. The solute leakage from liposomes in the dry state increased exponentially when the storage temperature was near and above T_g, indicating that the glassy state was essential for dry liposomes to retain trapped solutes during storage (Figs. 3, 6, and 8). The measurement of liposomal sizes found that average liposome diameter increased significantly at temperatures near T_g and above, showing that liposome fusions occurred as the storage temperature was raised (Fig. 4). These results show a direct relationship between liposome fusion and solute leakage of dry liposomes and the glass transition. Second, the T_g of dry liposomal samples was manipulated through the sorption of water vapor in specific relative humidities. The isothermal experiments yielded the same results as storage experiments and helped us to rule out a temperature-dependent effect on liposome fusion and solute leakage independent of the glass state transition. One might consider the shift of hydrogen bonding between sugar molecules and the headgroups of membrane phospholipids to the hydrogen bonding between water and phospholipids as a possible candidate for the destabilizing effect of water vapor sorption, because of the possibility that the breaking of the appropriate hydrogen bonding during hydration may destabilize the membranes. It is unlikely that the sorption of water vapor would destabilize the membranes by breaking up the hydrogen bonding between sugar molecules and phospholipids. In fact, the hydration with water vapor was found to decrease the T_m of phospholipids and was used to pre-rehydrate membranes to prevent imbibitional damage

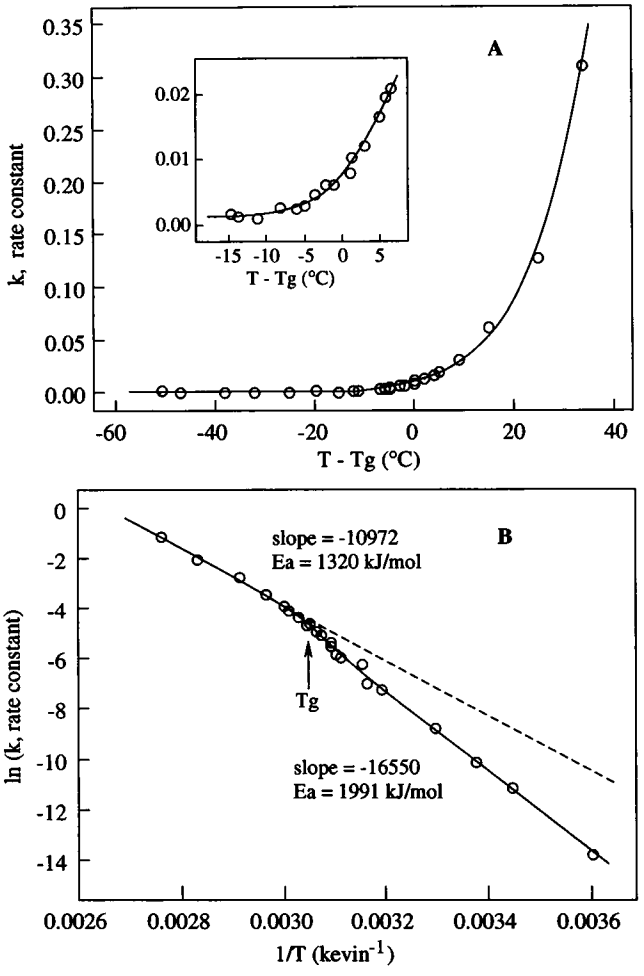

FIGURE 6 (*A*) Rate constants (data points) of carboxyfluorescein leakage from dry liposomes at temperatures below and above glass transition temperature (T_g). The data points below T_g and above T_g were separately fitted with the Arrhenius equation. Solid curve shows the values predicted from the Arrhenius equations. (*Inset*) the change near glass transition temperature. (*B*) The Arrhenius plot of carboxyfluorescein leakage (liposome fusion) in dry liposomes. The plot shows a break to the linear relationship between $1/T$ and ln k (rate constant) near glass transition. In the glassy state, the activation energy increased by 51% to 1991 kJ/mol.

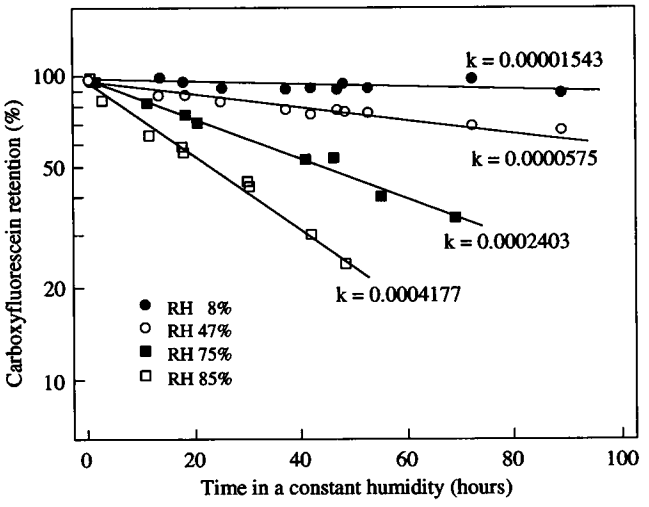

FIGURE 7 First-order plots of carboxyfluorescein retention inside egg PC-sucrose liposomes at 23°C during water vapor rehydration in four relative humidities. k, rate constants.

(solute leakage) on pollen membranes by liquid water rehydration (Crowe et al., 1989; Hoekstra et al., 1992).

The observed relationship between liposome fusion, solute leakage, and the glass transition is not a simple temperature-dependent effect. Such a relationship is not found for liposome in solution, which does not undergo a glass transition from 4°C to 90°C. The temperature effect on solute leakage and liposome fusion of egg PC liposomes in solutions has previously been published (Anchordoguy et al., 1992; Torchilin et al., 1992; Kono et al., 1994). Those studies showed that egg PC liposomes in solutions did not aggregate or undergo fusion at all during incubation between 5 and 80°C. CF leakage from liposomes during incubation was small, only about 10–15% at high incubation temperatures (Anchordoguy et al., 1992). The presence of sugars significantly reduced the solute leakage of liposomes during the incubation of solutions (Fabrie et al., 1990). However, we were unable to examine the temperature effects on liposome fusion and solute leakage of partially hydrated liposomes or dry liposomes in the absence of sugars. Partially hydrated liposomes (i.e., 0.2–1.0 g H_2O/g dry sample) are extremely unstable; liposome fusion and solute leakage occur so rapidly that it is difficult to make accurate measurements. Drying of liposomes in the absence of sugars resulted in complete membrane damage and 100% solute leakage. Therefore it is impossible to proceed with such an experiment.

In the dry state and in solutions, solute leakage of liposomes occurs when the phospholipids are undergoing a phase transition from the gel state to the liquid crystalline state (Crowe et al., 1986, 1989, 1994; McKersie et al., 1989; Fabrie et al., 1990; Torchilin et al., 1992). However, the observed relationship between liposome fusion, solute leakage, and the glass transition cannot be attributed to the phase transition. In the present study, we observed the egg PC phase transition at −20°C (Fig. 3 A), which was about 70°C below the glass transition temperature of the sucrose glass matrix. This result was consistent with our previous study, which showed that the presence of sugars (i.e., trehalose and sucrose) during drying depressed the T_m of egg PC to below −5°C in the dry state (Crowe et al., 1994).

Contribution of liposome fusion to solute leakage of dry liposomes

The increase in liposome size due to liposome fusion appears to be highly correlated with solute leakage in dry liposomes (Figs. 3 and 4). Similar correlations have been observed repeatedly (Crowe et al., 1984, 1986, 1994). However, a cause-and-effect relationship between liposome fusion and solute leakage is always difficult to establish. The same phenomena that induce liposome fusion may also

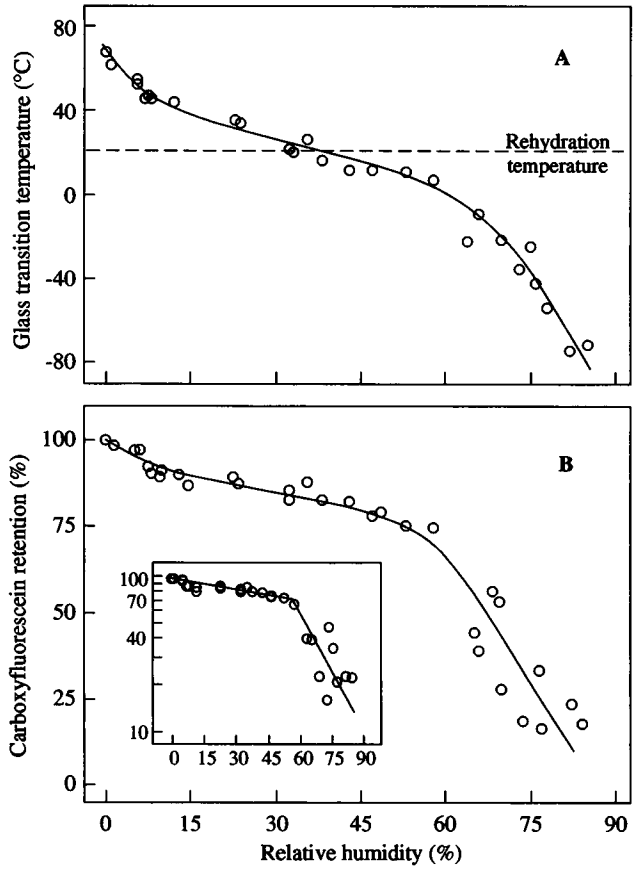

FIGURE 8 (*A*) Glass transition temperatures (T_g) of liposomal samples rehydrated in different relative humidities. Water contents of samples was determined after water vapor rehydration of 48 h. T_g was calculated according to the data in Figs. 1 and 2. (*B*) Carboxyfluorescein retention inside liposomes after water vapor rehydration at 23°C for 48 h. The same data set was plotted in logarithmic scale (inset).

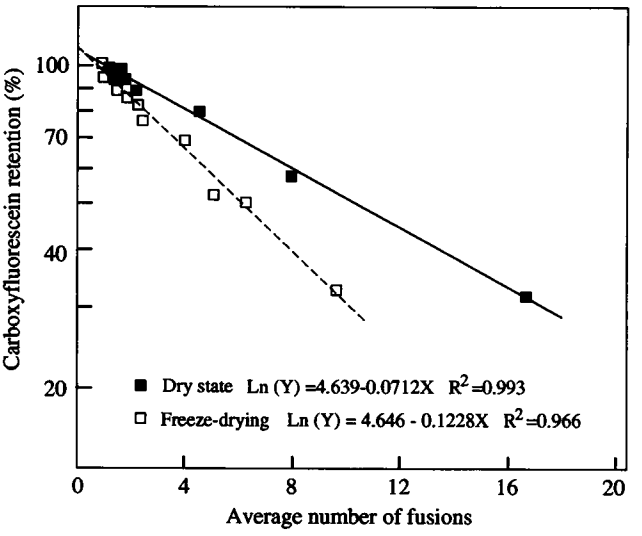

FIGURE 9 Pseudo-first-order plots showing a linear relationship between the number of liposome fusions and leakage of carboxyfluorescein from egg PC-sucrose liposomes. The average number of fusions occurring in a particular sample was calculated according to the surface area of fully rehydrated liposomes (see text for details). The data collected during freeze-drying were adopted from a previous study (Crowe et al., 1994).

independently make liposomes leaky. We investigated this effect further by approximating the number of fusion events per liposome during storage at various temperatures. For this purpose we calculated changes in surface area from the measurement of diameters of the liposomes made by laser light scattering (Fig. 4). The number of fusions that dry liposomes in samples had been involved in during storage could be calculated according to the change of surface area of rehydrated liposomes after storage. The number of dry liposomes that formed the larger liposomes after a certain period of storage is given by dividing the liposomal surface area after storage by that before storage. This number indicated the degree of fusions during storage. It should be pointed out that liposomal sizes such as the diameter and volume of rehydrated liposomes were not good measurements of the degree of liposome fusions because of the different number of dimensions. When the data of solute retention were plotted against the number of liposome fusions, the pseudo-first-order plot showed a linear relationship between liposome fusion and solute leakage during storage (Fig. 9, *filled squares*).

It is interesting to note that such a relationship also exists for liposomes during freeze-drying. In a deep freezing condition, liposomes are present in the concentrated amorphous matrix (glass). Crowe et al. (1994) studied the stabilization effect of the glassy state on frozen liposomes. From the published data we are able to establish a pseudo-first-order relationship similar to that for dry liposomes in the sugar glass (Fig. 9, *open squares*). The difference in slopes between dry liposomes and frozen liposomes can be easily explained, because freezing itself is another type of stress. This result suggests that the glassy state may use similar stabilization mechanisms to prevent liposome fusion during freezing and during dry storage.

Membrane leakiness due to stress can cause solute leakage without liposome fusion. For example, the vesicle-to-vesicle adhesion strains the membranes, leading to solute leakage outside the points of liposome contact. Some solute leakage with no liposome fusion has frequently been observed for egg PC liposomes in aqueous solution as temperature increases (Anchordoguy et al., 1992; Kono et al., 1994). In the present study, we have detected a 22% CF leakage from liposomes during drying with an initial sucrose concentration of 55 mg/ml, and the solute leakage was not accompanied by an increase in the mean diameter of liposomes (see Fig. 4). With scanning electron microscopy, Mobley and Schreier (1994) also showed fusion of unprotected dry liposomes and small holes and cracks on the dry membranes after freeze-drying. These data showed that a considerable portion of solute leakage during drying or in solution might not be caused by liposome fusion. However, solute leakage due to such influences had been taken into account before storage experiments started. It should be

pointed out that the situation in a liquid state might be completely different from that in the dry state (in a glass solid). According to our data presented in this paper, solute leakage of dry liposomes during storage appears to be mainly associated with liposome fusions.

Stabilization mechanisms of the glassy state

The role of the glassy state in the stability of dry systems is poorly understood. Currently the stabilization effect of the glassy state is explained by its high viscosity (Sun and Leopold, 1993, 1994). In the present study, we found that the kinetics of liposome fusion and solute leakage were first-order in both the glassy state and nonglassy state (Fig. 5). The Arrhenius plot clearly showed a change near the glass transition, with significantly different slopes above and below the T_g (Fig. 6 *B*). The activation energy, calculated from the slopes of the Arrhenius plot, was 1320 kJ/mol above the T_g, but increased by 51% to 1991 kJ/mol at temperatures below T_g. Therefore, the kinetics of solute leakage in the glassy state appears to be different from that in the nonglassy state. The increased stability of dry liposomes in the glassy state may be associated with this elevated energy barrier for liposome fusion.

It is surprising to us that kinetics of liposome fusion and solute leakage show an excellent fit with the Arrhenius equation at temperatures both above and below the T_g, with a transitional break near the T_g (Fig. 6 *B*). Previous studies have observed that relaxation processes in a supersaturated liquid cannot be described by the Arrhenius relationship. As the glass transition is approached, rates of physical and chemical processes deviate from the Arrhenius behaviors and follow the Vogel-Tammann-Fulcher (VTF) and Williams-Lendel-Ferry (WLF) equations (Franks, 1993). However, biological processes such as liposome fusion and solute leakage in the glassy state may be significantly different from relaxation processes of the glassy state themselves.

It had been suggested previously that the protective effect of carbohydrates during freeze-drying might result from their spacing role, which prevented liposome fusion. This was found to be incorrect because not all sugars were equally effective. The prevention of liposome fusion during freeze-drying was directly related to sugar's ability to interact with the phospholipids (Womersley et al., 1986; Anchordoguy et al., 1987). However, the physical separation of dry liposomes by bulky sugar glass could be an important factor in preventing liposome fusion in the glassy state, because dynamic properties of the glassy state depend mainly on high viscosity, which lowers the mobility of molecules, and the physical separation of dry liposomes could certainly inhibit the fusion in the glass state. In the present study, sucrose was used at 55 mg/ml before air-drying, a level that was sufficient to prevent liposome fusion during air-drying (Crowe et al., in press). As the dried samples were heated to near and above T_g (55°C), liposome fusion was observed (Fig. 4). However, in a separate experiment we increased sucrose concentration to 190 mg/ml before air-drying, and the dried liposome samples were then subjected to the same storage treatment as that for liposomes dried at a sucrose concentration of 55 mg/ml. The higher sucrose level effectively prevented liposome fusion and aggregation, even after the liposome samples were heated at 75°C for 1 h. With the higher sucrose level, dry liposomes are sufficiently separated that fusion would hardly occur.

Shelf-life of a liposome product in the glassy state

The longevity of dry liposomes for solute retention is of particular interest for practical application. As discussed above, the glassy state is thermodynamically unstable, and the "real time" stability of dry liposomes in the sugar glass depends on the kinetic stability of the glassy state. It was shown recently that dried liposomes remained intact after annealing when they were in a sugar glass matrix (Mobley and Schreier, 1994). It is commonly believed that dry liposomes are quite stable in the glassy state. Our kinetic study showed, however, that liposome fusion and leakage could occur even in the glassy state, although at quite slow rates (Figs. 3 *B*, 5, and 6 *A*). Fig. 10 is a plot of the half-life of solute retention, the time by which dry liposomes lose 50% of the initially trapped solutes. The data of half-life of solute retention were collected from experiments in Fig. 5. Fig. 10

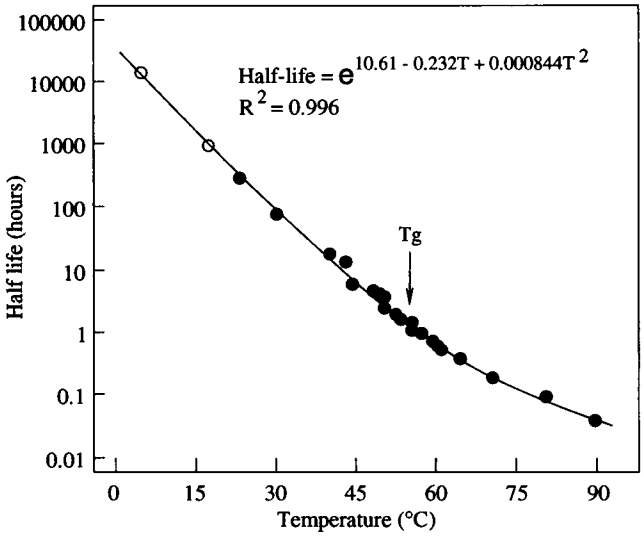

FIGURE 10 Half-life of carboxyfluorescein (CF) retention in egg PC-sucrose liposomes. Half-life was the time by which vesicles lost 50% of CF initially trapped inside. ●, actual experiment data points. ○, data points extrapolated with the rate constants (in Fig. 6) because solute retention of these samples had not decreased to 50% during the period of this study. According to the law of first-order kinetics, $\ln [A] - \ln [A_0] = -kt$, where A is solute retention at a given time (t), A_0 is the retention at $t = 0$, and k is the rate constant. From this equation, half-life (t_{50}) is calculated to be $\ln [A_0/A]/k = 0.69315/k$ ($A/A_0 = 0.5$).

shows that the half-life of solute retention continuously increases exponentially as storage temperature decreases below the T_g. A similar relationship is also derived between the half-life of solute retention and $T - T_g$ ($T = 23°C$) from isothermal experiments, in which the T_g of dry liposome samples was altered through the sorption of water vapor in various relative humidities (data not shown; see original data in Figs. 7 and 8). These results indicate that the half-life of solute retention can be prolonged by storing dry liposomes at temperatures below the T_g and by increasing the T_g of the dry liposome preparation.

These results are consistent with the thermodynamically unstable nature of the glassy state and indicate that the glassy state itself is not the only factor determining the stability of dry liposomes during long-term storage. We wish to emphasize that, while maintenance of the glassy state may be required for stabilization of liposomes in the dry state, it is not in itself sufficient. For instance, dextrans form glasses at relatively high temperatures and would thus be expected to be particularly effective at stabilizing liposomes if glass formation were the only requirement. In fact, dextrans and other polymers are totally ineffective with liposomes that pass through the liquid crystalline to gel phase transition during dehydration and rehydration (Crowe et al., 1994). In such a case, which applies to many phospholipids, depression of T_m in the dry phospholipids is also required for the stabilization of dry liposomes. Dextrans do not depress T_m in dry phospholipids (Crowe et al., 1994). It appears that the stability of dry liposome products depends on both glassy-state transition and phospholipid phase transition.

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