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EFFECT OF SUGARS ON FREEZE-THAW AND LYOPHILIZATION OF LIPOSOMES

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ABSTRACT

Various sugars were investigated for their ability to protect liposomes against fusion and leakage during freeze-and-thaw or lyophilization processes. Size of liposome was measured before and after

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the events with a light scattering technique. Leakage of the content of the sulfate, was encapsulated in the liposome which was made of egg phosphatidylcholine (EPC)/egg phosphatidylglycerol (EPG)/cholesterol (5:1:2). Addition of 1% lactose to the liposome suspension prevented the fusion between liposome but not the leakage of the content. Freeze-thawing caused more damage to the liposomes than the freeze-drying/reconstitution. After freeze-thawing, one-third of the encapsulated drug leaked out from the liposome. The freeze-drying did not cause additional leakage.

INTRODUCTION

Liposomes have received considerable attention as a drug delivery system since its discovery (1 - 6) two decades ago. The ability to encapsulate a drug in a unilamellar vesicle (ULV) or multilamellar vesicle (MLV) offers several advantages over conventional drug delivery systems. Sustained release and target delivery are good examples, to name a few. However, water soluble drugs entrapped in liposomal vesicles tend to leak out during preparation and upon storage. The leakage problem is one of the limiting factors in commercial development of liposome products. Nevertheless, limited success was attained to improve the long-term

retention of encapsulated solutes by freeze-drying (1 - 5, 7, 8). Crowe et al. (8) found that only trehalose provided satisfactory cryoprotection in their laboratory experiments. The purpose of this study was to re-examine the ability of commonly used cryoprotectants to protect liposomes vesicles from fusion and leakage during freeze-and-thaw or lyophilization processes. An effort was also made to distinguish the freezing damage from the drying damage. Since frozen liposomes have to be thawed and lyophilized liposomes have to be reconstituted before analysis, the study results represent a combined effect of freezing and thawing, and lyophilization and reconstitution.

MATERIALS

Egg phosphatidylcholine, 95% (Asahi Chemical Industry Company, Ltd., Japan), egg phosphatidylglycerol, 95% (Avanti Polar Lipids, Inc., Birmingham, AL), cholesterol (Croda, Inc., Mill Hall, PA), metaproterenol sulfate, USP (Vinchem, Inc., Chatham, NJ) were purchased and tested for purity before use. dl-Alpha-tocopherol, disodium phosphate dibasic, heptahydrate, edetate disodium, lactose monohydrate, and sucrose were obtained from Sigma Chemicals (St. Louis, MO). Sodium phosphate monobasic monohydrate, USP, and mannitol. USP were supplied by

Mallinckroft Chemical Company. Trehalose was purchased from Pfanstiehl Laboratories, Inc. (Waukegan, IN).

METHODS

Preparation of Liposomes

Egg phosphatidylcholine (EPC), egg phosphatidylglycerol (EPG), cholesterol, and dl-alpha-tocopherol were dissolved in chloroform. Subsequently, the solvent was removed by rotary evaporation. The resultant dry phospholipid mixture was slowly hydrated with an aqueous solution of metaproterenol sulfate (125 mg/ml, pH 7.0). The liposome suspension was sized to 0.2 micron mean diameter by a membrane filtration technique (9) and then concentrated to a paste consistency by ultrafiltration using a 100 K polysulfone membrane (Sartocon II, Sartorius, Hayward, CA). The resultant liposome paste is a mixture of encapsulated and unencapsulated forms of drug.

Cryoprotectant Selection Study

The liposome paste was suspended on 0.02 M sodium phosphate buffer to obtain a 10% (W/V) suspension of lipids. The lipid

concentration was about 20 mg/ml. Various solutions of 10% and 20% (W/V) lactose, dextrose, sucrose, and trehalose solutions were prepared in 0.02 M sodium phosphate buffer. Three ml of the liposome suspension and 3 ml of the above sugar solutions were mixed in vials.

The vials were placed in a dry ice isopropanol mixture to freeze the samples prior to lyophilization. Lyophilization was carried out in a freeze dryer (Freezemobile 12, Virtis Company) under 150 millitorr vacuum, at -43°C to -45°C for 17 to 18 hours.

Two grams of liposome paste was weighed into a 30 ml vial. Four grams of the 0.02 M sodium phosphate buffer (pH 7.4) containing a given amount of lactose was added to the above vial. The concentration of lactose in the buffer varied from 0 to 43%.

Freezing Rate Study

Two grams of liposome paste was weighed into a 30 ml vial. Four grams of the 0.02 M sodium phosphate buffer (pH 7.4) containing a given amount of lactose was added to the above vial. The concentration of lactose in the buffer varied from 0 to 43%. Freezing of the samples was

conducted at three different conditions. The "one-minute freeze" was achieved by dipping the vial into liquid nitrogen (-196°C) for one minute to freeze the sample. The "five-minute freeze" was achieved by dipping the vial into a dry ice-acetone mixture (-50°C) for five minutes to freeze the sample. The "thirty-minute freeze" was done in a lyophilizer (Dura Dry, FTS Systems, Inc., Stone Ridge, NY) by allowing the lyophilizer to reach -20°C before introducing sample vials. It required 30 minutes to freeze the samples. The frozen samples were kept at -20°C for 1-1/2 hours, thereafter.

The samples were divided into two groups after freezing. One group of samples was allowed to thaw at room temperature for analysis, and the other group was subjected to freeze-drying in the lyophilizer (Dura Dry, FTS System, Inc.). Each vial of the freeze-thawed samples (0.2 gm liposome plus 4 gm lactose solution) was diluted to 50 ml with 0.9% NaCl solution prior to drug encapsulation analysis. This liposome suspension was further diluted with distilled water prior to vesicle size analysis.

Lyophilization Study

The lyophilizer was pre-cooled to -30°C. The stoppers on the frozen vials from the above study were partially re-opened and then placed

in the lyophilizer. Freeze-drying was carried out under 150 millitorr vacuum at -30°C for 20 to 21 hours. The lyophilized samples were stored in a refrigerator before analysis. Prior to analysis, each vial of the lyophilized samples were reconstituted with 0.9% NaCl solution to a total volume of 50 ml.

Determination of Liposome Size Distribution

Liposome size distributions were determined by dynamic light scattering analysis, using Nicomp 270 submicron particle sizer. The technique is also referred to as quasi-elastic light scattering or photon correlation spectroscopy. Nicomp 270 employs a digital autocorrelator to analyze the fluctuations in scattered light intensity generated by the diffusion of vesicles in solution. The measured diffusion coefficient is used in the Stokes-Einstein equation to obtain the average vesicle radius and, hence, the mean diameter of liposome vesicles.

Quantitation of Encapsulated Drug

One ml of the diluted freeze-thawed sample or the reconstituted lyophilized cake (see previous sections for details) was transferred into a

centrifuge tube and spun at 100,000 rpm (4°C) for 30 minutes (Ultracentrifuge, Model TL100, Beckman Instruments, Inc., Palo Alto, CA). The supernatant and the pellet were separated by decantation. The supernatant was further diluted 1 to 10 with normal saline. This diluted solution was then analyzed by HPLC.

The pellet was reconstituted to 10 ml with normal saline. Five hundred microliters of this solution was mixed with 250 microliters of normal saline and 250 microliters of a 1% Triton X-100 (a detergent) solution to render a clear solution. The final clear solution was analyzed by HPLC. The percentage of the drug encapsulated was calculated by the ratio of the drug in the pellet to the sum of the drug in the pellet and the drug in the supernatant.

HPLC System

The HPLC system (Nicolet Analytical Instruments, Madison, WI), consisted of a liquid chromatograph (LC/9560), and a variable wavelength UV detector (LC/9563) and an autosampler (LC/9505). The output of the detector is analyzed by an IBM 9000 data system. A Whatman C-18 ODS analytical column, 10 micron particle size was used. The flow rate was

1 ml/min. The mobile phase consisted of 70% phosphate buffer (pH = 7.0) and 30% methanol. The UV dector's wavelength was set at 278 nm.

Determination of Osmolality

Two grams of liposome paste was weighed into several glass vials. Four ml of a lactose solution (0, 1, 2, 3, 4, or 5% lactose, W/V) was added to each vial. The final concentrations of lactose in the resultant solutions were 0.00%, 0.67%, 1.33%, 2.00%, 2.67%, and 3.33% (W/V).

Osmolality measurements were performed on the Wescor 5500 vapor pressure osmometer immediately after the above solutions were made. The instrument was standardized with 100, 290, and 1000 mOsm solutions. A portion of each sample was then slowly frozen in a dura-dry freeze dryer over a period of four hours. The frozen samples were thawed at room temperature before the osmolality measurements were taken.

RESULTS AND DISCUSSION

Table 1 summarizes results of a preliminary screening study for the cryoprotective effects of various sugars. Dextrose, lactose, mannitol,

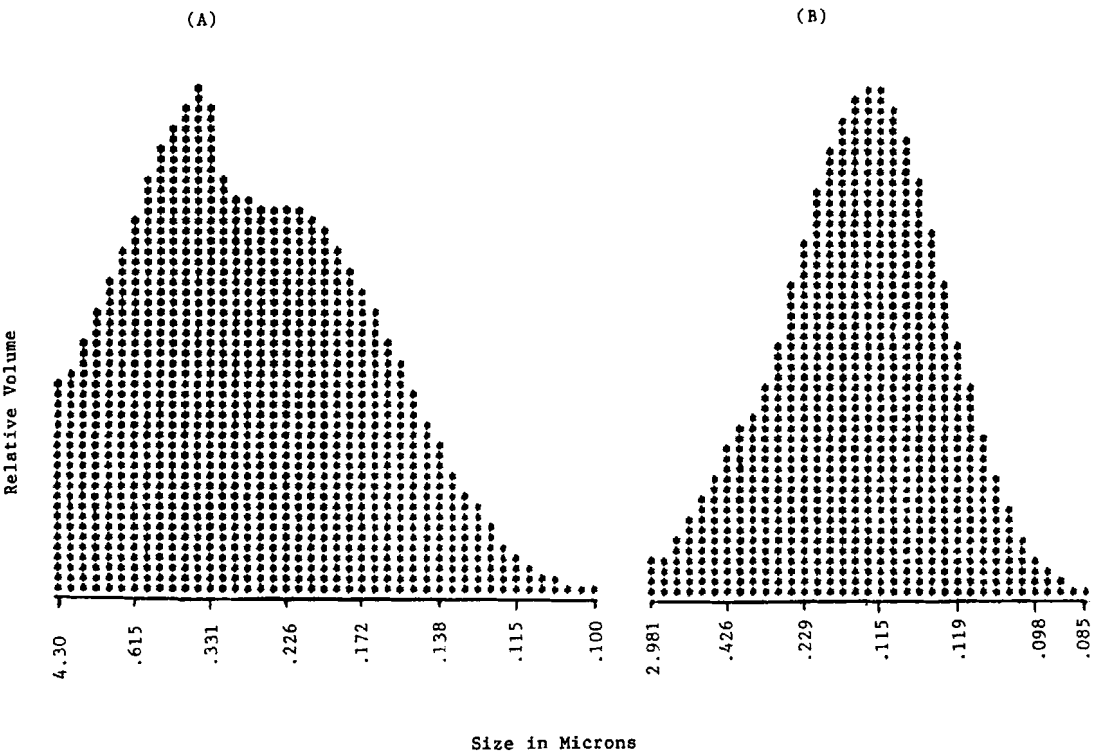

Figure 1

Typical Gaussian analysis using a Nicomp laser particle sizer. (a) Liposome size distribution without lactose in freezing media, and (b) Liposome size distribution upon freeze-thaw. The freezing media contain about 3% lactose. Freezing was carried out by dipping the vial in dry-ice acetone mixture.

sucrose, and trehalose were studied at 5% and 10% concentration for their abilities to preserve the vesicle size and percent of drug encapsulation after lyophilization. Figure 1 shows the vesicle size distribution with and without lactose (3.33% W/V) in the freezing media. As can be seen in Table 1, none of the sugars prevented drug leakage caused by lyophilization. As indicated by Crowe et al. (7), it may require higher concentrations of sugars to prevent leakage. It was also pointed out that the concentration of trehalose required to prevent leakage is more than 10-fold that required to prevent fusion. On the contrary, the control, which was lyophilized without any added sugar, retained the highest level of drug encapsulation. However, the sugars appeared to increase in apparent liposomes size, as evidenced by the comparable vesicle sizes before and after lyophilization. The control showed an increase in vesicle size after lyophilization.

Since none of the sugars offered protection against leakage and all of them provided comparable protection against increase in apparent liposomes size, lactose was selected to be the representative sugar for the remainder of the study.

Figure 2 depicts results of a "range finding" study for the concentration of lactose. Lactose ranging from 0 to 43% (W/V) in

Table 1

Effect of Various Sugars on the Vesicle Size and % Encapsulation of the Lyophilized[1] Liposome

Sugar Solution Added	5% (W/V Sugar) Encapsulation (%)	5% (W/V Sugar) Liposome Size[2] (Microns)	10% (W/V) Sugar Encapsulation (%)	10% (W/V) Sugar Liposome Size[2] (Microns)
None	39.5	0.32	38.0	0.28
Dextrose	27.5	0.25	21.3	0.26
Lactose	28.6	0.26	20.6	0.27
Mannitol	24.2	0.28	21.2	--
Sucrose	32.0	0.24	20.2	0.25
Trehalose	24.5	0.27	24.0	0.27
Before Lyophilization	64.3	0.27	59.6	0.27

[1] Freeze Mobile 12, Vertis Co.

[2] Nicomp 370

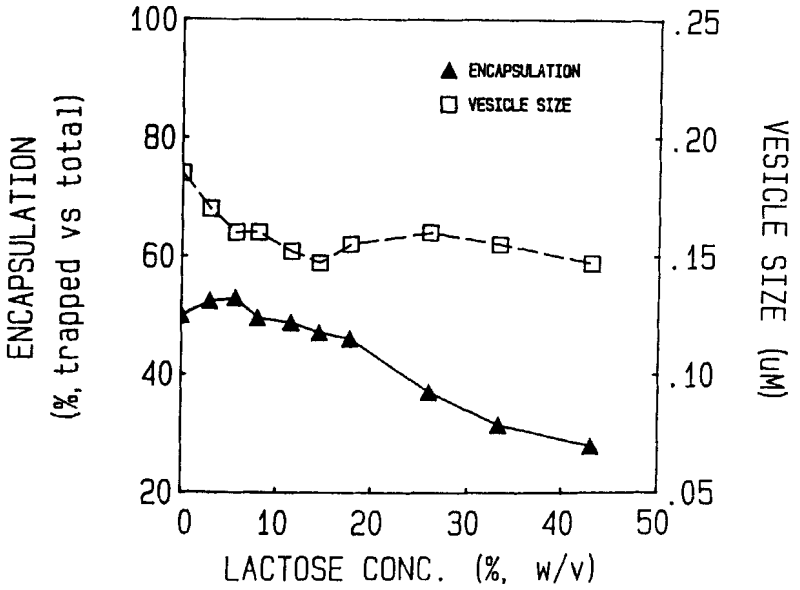

Figure 2

Effect of various concentrations of lactose on the ability of liposome to preserve its vesicle size and the percent encapsulation of drug during lyophilization. Lactose was added to the liposome suspension prior to lyophilization.

concentration was added to a liposome suspension prior to lyophilization. As can be seen in Figure 2, there is a clear trend of loss of encapsulation with increasing concentration of the added lactose. A similar trend was observed for the vesicle size: the higher the lactose concentration, the smaller the vesicles. The effect of lactose on the vesicle size may explain

the effect of lactose on the loss of encapsulation. Small vesicles require more surface area to entrap a given volume. Thereby, the smaller the vesicle it becomes, the lower the entrapment efficiency it will have. However, it is unknown why the higher the lactose concentration, the smaller the vesicle it becomes after lyophilization. From the "range finding" study shown in Figure 2, a range of 1 - 5% lactose was selected for the remainder of this investigation.

Figures 3 - 6 summarize the results of the freezing rate study. Liposome suspensions were frozen at three freezing rates (see the experimental section for details). After thawing at room temperature, the samples were analyzed for vesicle size and percent encapsulation. Results are shown in Figures 3 and 4. A number of the above frozen samples were subject to freeze-drying. The lyophilized samples were then reconstituted for analysis subsequently. Results are shown in Figures 5 and 6. From Figures 3 and 4, it can be concluded that freezing (and possibly the subsequent thawing) is the predominant cause of the leakage of liposome content during lyophilization. The percent encapsulation dropped from 65% to about 45% after freezing (Figure 3) and remained at about the same level of encapsulation after subsequent freeze-drying (Figure 5), regardless with or without lactose. Various freezing rates do

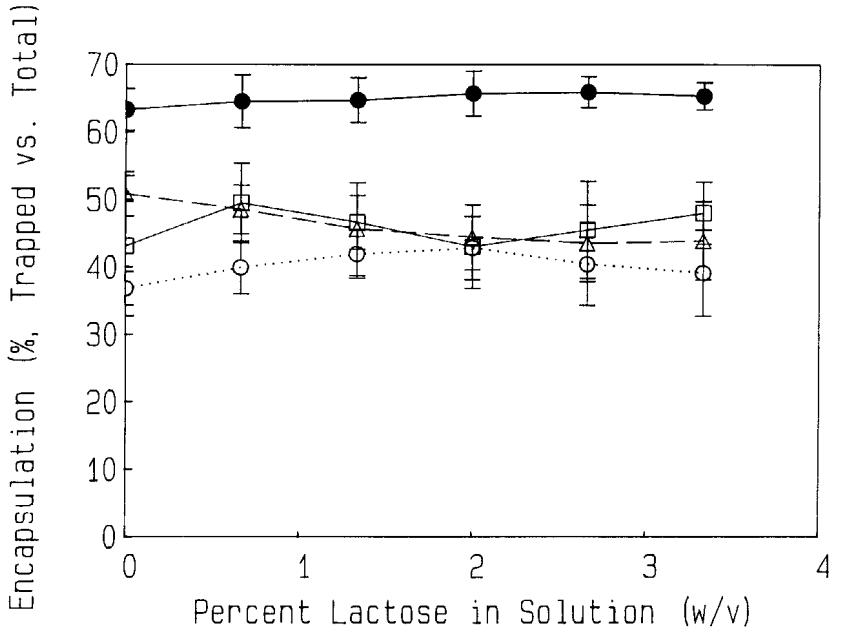

Figure 3

Effect of various freezing rates on the freeze-thaw damage to percent encapsulation of liposomes in the presence of lactose. Lactose was added to the liposome suspension prior to freezing. The frozen samples were then thawed at room temperature for analysis. Key: , frozen in one minute; , frozen in five minutes; , frozen in 1/2 hour; , control, before freezing. Each of the data points is an average of three values obtained on three different lots. The vertical bars denote one standard deviation.

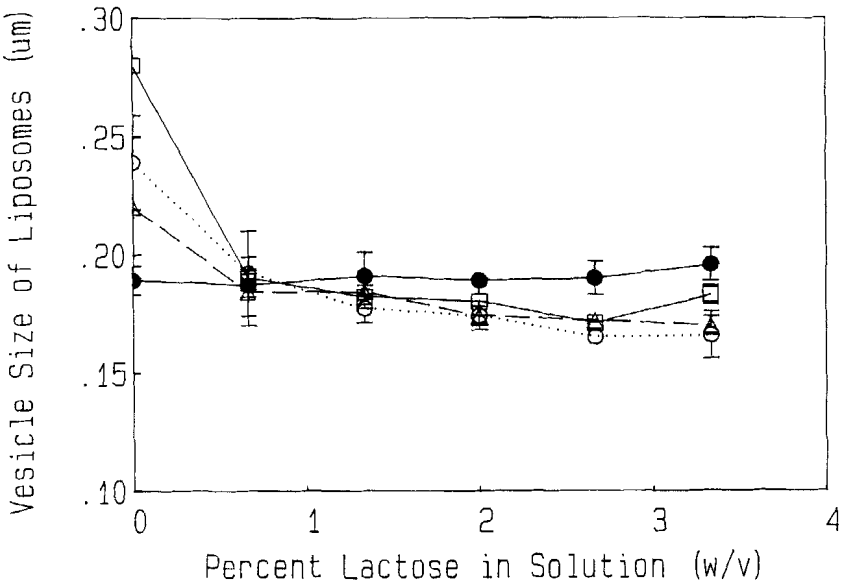

Figure 4

Effect of various freezing rates on the freeze-thaw damage to the vesicle size of liposomes in the presence of lactose. Lactose was added to the liposome suspensions prior to freezing. The frozen samples were then thawed at room temperature for analysis. Key: , frozen in one minute; , frozen in five minutes; , frozen in 1/2 hour; , control, before freezing. Each of the data points represent an average of three values obtaining on three different lots. The vertical bars denote one standard deviation.

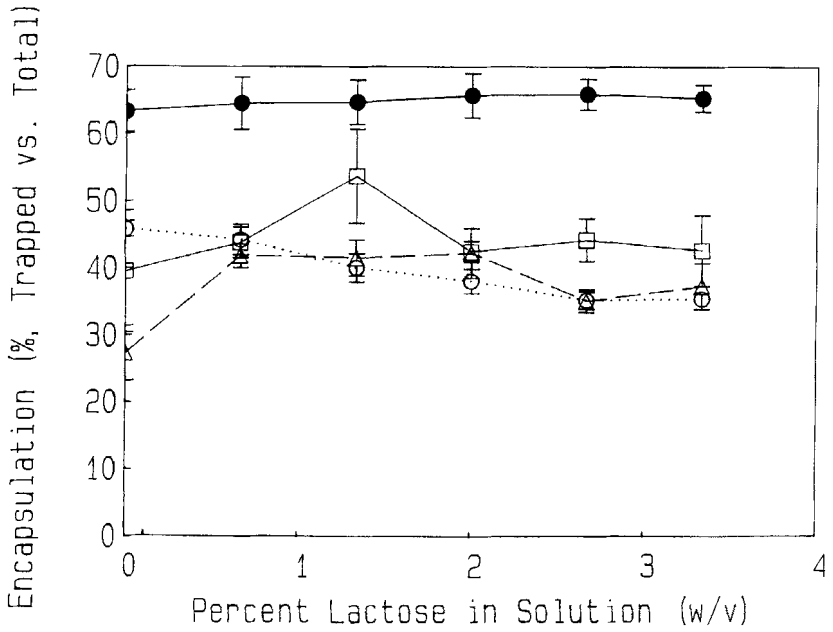

Figure 5

Effect of various freezing rates on the lyophilization damage to the percent encapsulation of liposome in the presence of lactose. Lactose was added to the liposome suspension prior to freezing. The frozen samples were then lyophilized and reconstituted for analysis. Key: , frozen in one minute; , frozen in five minutes; , frozen in 1/2 hour; , control, before freezing. Each of the data points is an average of three values obtained on three different lots. The vertical bars denote one standard deviation.

not seem to alter the extent of the freezing damage. The freezing damage to the percent encapsulation was obviously not preventable by lactose. However, Figures 4 and 6 reveal that all concentrations of lactose (ranging from 1 to 5%) prevent two or more liposome vesicles from fusing into larger vesicles. Without lactose, the vesicles grew from 0.19 microns to 0.24, 0.22, and 0.28 microns after freezing at the thirty-minute, five-minute, and one-minute freezing rates, respectively (Figure 4). Similarly, after subsequent freeze-drying, the vesicle sizes of the liposome without lactose were found to be 0.19 (thirty-minute), 0.27 (five-minute), and 0.28 (one-minute). Freeze-drying (and possibly the subsequent reconstitution) apparently further altered the vesicle size of liposome. A comparison of Figures 4 and 6 reveals that the vesicle sizes of the lactose-protected liposomes differ very little among the three freezing rates after freeze-thawing (Figure 4) while the vesicle sizes of the same liposomes differ a lot more after the subsequent freeze-drying and reconstitution (Figure 6).

Figure 7 shows the effect of lactose concentration (W/V) on osmolality of liposome suspension before and after freeze-thaw. Each point in this graph is an average of three determinations. The top curve represents the osmolarity of liposomes after freeze-thawing, while the bottom curve is for before freeze-thawing. As the concentration of lactose

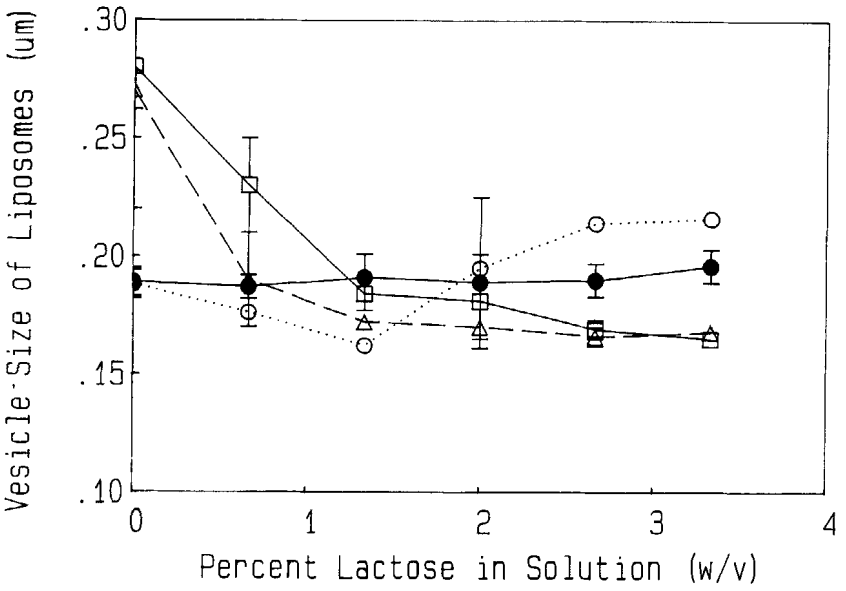

Figure 6

Effect of various freezing rates on the lyophilization damage to the vesicle size of liposome in the presence of lactose. Lactose was added to the liposome suspension prior to freezing. The frozen samples were then lyophilized and reconstituted for analysis. Key: , frozen in one minute; frozen in five minutes; frozen in 1/2 hour; , control, before freezing. Each of the data points obtained on three different lots. The vertical bars denote one standard deviation.

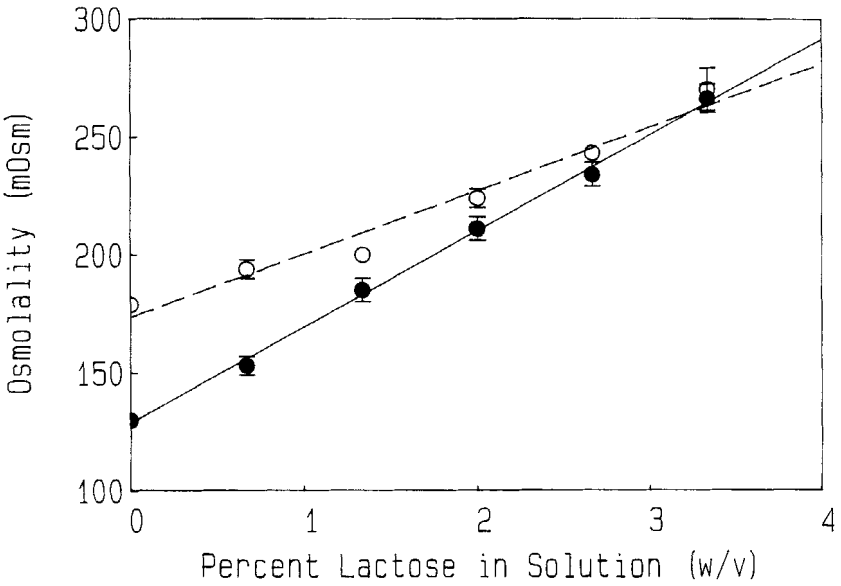

Figure 7

Effect of lactose concentration on osmolality of liposome before and after freeze-thawing. Key: , before freeze-thawing; and , after freeze-thawing. Each point is an average of nine determinations. The vertical bars on the points denote one standard deviation.

in solution increased, the difference in osmolality for before and after freeze-thawing samples was narrowed. Although there are detectable differences in osmolality at different concentrations of lactose in the formulation were found, it did not show significant variations in drug encapsulation and vesicle size. However, inclusion of lactose in freezing media, indeed, protected the liposome vesicles from fusion.

In conclusion, leakage of the liposome content after lyophilization is primarily caused by the freezing step of the process. Freezing rate does not affect the extent of the leakage. None of the sugars studied prevented the freezing damage. However, 1 - 5% lactose adequately prevents the relative increase in apparent liposome size seen following the freezing step of the lyophilization.

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