Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochimica et Biophysica Acta 1661 (2004) 125–134
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
www.bba-direct.com

Preservation of dried liposomes in the presence of sugar and phosphate

Willem F. Wolkers[a,b,*], Harriëtte Oldenhof[c], Fern Tablin[a,d], John H. Crowe[a,b]

[a] Center for Biostabilization, University of California, Davis, CA 95616, USA
[b] Section of Molecular and Cellular Biology, University of California, Davis, CA 95616, USA
[c] Department of Plant Biology, University of California, Davis, CA 95616, USA
[d] Department of Anatomy, Physiology, and Cell Biology, University of California, Davis, CA 95616, USA

Received 17 September 2003; received in revised form 4 December 2003; accepted 5 December 2003

Abstract

It has been well established that sugars can be used to stabilize liposomes during drying by a mechanism that involves the formation of a glassy state by the sugars as well as by a direct interaction between the sugar and the phospholipid head groups. We have investigated the protective effect of phosphate on solute retention and storage stability of egg phosphatidylcholine (egg PC) liposomes that were dried (air-dried and freeze-dried) in the presence of sugars and phosphate. The protective effect of phosphate was tested using both glucose (low T_g) and sucrose (high T_g) by measuring leakage of carboxyfluorescein (CF), which was incorporated inside the vesicles. Liposomes that were dried with glucose or phosphate alone showed complete leakage after rehydration. However, approximately 30% CF-retention was obtained using mixtures of phosphate and glucose. Approximately 75% CF-retention was observed with liposomes that were dried with sucrose. The solute retention further increased to 85% using mixtures of phosphate and sucrose. The pH of the phosphate buffer prior to drying was found to have a strong effect on the solute retention. Fourier transform infrared spectroscopy studies showed that phosphate and sugars form a strong hydrogen bonding network, which dramatically increased the T_g. The HPO_4^{2-} form of phosphate was found to interact stronger with sugars than the $H_2PO_4^-$ form. The increased solute retention of liposomes dried in the sugar phosphate mixtures did not coincide with improved storage stability. At temperatures below 60 °C the rate of solute-leakage was found to be strikingly higher in the presence of phosphate, indicating that phosphate impairs storage stability of dried liposomes.

Keywords: Air-drying; Carbohydrate; Freeze-drying; FTIR; Glass; Liposome

1. Introduction

Drying of cells generally leads to irreversible damage to cellular membranes and proteins, leading to cell death. Anhydrobiotic organisms, however, have the ability to cope with such problems and are able to survive almost complete dehydration [1,2]. These organisms often accumulate high concentrations of disaccharides such as sucrose and trehalose before they enter the anhydrobiotic state. These sugars are thought to convey protection to intracellular biomolecules and organelles in the dried state.

The stabilizing properties of sugars in nature have been inferred from model systems of isolated proteins [3,4] and liposomes [5,6]. The mechanism by which sugars stabilize liposomes during drying involves the formation of a glassy state by the sugars as well as a direct interaction between the sugars and the phospholipid head groups [6]. In addition to serving as a model system for how sugars in anhydrobiotic organisms aid protection to intracellular organelles, liposomes can also be used for practical applications such as intravenous delivery of drugs. This has led to many studies over the past decades on solute retention and storage of liposomes in the dried state [5–8].

Liposomes can be stored effectively by freezing using a wide variety of cryoprotectants, including dimethylsulfoxide, glycerol, quarternary amines, and carbohydrates. The requirements for drying are much more stringent, and only disaccharides seem to be effective [9]. The protective effect of carbohydrates during drying of liposomes is

* Corresponding author. Center for Biostabilization, University of California, 1 Shields Avenue, Davis, CA 95616, USA. Tel.: +1-530-297-4693; fax: +1-530-297-4697.
E-mail address: wfwolkers@ucdavis.edu (W.F. Wolkers).

0005-2736/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2003.12.006

based on a narrow balance between the interaction between the sugar and the lipid and the glass-forming properties of the carbohydrate. A direct interaction between the sugar and the phospholipid head groups is pivotal to prevent leakage through the bilayers [6], whereas vesicle fusion can be prevented by the formation of a stable glassy state. The T_g of monosaccharides such as glucose is generally too low to stabilize liposomes in the dried state, whereas oligo- and polysaccharides are able to form a stable glass, but, due to their large size, are unable to interact with lipid head groups. Fructans are an interesting exception to this general rule [8]. Disaccharides are small enough to be able to interact with vesicles and have a sufficiently high T_g. Thus, a good stabilizer during drying of liposomes should: (1) have a sufficiently high T_g, and (2) provide good interaction with the lipid bilayer. These requirements can also be met using combinations of protectants. Egg PC liposomes, for example, cannot be stabilized using glucose or hydroxyl ethylene starch (HES) alone, but stabilization can be accomplished using a combination of the two [6]. In this case, interaction is provided by the glucose, whereas the HES, which cannot interact with the lipid bilayer due to its large size, provides a sufficiently high T_g. Red blood cells have been successfully freeze-dried using a combination of glucose and HES [10]. Such synergism of protective compounds has also been reported using mixtures of trehalose and borate [11,12]. Borate ions increase the T_g of trehalose, by forming a complex with trehalose in the dried state. Storage stability of lactate dehydrogenase and *Lactobacillus acidophilus* was shown to be considerably improved under conditions of high temperature or high humidity using mixtures of trehalose and borate. Just like borate, inorganic phosphate also interacts with sugars in the dried state, thereby increasing the T_g of the sugars [13]. It was shown that phosphate, in combination with sugars, improves long-term stability of fragile proteins and bacteria in the dried state.

In this study, we have investigated the protective effect of phosphate during air-drying and freeze-drying of egg phosphatidylcholine (egg PC) liposomes in mixtures of phosphate and sugars. The protective effect was measured by the retention of a fluorescent dye, carboxyfluorescein (CF), which was incorporated inside the vesicles. The effect of phosphate was tested using both glucose, which does not protect liposomes during drying due to its low T_g (36 °C [14]), and sucrose, which has a sufficiently high T_g (67 °C [14]) to protect liposomes. Fourier transform infrared spectroscopy was used to study the interaction between phosphate and sugars through hydrogen bonding, and to determine the glass transition temperature of the sugar–phosphate mixtures. We show that phosphate in combination with glucose or sucrose increases the initial protection during drying of liposomes, but that storage stability of the dried liposomes at ambient temperatures is decreased in the presence of phosphate.

2. Materials and methods

2.1. CF-retention studies

Sucrose and glucose were purchased from Pfahnstiel (Waukegan, IL, USA). Egg PC was obtained from Avanti Polar lipids (Alabaster, AL, USA). CF was obtained from Molecular Probes (Eugene, OR, USA), and purified using a lipopohilic sephadex LH20 column (Sigma, MO, USA). Dried lipid (20 mg/ml) was mixed with 250 mM sugar (glucose or sucrose) and 100 mM CF in TES buffer (1 mM, pH = 8.0). The lipid suspension was extruded through membrane filters (pore size, 100 nm) 19 times, using a Lipofast extruder (Avestin, Ottowa, ON, Canada) [15]. CF outside was removed by passage through a 15-cm Sephadex G-50 (Sigma) column. Air-drying of vesicles was done as described previously [5,7]. Vesicle solution was mixed with an equal volume protection buffer (final volume 20 μl) of varying molar ratios of sugar and phosphate. The samples were placed inside the caps of Eppendorf microfuge tubes, and dried for approximately 4 h in a dry box that was continuously flushed with dry air of 0% relative humidity at 22 °C. This results in a residual water content of 0.02 to 0.04 g H_2O/g dry weight as was determined by gravimetric analysis of the samples on a Cahn microbalance (Madison, WI, USA). Freeze-drying was done overnight in Eppendorf vials using a Virtis lyophilizer (Gardiner, NY, USA) following freezing in a −80 °C freezer. The final lipid concentration in the samples prior to drying was approximately 5 mg/ml. The protection buffers were composed of sugar (glucose or sucrose at a final concentration of 250 mM) and sodium phosphate (between 0 to 250 mM). Thus, the glucose/lipid and sucrose/lipid mass ratios were 9 and 17, respectively. The pH was varied by mixing solutions of Na_2HPO_4 and NaH_2PO_4.

CF-retention inside the liposomes was determined by spectrofluorometry as described previously [5,16]. Fluorescence of CF is strongly quenched at the high concentration inside the vesicles and is increased when CF is released into the medium. The total CF content of the vesicles was determined after lysis of the membranes with Triton X-100. CF-retention of rehydrated vesicles was determined from the fluorescence before and after addition of Triton X-100. The dried vesicles were rehydrated in 3-ml TES buffer (10 mM TES, pH = 8.0), and Triton X-100 was added at a final concentration 0.02%.

Size of the vesicles was measured with a Brookhaven particle size analyzer (Holtsville, NY, USA) using 2500 scans. The extent of vesicle fusion was estimated by comparison of the vesicle diameter, before and after drying.

The kinetics of solute leakage in the dried state was studied by storage of the air-dried liposomes at constant temperatures from 10 to 80 °C. After air-drying as described above, samples were sealed inside the dry box (0% RH) and placed in incubation chambers of varying temperatures. Samples were taken as a function of time and rehydrated

to determine CF-retention. CF-retention was plotted against storage time, and the rate constants were determined by fitting the data using a single exponential decay function. Arrhenius plots were constructed by plotting the reciprocal temperature versus the natural logarithm of the rate of CF-leakage. The activation energy was obtained from the slope in these plots.

2.2. Fourier transform infrared spectroscopy studies

Infrared absorption measurements were carried out with a Perkin-Elmer series 2000 Fourier transform infrared spectrometer (Perkin-Elmer, Norwalk, CT, USA). The instrument was equipped with a narrow band Mercury/Cadmium/Telluride LN_2-cooled IR-detector. The temperature of the FTIR cell was regulated by a computer-controlled device and the temperature of the sample was recorded separately using a thermocouple located very close to the sample windows. The temperature dependence of the FTIR spectra was studied starting with the lowest temperature, with a scanning rate of 2 °C/min. The optical bench was purged with dry CO_2-free air. The acquisition parameters were: 4 cm^{-1} resolution, 32 coadded interferograms, with a 3600–900 cm^{-1} wave number range.

The interaction between phosphate and glucose, and that between egg PC liposomes and glucose was studied using the band position of the OH stretching vibration arising from the sugar OH groups (νOH). The thermotropic response of the OH stretching band was used to determine the glass transition temperature of the dry samples as described in detail elsewhere [17]. Spectral analysis and display were carried out using the interactive Perkin-Elmer software. The spectral region between 3600 and 3000 cm^{-1} was selected and normalised. The band position was calculated as the average of the spectral positions at 80% of the total peak height. T_g was determined by linear regression of the wave number of the OH stretching band as a function of the temperature in both the liquid and the solid-like regions of the plot. The point of intersection of these two regression lines was defined as the glass transition temperature T_g.

3. Results

CF-retention of egg PC liposomes following drying and rehydration was studied using both glucose–phosphate and sucrose–phosphate mixtures. Liposomes were dried with sugar present both inside and outside the vesicles. The sugar and lipid concentrations were chosen such that a sugar to lipid mass ratio greater than 5 was obtained. It was previously reported that this results in maximal CF-retention following drying and rehydration when sucrose is used to stabilize the liposomes [7]. CF-leakage cannot be prevented using glucose, but at a glucose/lipid mass ratio of 9 there is sufficient glucose to provide maximal interaction with the phospholipid head groups [6].

Table 1
CF-retention and mean diameter (± S.E.) of egg PC liposomes dried in water, glucose, or phosphate

	Mean diameter (nm)	CF-retention
Fresh control vesicles prior to drying	133 ± 17	–
Dried in water	875 ± 277	6.4 ± 1.4
Dried in phosphate (250 nm)	2065 ± 661	7.1 ± 1.2
Dried in glucose (250 mm)	122 ± 11	7.9 ± 0.5

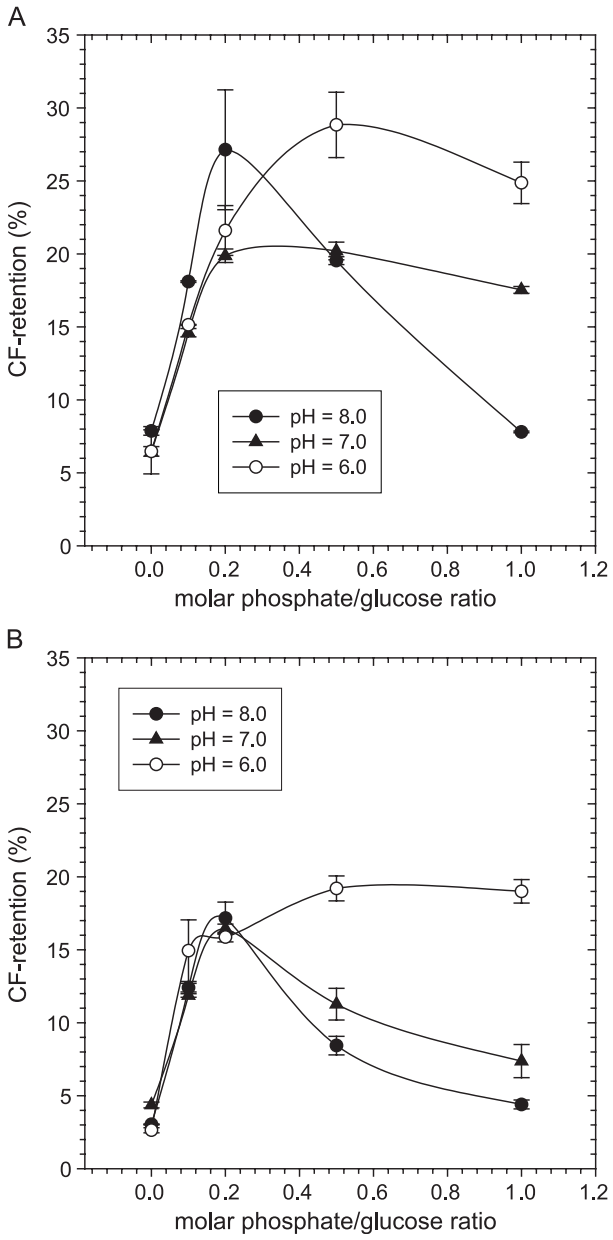

Fig. 1. CF-retention of egg PC liposomes after (A) air-drying and (B) freeze-drying. Egg PC liposomes contained 250 mM glucose inside and were dried with 250 mM glucose outside. Phosphate was added to the outside of the liposomes at the indicated molar ratios. The CF-retention was studied at pH 6.0 (open circles), 7.0 (filled triangle) and 8.0 (filled circles). The data points are means of three measurements with standard error.

3.1. CF-retention using glucose, or phosphate alone

The CF-retention of vesicles that were dried in the absence of glucose or phosphate or with phosphate or glucose alone was around 7% (Table 1), indicating that neither phosphate nor glucose was able to prevent leakage. The mean diameter of the vesicles was 133 nm before drying, and increased to 875 nm following drying and rehydration in the absence of glucose or phosphate. This increased diameter of the rehydrated vesicles is indicative of fusion. The mean diameter of vesicles that were dried with phosphate alone was 2065 nm, whereas the diameter of vesicles dried with glucose (at a glucose/lipid mass ratio of 9) was 122 nm, similar to the size of the vesicles prior to drying. Thus, drying at a glucose/lipid mass ratio of 9 results in complete leakage of solutes, but fusion is prevented. This implies that at such high sugar/lipid mass ratios, fusion is not the mechanism by which CF leakage occurs. The fact that the diameter of vesicles dried in the presence of phosphate is greater than those dried in water is likely due to the increase in ionic strength in the presence of phosphate.

3.2. Solute retention of liposomes using glucose–phosphate mixtures

Fig. 1 shows that CF-retention increased from 7% using glucose alone to approximately 30% using glucose–phosphate mixtures at molar ratios between 0.1 and 0.5. The stabilizing effect of phosphate on CF-retention was observed both after air-drying and freeze-drying. The protective effect of phosphate is dependent on the pH of the buffer prior to drying, which is particularly evident at the higher phosphate/glucose ratios. At a phosphate/glucose ratio of 1, the CF-retention sharply decreases with increasing pH. A possible explanation for this is that at high pH with phosphate mostly in the single protonated HPO_4^{2-} form, glucose interacts more strongly with phosphate than with the phospholipid head groups. We provide evidence that this is indeed the case (see Figs. 4 and 5).

3.3. Size of vesicles following rehydration

The effect of drying and temperature on vesicle fusion was studied by particle size analysis. Fusion was prevented at a glucose/lipid mass ratio of 9 both in the presence or absence of phosphate, even after storage for 2 h at 80 °C (data not shown). Apparently the vesicle–vesicle interactions that lead to fusion are prevented at such high sugar/lipid ratios even when the sugar does not form a glassy state.

In order to determine if glucose–phosphate mixtures can reduce fusion a glucose/lipid mass ratio of 2 was used. When liposomes were dried with glucose alone at this ratio the mean diameter of the vesicles after rehydration was 311 nm (see Fig. 2), indicating that vesicle fusion had occurred. Fusion was reduced in the glucose phosphate mixture as was evident from the mean diameter of the vesicles; 160 nm. The diameter of liposomes that were dried using glucose alone further increased after an additional 2-h incubation period of the dried samples at elevated temperatures (Fig. 2). Liposomes that were dried in the glucose–phosphate mixture and incubated at ele-

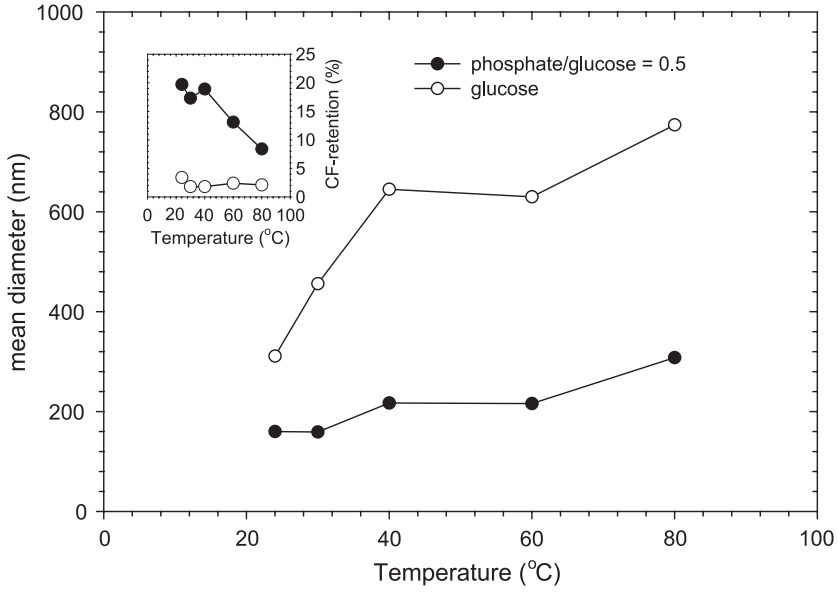

Fig. 2. Mean diameter and CF-retention (insert figure) of egg PC vesicles that were air-dried and incubated for an additional 2 h at the indicated temperatures. Vesicles were air-dried at a glucose/lipid mass ratio of 2 in the absence (open circles) and presence (filled circles) of phosphate. The phosphate/glucose molar ratio was 0.5.

vated temperatures were significantly smaller than those dried with glucose alone, indicating that phosphate reduces fusion. This reduction in fusion cannot be attributed to the higher protectant/lipid mass ratio of the glucose–phosphate samples compared to the glucose samples, since phosphate alone causes massive fusion (see Table 1). The CF-retention of liposomes dried in the glucose–phosphate mixture progressively decreased with increasing incubation temperature (see insert in Fig. 2) coincident with the increase in mean diameter of the liposomes.

3.4. Solute retention of liposomes using sucrose–phosphate mixtures

The stabilization of egg PC liposomes by sucrose has been thoroughly investigated [7]. Sucrose stabilizes egg PC liposomes both during air-drying and freeze-drying. We observed 75% CF-retention in vesicles that were air-dried with sucrose alone (sucrose/lipid mass ratio 17). The beneficial effects of a sucrose–phosphate mixture were only evident with air-drying (Fig. 3A). The CF-retention increased to 85% using mixtures of sucrose and phosphate. By contrast, phosphate did not increase CF-retention of freeze-dried liposomes (Fig. 3B). Thus, the additional protection of phosphate in a sucrose–phosphate mixture is small compared to that in a glucose–phosphate mixture. Similar to the finding with the glucose–phosphate mixtures, a sharp drop in CF-retention with increasing pH was observed at a phosphate/sucrose molar ratio of 1, indicating that protection is dependent on the protonation of the phosphate.

3.5. Molecular interactions in dried glucose–phosphate–egg PC

In order to explain the stabilizing effect of phosphate, the molecular interactions in the air-dried phosphate–sugar–egg PC system were studied using FTIR. The interaction between sugar and egg PC through hydrogen bonding and that between sugar and phosphate were studied separately in order to distinguish the interactions that take place in the phosphate–sugar–egg PC mixture. For this purpose, the effects of both phosphate and egg PC on νOH of the sugar OH groups were studied. The thermotropic response of the OH stretching mode arising from the sugar OH groups was used to determine T_g of the air-dried liposomal samples.

3.6. Interaction between phosphate and sugars

Fig. 4 depicts the effect of phosphate on νOH of glucose. The wave number of the OH stretching vibration decreases with increasing amounts of phosphate, indicating that phosphate decreases the average length of hydrogen bonds in the dried state. At phosphate/glucose

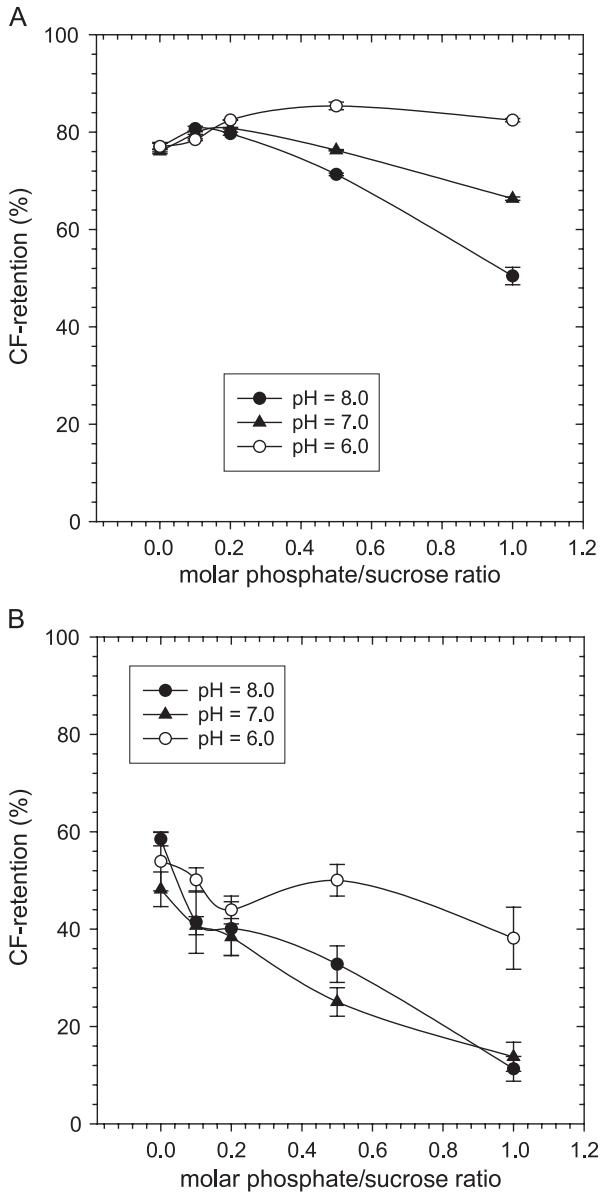

Fig. 3. CF-retention of egg PC liposomes after (A) air-drying and (B) freeze-drying. Egg PC liposomes contained 250 mM sucrose inside and were dried with 250 mM sucrose outside. Phosphate was added to the outside of the liposomes at the indicated molar ratios. The CF-retention was studied at pH 6.0 (open circles), 7.0 (filled triangle) and 8.0 (filled circles). The data points are means of three measurements with standard error.

ratios greater than 1, νOH reaches a minimum. The effect of phosphate on νOH is dependent on the pH of the phosphate buffer prior to drying. At a phosphate/glucose ratio of 1, νOH strongly decreases with increasing pH of the buffer (Fig. 4B), indicating that the HPO_4^{2-} form of phosphate interacts stronger with the sugar OH groups than the $H_2PO_4^-$ form. The νOH versus pH plot shows an inflection point at the pK_a (7.2) of the phosphate buffer. Phosphate has similar effects on the νOH of sucrose (data not shown). In the formation of the hydrogen bonds between sugars and phosphate, the OH groups of the

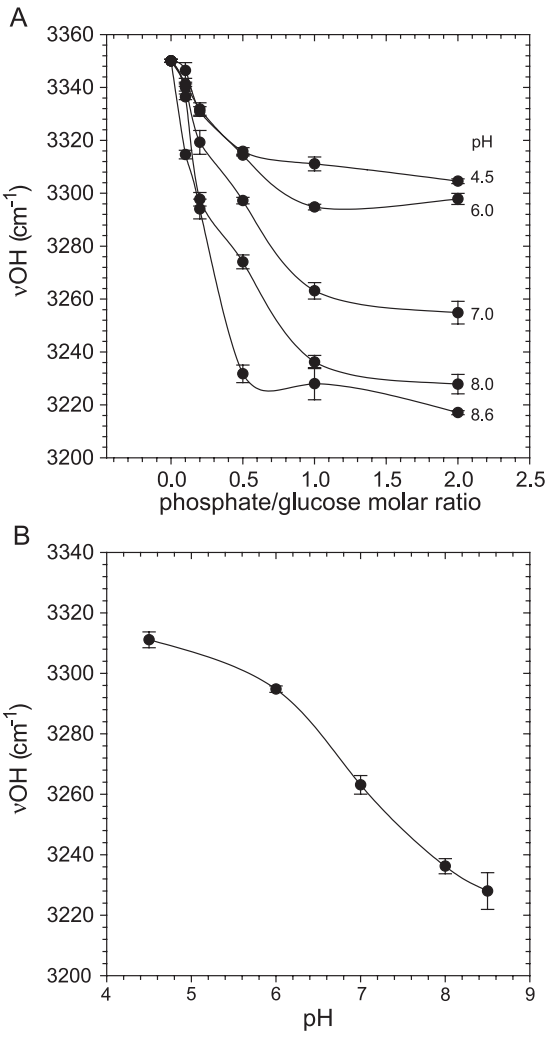

Fig. 4. Interaction between phosphate and glucose through hydrogen bonding: (A) *v*OH versus molar phosphate/glucose ratio; (B) correlation between *v*OH and the pH of the phosphate buffer at a molar phosphate/glucose ratio of 1. The data points are means of three measurements with standard error.

sugars act as proton donors (i.e. acids) and the phosphate as the proton acceptor (i.e. base). Since the pK_a is 13.2 for the HPO_4^{2-} form and 7.2 for $H_2PO_4^-$, the HPO_4^{2-}, which is the stronger base, will form stronger interactions with the sugar OH groups.

3.7. Interaction between egg PC and sugars

The effect of egg PC on *v*OH of glucose, sucrose and dextran was studied as a measure for hydrogen bonding between sugars and liposomes in the dried state. Fig. 5 shows that egg PC liposomes decrease the *v*OH of glucose, indicating that glucose and egg PC interact through hydrogen bonding. The *v*OH reaches a minimum at egg PC/sugar mass ratios greater than 1. The sugar OH groups interact directly with the phosphate of the phospholipid head groups [9]. Egg PC liposomes have a similar effect on the OH stretching mode of sucrose. By contrast, the OH stretching mode of dextran, a polysaccharide, does not change upon addition of liposomes, which indicates that dextran has minimal interaction with liposomes in the dried state.

It is interesting to note that the minimum *v*OH of glucose that is observed using saturating amounts of egg PC is higher than the minimum *v*OH at saturating amounts of phosphate (compare Figs. 4 and 5). At a pH of 8.6, for example, the minimum *v*OH of glucose in the glucose–phosphate mixture is 3220 cm^{-1}, whereas *v*OH in the egg PC–glucose mixture reaches a minimum at 3320 cm^{-1}. This implies that the interaction between glucose and phosphate is much stronger than that between glucose and egg PC. This could explain the observed decrease in CF-retention (Fig. 1) at phosphate/glucose ratios greater than 0.5. Because at high pH glucose interacts more strongly with free phosphate than with egg PC, glucose thus becomes unavailable to the liposomes, which results in additional CF-leakage; in other words, we suggest that the effect is due to competition between free phosphate and phospholipid for the available glucose. At a pH of 6 the interaction between glucose and phosphate (*v*OH reaches a minimum at 3300 cm^{-1}) is only slightly stronger than that between egg PC and glucose. In this case, CF-retention increases (Fig. 3B) as predicted.

3.8. Effect of phosphate on T_g

The glass transition temperature, T_g, can be measured using FTIR by monitoring *v*OH as a function of temperature. The glass transition of carbohydrates is manifested as an abrupt change in the wave number temperature

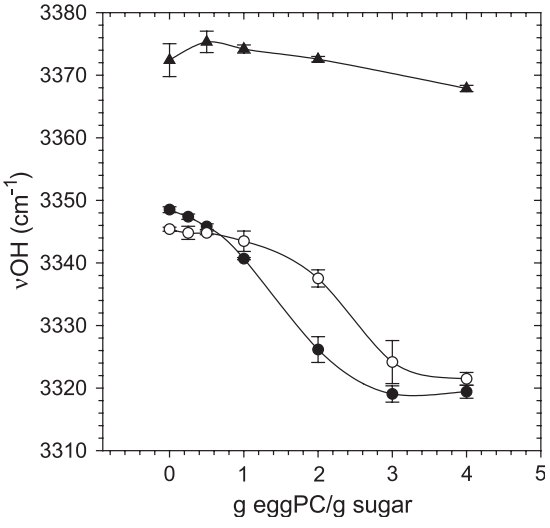

Fig. 5. Interaction between egg PC and glucose (filled circles), egg PC and sucrose (open circles), and egg PC and dextran (filled triangles) through hydrogen bonding. The plots reflect the *v*OH versus mass ratio of eggPC/sugar. The data points are means of three measurements with standard error. The pH of the samples prior to drying was 8.0.

coefficient (WTC) of the OH stretching mode [17]. The WTC of *v*OH reflects changes in hydrogen bonding with changes in temperature, and can be considered as a measure of the strength of hydrogen bonding. The insert in Fig. 6 shows a *v*OH versus temperature plot of liposomes that were air-dried with glucose alone: the break in the plot denotes the glass transition temperature of the system. Fig. 6 shows the thermotropic response of

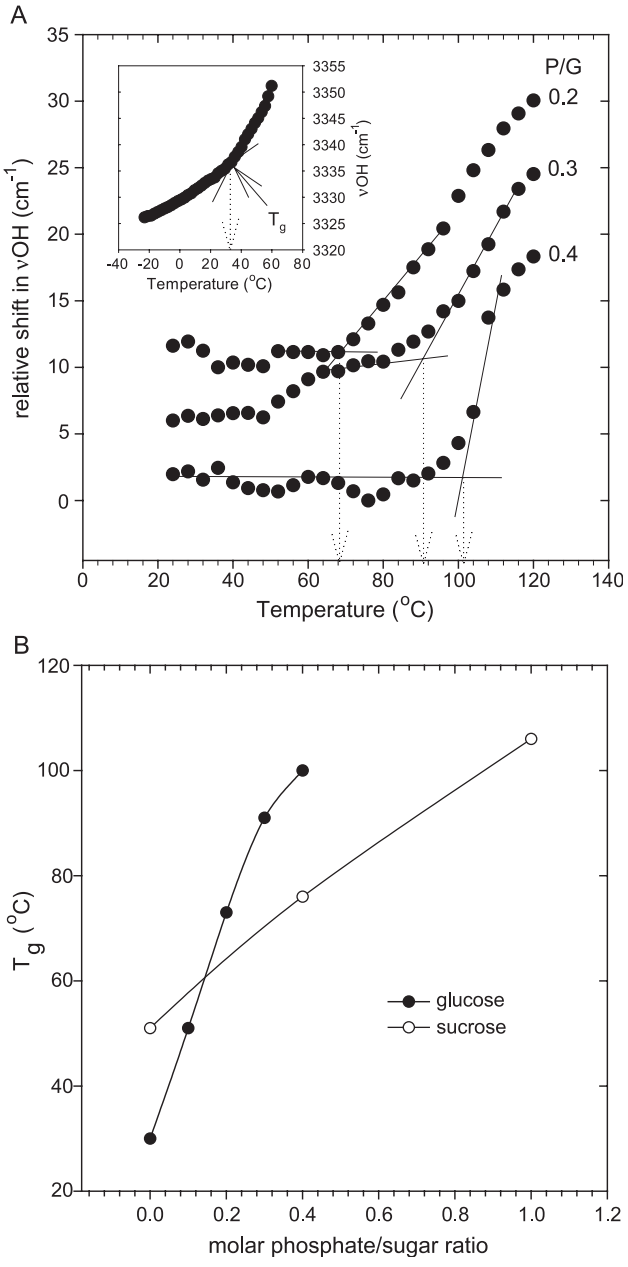

Fig. 6. (A) *v*OH versus temperature plots of liposomes that were air-dried in glucose–phosphate mixtures at the indicated molar ratios at pH 8.6. The data points reflect the relative change in *v*OH with increasing temperature. The insert figure represents a *v*OH versus temperature plot of liposomes dried with glucose alone. The glass transition temperatures are indicated with arrows. (B) Effect of phosphate/sugar molar ratio on T_g of glucose (filled circles) and sucrose (open circles).

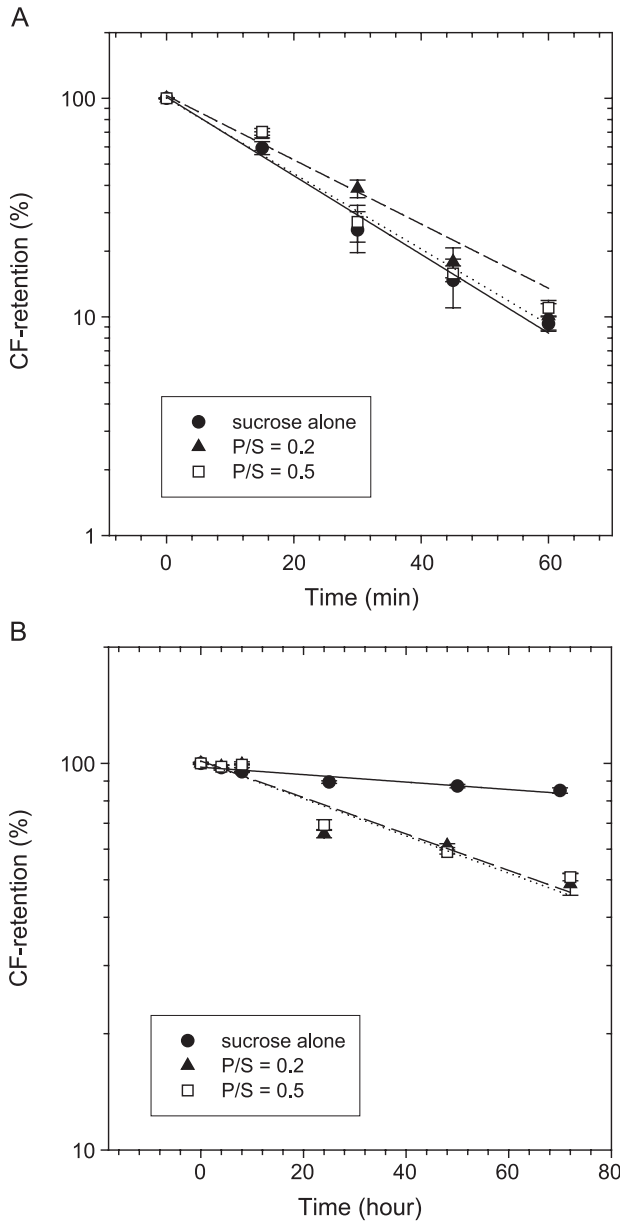

Fig. 7. CF-retention of air-dried egg PC liposomes dried with mixtures of phosphate and sucrose at: (A) 80 °C and (B) 30 °C. The data points of sucrose alone (filled circles, solid line), and those of phosphate/sucrose at molar ratios 0.2 (filled triangles, dotted line) and 0.5 (open squares, broken line) were fitted using a single exponential decay. The data points are means of three experiments, and the error bars reflect the standard error.

*v*OH of liposome samples dried with differing mixtures of phosphate and glucose. The relative shift in *v*OH is shown instead of the absolute value of *v*OH, because the starting wave number of the different glucose–phosphate mixtures showed great differences. The T_g of the air-dried liposomal samples increased from 30 °C using glucose alone to 100 °C using a glucose–phosphate mixture at a molar ratio of 0.4. In addition to increasing the T_g of glucose, phosphate strongly decreased the WTC of the OH stretching band at temperatures below T_g. The WTC in the glassy state, WTC_g, dropped from 0.15

$cm^{-1}/°C$ with glucose alone to 0.0 $cm^{-1}/°C$ at a phosphate/glucose ratio of 0.4. This indicates that phosphate increases the average strength of hydrogen bonding. The higher WTC_g of glucose compared to that of the glucose–phosphate mixtures is also indicative of a higher degree of freedom for rearrangements in intermolecular hydrogen bonding as temperature changes. The T_g of liposomes that were dried with sucrose increased from 50 °C using sucrose alone to 106 °C using a phosphate/sucrose molar ratio of 1.

3.9. Storage stability of liposomes in sugar–phosphate glasses

Storage stability of the air-dried liposomal samples was studied using sucrose alone and in sucrose–phosphate and glucose–phosphate mixtures by determining the CF-retention after storing the samples that were dried for 4 h at room temperature, for an additional time at varying temperatures. The kinetics of CF-retention during storage followed first-order kinetics as described previously [7]. The rate constants of CF-leakage were determined by fitting the data with a single exponential decay. Storage at 80 °C resulted in 90% reduction of CF-retention within 60 min (Fig. 7). The rate of CF-leakage was approximately the same at varying molar ratio of phosphate and sucrose. At 30 °C, however, the rate of CF-leakage of liposomes dried in sucrose–phosphate mixtures was strikingly greater than in liposomes dried with sucrose alone. In this case, phosphate causes a fivefold increase in the rate of CF-leakage (Table 2). The destabilizing effect of phosphate on storage stability is already evident at phosphate/glucose molar ratios as low as 0.1. The rate constants of CF-leakage during storage showed good fits with the Arrhenius equation (Fig. 8). The activation energy in this equation can be obtained from the slope in a plot of the reciprocal temperature versus the natural logarithm of the rate of CF leakage. The activation energy of CF-leakage with sucrose alone was determined to be 35.3 kcal/mol at temperatures below 60 °C (below the T_g of sucrose), in good agreement with previous findings in our laboratory (33 kcal/mol; recalculated from Sun et al. [7]). Phosphate decreased the activation energy to 22.7, 22.4, and 20.6 kcal/mol at phosphate/sucrose molar ratios of 0.1, 0.2, and 0.5, respectively. The decreased activation energy in the presence of phosphate implies that there is less effect of temperature on the rate of CF-leakage in the dried state. Liposomes that were dried in glucose–phosphate mixtures demonstrated similar decay curves of CF-leakage with similar activation energies compared to those dried in sucrose–phosphate mixtures. Activation energies in glucose–phsophate mixtures were determined to be 21.8, 20.9, and 18.2 kcal/mol at phosphate/glucose ratios of 0.1, 0.2, and 0.5, respectively (Table 3).

We conclude that phosphate reduces the effect of temperature on the rate of solute-leakage of dried lip-

Table 2
Rate constants (± S.E.) of CF-leakage of liposomes dried in sucrose alone and in sucrose–phosphate mixtures

Phosphate/sucrose (molar ratio)	k at T=80 °C (s^{-1})	k at T=30 °C (s^{-1})
0	$6.83 \pm 0.5 \times 10^{-4}$	$0.60 \pm 0.1 \times 10^{-6}$
0.1	$6.54 \pm 0.9 \times 10^{-4}$	$2.89 \pm 0.5 \times 10^{-6}$
0.2	$6.59 \pm 0.1 \times 10^{-4}$	$2.83 \pm 0.4 \times 10^{-6}$
0.5	$6.56 \pm 0.5 \times 10^{-4}$	$3.47 \pm 0.3 \times 10^{-6}$

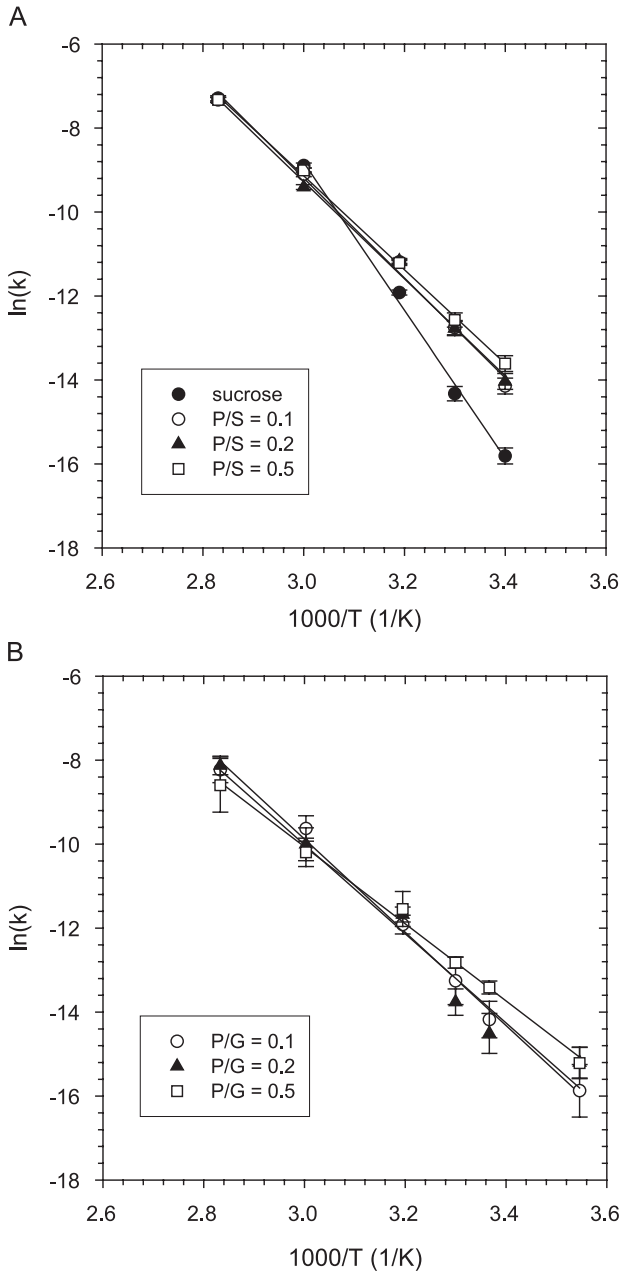

Fig. 8. Arrhenius plots of CF-leakage of air-dried egg PC liposomes protected with: (A) phosphate/sucrose (P/S) and (B) phosphate/glucose (P/G) at the indicated molar ratios. The data points are means of three experiments, and the error bars reflect the standard error.

Table 3
Activation energies of CF-leakage (± S.E.) in liposomes dried in sucrose–phosphate and glucose–phosphate mixtures

	Activation energy (kcal/mol)
Sucrose	35.3 ± 1.0
Phosphate/sucrose = 0.1	22.7 ± 1.0
Phosphate/sucrose = 0.2	22.4 ± 0.9
Phosphate/sucrose = 0.5	20.6 ± 0.8
Phosphate/glucose = 0.1	21.8 ± 0.5
Phosphate/glucose = 0.2	20.9 ± 0.5
Phosphate/glucose = 0.5	18.2 ± 0.5

The phosphate/sucrose and phosphate/glucose molar ratios are indicated.

osomes. However, the rate of solute-leakage at ambient temperature is increased in the presence of phosphate.

4. Discussion

In this study we have investigated the protective effect of phosphate during air-drying and freeze-drying of liposomes using mixtures of phosphate and sugars. The protective effect of phosphate was more pronounced when phosphate was used in combination with glucose (low T_g) than in combination with sucrose (high T_g). The protonation of the phosphate prior to drying was found to have a strong effect on the CF-retention and on the sugar phosphate interaction. Despite its effect on the initial CF-retention after drying, phosphate decreased storage stability of dried liposomes at ambient temperatures.

Neither phosphate nor glucose alone is able to stabilize liposomes during drying. However, solute retention is clearly increased using a glucose–phosphate mixture both during air-drying and freeze-drying. This indicates that phosphate and glucose stabilize liposomes in a synergistic manner. Using the glucose–phosphate mixture to protect liposomes, interaction is provided by glucose, whereas phosphate increases the T_g. The phosphate-induced increase in the T_g is mediated by a strong interaction between phosphate and glucose through hydrogen bonding. The strength of hydrogen bonding between glucose and phosphate increases with increasing pH of the phosphate prior to drying. Glucose interacts stronger with the single-protonated HPO_4^{2-} form of phosphate than with the double-protonated $H_2PO_4^-$ form.

The stronger glucose–phosphate interaction with increasing pH may explain the effect of pH on CF-retention. The reduction in CF-retention with increasing pH at phosphate/glucose ratios greater than 0.5 is likely due to competition between phosphate and the phospholipid head groups in the interaction with glucose. At high pH with phosphate mostly in the HPO_4^{2-} form, the glucose–phospholipid interaction is relatively weak compared to the strong glucose–phosphate interaction, which leads to preferential interaction of glucose with phosphate at the expense of the glucose phospholipid interaction. Thus, despite the fact that the T_g is sufficient to prevent fusion, CF-leakage occurs due to insufficient interaction between glucose and the lipid. At lower pH, glucose does not have a preferential interaction with phosphate or the phospholipid head groups. In this case, CF-retention initially increases due to the phosphate-induced increase in T_g, and then decreases due to noncompetitive-like inhibition of the interaction of glucose with phosphate and egg PC.

Our kinetic study shows that phosphate decreases the activation energy for solute leakage from liposomes. However, solute leakage during storage at room temperature is increased by phosphate. Phosphate slightly reduced solute leakage at temperatures above 60 °C. At temperatures below 60 °C, however, solute leakage is increased in the sugar–phosphate glasses. The explanation for this may lie in the molecular structure of the amorphous matrix that is formed by sugars and phosphate. One striking difference between the sugar glasses and the sugar–phosphate glasses is the thermotropic response of the OH stretching mode. The WTC_g of the sugar phosphate glasses is strongly decreased with the addition of small amounts of phosphate, indicating that phosphate increases the average strength of hydrogen bonding of the amorphous sugar matrix. We previously reported that WTC_g increases with increasing T_g of carbohydrates: oligosaccharides with high T_g generally have a higher WTC_g than monosaccharides [17]. Phosphate apparently has the opposite effect: with increasing phosphate/sugar ratio, T_g increases, while WTC_g decreases. The WTC of the OH stretching mode in the glassy state is a measure for the degree of freedom to rearrange hydrogen bonds as temperature changes. We speculate that it is this decreased freedom in hydrogen bonding that causes the poor storage stability of liposomes in sugar–phosphate glasses. The hydrogen bonding interactions between sugars and phosphate are stronger than the intermolecular hydrogen bonds between sugars. The sugar–phosphate hydrogen bonds, however, are likely of more defined geometric orientations. We argue that the stabilizing effect of sugars can be attributed to the fact that these molecules have fluid-like interactions in the dried state. Thus, a certain level of molecular disorder is retained while molecular mobility is strongly reduced. Phosphate may interfere with the random molecular interactions in glasses of pure carbohydrates and induce more defined intermolecular interactions with more defined hydrogen bonds. We speculate that this increase in order in the dried state may impair storage stability.

Stability of liposomes in the dried state provides insight into macromolecular stability of anhydrobiotic organisms in the dried state, but is also of interest for practical applications. We conclude that phosphate improves solute retention during drying of liposomes in sugar–phosphate mixtures, which is likely due to the increase in T_g of the sugar by phosphate. Storage stability of dried liposomes at ambient temperature, however, is decreased by phosphate, which could be due to the ordering effect of phosphate on the molecular organization of the amorphous sugar matrix in the dried state.

Acknowledgements

We acknowledge Ann Oliver for carefully reading the manuscript. This project was financially supported by grants HL57810 and HL61204 from NIH, and 981711 from DARPA.

References

[1] D. Billi, M. Potts, Life and death of dried prokaryotes, Res. Microbiol. 153 (2002) 7–12.
[2] J.H. Crowe, L.M. Crowe, J.F. Carpenter, S.J. Prestrelski, F.A. Hoekstra, Anhydrobiosis: cellular adaptations to extreme dehydration, in: W.H. Dantzler (Ed.), Handbook of Physiology Section 13, Comparative Physiology, vol. II, Oxford Univ. Press, Oxford, 1997, pp. 1445–1477.
[3] P. Davidson, W.Q. Sun, Effect of sucrose/raffinose mass ratios on the stability of co-lyophilized protein during storage above T_g, Pharma. Res. 18 (2001) 474–479.
[4] S.J. Prestrelski, N. Tedeschi, T. Arakawa, J.F. Carpenter, Dehydration-induced conformational transitions in proteins and their inhibition by stabilizers, Biophys. J. 65 (1993) 661–671.
[5] L.M. Crowe, L.M.C. Womersley, J.H. Crowe, D. Reid, L. Appel, A. Rudolf, Prevention of fusion and leakage in freeze-dried liposomes by carbohydrates, Biochim. Biophys. Acta 861 (1986) 131–140.
[6] J.H. Crowe, A.E. Oliver, F.A. Hoekstra, L.M. Crowe, Stabilization of dry membranes by mixtures of hydroxyethyl starch and glucose: the role of vitrification, Cryobiology 35 (1997) 20–30.
[7] W.Q. Sun, A.C. Leopold, L.M. Crowe, J.H. Crowe, Stability of dry liposomes in sugar glasses, Biophys. J. 70 (1996) 1769–1776.
[8] D.K. Hincha, E. Zuther, E.M. Hellwege, A.G. Heyer, Specific effects of fructo- and gluco-oligosaccharides in the preservation of liposomes during drying, Glycobiology 12 (2002) 103–110.
[9] J.H. Crowe, J.F. Carpenter, L.M. Crowe, T.J. Anchordoguy, Are freezing and dehydration similar stress vectors? A comparison of modes of interaction of stabilizing solutes with biomolecules, Cryobiology 27 (1990) 219–231.
[10] R.P. Goodrich, S.O. Sowemimo-Coker, C.R. Zerez, K.R. Tanaka, Preservation of metabolic activity in lyophilized human erythrocytes, Proc. Natl. Acad. Sci. U. S. A. 89 (1992) 967–971.
[11] P.B. Conrad, D.P. Miller, P.R. Cielenski, J.J. de Pablo, Stabilization and preservation of *Lactobacillus acidophilus* in saccharide matrices, Cryobiology 41 (2000) 17–24.
[12] D.P. Miller, R.E. Anderson, J.J. De Pablo, Stabilization of lactate dehydrogenase following freeze-thawing and vacuum-drying in the presence of trehalose and borate, Pharma. Res. 15 (1998) 1215–1221.
[13] P.B. Conrad, Preservation of biological molecules and living cells, PhD thesis, University of Wisconsin (2000).
[14] Y.H. Roos, Phase Transitions in Foods, Academic Press, London, 1995, pp. 1–360.
[15] R.C. MacDonald, R.I. MacDonald, B.P.M. Menco, K. Takeshita, N.K. Subbarao, L. Hu, Small-volume extrusion apparatus for preparation of large unilamellar vesicles, Biochim. Biophys. Acta 1061 (1991) 297–304.
[16] J. Wilschut, N. Düzgünes, K. Hong, D. Hoekstra, D. Papahadjopoulos, Retention of aqueous contents during divalent cation induced fusion of phospholipid vesicles, Biochim. Biophys. Acta 734 (1983) 309–318.
[17] W.F. Wolkers, H. Oldenhof, M. Alberda, F.A. Hoekstra, A Fourier transform infrared microspectroscopy study of sugar glasses: application to anhydrobiotic higher plant cells, Biochim. Biophys. Acta 1379 (1998) 83–96.