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Stabilization of lipid/DNA complexes during the freezing step of the lyophilization process: the particle isolation hypothesis

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Abstract

The instability of nonviral vectors in aqueous suspensions has stimulated an interest in developing lyophilized formulations for use in gene therapy. Previous work has demonstrated a strong correlation between the maintenance of particle size and retention of transfection rates. Our earlier work has shown that aggregation of nonviral vectors typically occurs during the freezing step of the lyophilization process, and that high concentrations of sugars are capable of maintaining particle size. This study extends these observations, and demonstrates that glass formation is not the mechanism by which sugars protect lipid/DNA complexes during freezing. We also show that polymers (e.g., hydroxyethyl starch) are not capable of preventing aggregation despite their ability to form glasses at relatively high subzero temperatures. Instead, our data suggest that it is the separation of individual particles within the unfrozen fraction that prevents aggregation during freezing, i.e., the particle isolation hypothesis. Furthermore, we suggest that the relatively low surface tension of mono- and disaccharides, as compared to starch, allows phase-separated particles to remain dispersed within the unfrozen excipient solution, which preserves particle size and transfection rates during freezing.

Keywords: Stabilization; Gene delivery; Nonviral vector; Lyophilization; Freezing; Formulation

1. Introduction

DNA-based therapeutics offer promising strategies for the prevention and treatment of diseases that are currently considered untreatable [1–3]. Although viruses have been employed in the majority of gene therapy clinical trials, the administration of these agents causes an immune reaction that can limit therapeutic benefit and compromise patient health [4–7]. Recently, adverse reactions to viral gene delivery vehicles have been implicated in the deaths of patients enrolled in clinical trials [8]. These tragic events have further stimulated an interest in developing efficient synthetic vectors that do not elicit a specific immune response. While contemporary nonviral vehicles have proven to be safe, their efficiency must be improved if therapeutic levels of heterologous gene expression are to be realized. Accordingly, nonviral studies to date have focused almost exclusively on increasing delivery efficiency, and little attention has been paid to other critical pharmaceutical aspects, e.g., stability.

Contemporary nonviral gene delivery systems typically employ cationic liposomes or polycations to collapse DNA via electrostatic interactions [4]. The resulting complex of DNA with cationic agent has

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been shown to facilitate gene delivery both in vitro and in vivo [4,9,10]. However, one of the major barriers to the widespread use of these novel pharmaceutical agents is their high instability in aqueous suspensions [11–15]. Early clinical trials circumvented this instability by preparing vectors at the bedside, immediately prior to injection [9,10]. While more stable nonviral vector preparations have been developed [16–18], it is doubtful that liquid formulations can be rendered sufficiently stable to resist stresses inherent in shipping and storage [15,19,20]. For example, a freeze-thaw cycle that might be encountered during shipping can dramatically reduce transfection rates [15,19–21]. Previous studies have demonstrated that high concentrations of sugars are able to protect nonviral vectors from freezing-induced damage [16,20–25]. Furthermore, frozen formulations have been shown to retain vector size and transfection rates during prolonged storage [16,21]. However, the high cost associated with maintaining the frozen state during shipping and storage is a significant drawback [15,19].

In contrast to frozen formulations, dehydrated preparations are lightweight, and can be shipped and stored at ambient temperatures. Lyophilization has proven to be an effective method for the large-scale production of dried pharmaceuticals. However, the lyophilization process subjects preparations to two distinct stresses that are known to damage nonviral vectors, i.e., freezing and drying [15,19]. Previous studies on the lyophilization of nonviral vectors have clearly demonstrated that transfection rates upon rehydration are closely correlated with the maintenance of particle size [22–26]. These findings are consistent with other reports indicating that vector size plays a critical role in determining rates of gene delivery [27]. Therefore, the development of lyophilized formulations requires that particle size be maintained throughout the lyophilization process.

Although both freezing and drying can promote aggregation, earlier studies indicate that increases in particle size are typically manifested during the freezing step of the lyophilization process [20,22,24,25]. Our previous work has shown that the slow cooling rates achieved during lyophilization cause significantly more aggregation than quick freezing in liquid nitrogen [20]. Presumably, slow cooling allows complexes sufficient time to diffuse away from ice crystals such that particles are concentrated with excipients in the unfrozen fraction. As the temperature is further reduced, the progressive growth of ice crystals causes vectors to be further concentrated in the unfrozen fraction, and aggregation is facilitated. The observation that sugars can be employed to maintain particle size during freezing indicates that these excipients are capable of minimizing interactions among individual particles [15]. However, the mechanism by which excipients exert this effect on nonviral vectors has yet to be clearly elucidated.

Earlier work by Levine and Slade [28] has suggested that protection can be achieved by immobilizing macromolecules in a glassy excipient matrix. These authors proposed that such a mechanism could inhibit protein unfolding and aggregation during both the freezing and drying steps of lyophilization. This hypothesis has become known as the 'vitrification hypothesis', and could potentially explain the observed maintenance of vector size during freezing in the presence of glass-forming carbohydrates (e.g., sucrose). This hypothesis is consistent with reports by Crowe et al. [29] showing that liposome fusion can be prevented by vitrification, but that warming of frozen samples above the glass transition temperature (T_g') allows fusion to occur. In addition, these authors show that the physical separation of dipalmitoylphosphatidylcholine (DPPC) liposomes within the glassy excipient matrix is sufficient to prevent fusion. We propose that a similar physical separation is responsible for the reported excipient-induced protection of nonviral vectors during freezing. However, we suggest that particle isolation may be achieved via an increase in the volume of the unfrozen fraction, and therefore vitrification is not required to maintain particle size.

To test this hypothesis, we employed complexes of cationic lipid (DMRIE-C) and plasmid DNA as a model nonviral vector system. Excipients with different glass-forming tendencies were assessed for their ability to inhibit aggregation during the freezing step of a typical lyophilization protocol. In addition, differential scanning calorimetry (DSC) was used to monitor glass formation in different formulations. Maintenance of particle size was compared to vitrification during freezing to ascertain whether the physical state of the excipient matrix is the sole determinant of protection. In contrast, our results dem-

onstrate that sugar/DNA ratios and the volume of the unfrozen fraction ultimately determine the extent of cryoprotection.

2. Materials and methods

2.1. Reagents

Sucrose and glucose were purchased from Pfanstiehl Laboratories (Waukegan, IL). Mannitol was purchased from Sigma (St. Louis, MO). Purified hydroxyethyl starch (HES) was obtained from Fresenius (Linz, Austria). DMRIE-C is a cationic lipid formulation consisting of a 1:1 molar ratio of the cationic lipid DMRIE (1,2-dimyristoyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide) and cholesterol. DMRIE-C was purchased as an aqueous suspension (2 mg/ml) from Gibco BRL (Grand Island, NY) and stored at 4°C. The DNA plasmid encoding green fluorescent protein (plasmid pGreen Lantern-1) was obtained from Gibco BRL. DNA was dissolved in sterile 2.5 mM Tris–HCl pH 8.5 and diluted to a concentration of 1 mg/ml prior to use.

2.2. Cell culture

African green monkey kidney cells (COS-7: ATCC No. CRL1651) were obtained from American Type Culture Collection (Rockville, MD). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 50 U/ml penicillin G, and 50 μg/ml streptomycin sulfate, and were propagated by reseeding at $1–3\times10^5$ cells/100-mm dish every 2–3 days. For use in our experiments, cultures were freshly seeded at 5×10^4 cells/60-mm dish 24 h before transfection [23].

2.3. Complex preparation and freeze-thawing protocols

The complexes were prepared with a 3:1 lipid/DNA weight ratio (50 μg DMRIE-C/16.7 μg DNA in 2.5 mM Tris–HCl pH 8.5) in polypropylene microcentrifuge tubes by gentle mixing, and incubated for 30 min at room temperature as previously described [20]. This method of preparation results in a heterogeneous suspension of particles with a calculated +/− charge ratio of 0.97. Aliquots of 12 μl, corresponding to 4 μg of DNA of the resulting suspension of lipid/DNA complexes, were diluted to 50 μl total with 2.5 mM Tris–HCl, pH 8.5 and mixed with an equal volume of excipient solutions in Tris buffer. To vary the excipient/DNA ratio, experiments utilized different dilutions of complexes and/or altered the total sample volume (up to 500 μl). Samples were then transferred to 1-ml flat-bottomed borosilicate lyophilization vials (West Co., Litiz, PA). Vials were placed on the shelf of an FTS Durastop lyophilizer (Stone Ridge, NY) and cooled to a shelf temperature of −40°C at 2.5°C/min. Thermocouples placed in vials consistently indicated sample temperatures of −38°C, presumably due to heat radiating from the walls of the lyophilization chamber. Samples were maintained at −38°C overnight, and rapidly thawed in a water bath of 37°C prior to analysis.

2.4. Transfection assay

Lipid/DNA complexes containing 2 μg DNA (thawed and freshly prepared) were diluted in 1 ml serum-free, antibiotic-free DMEM and applied to a 60-mm dish containing COS-7 cells freshly washed with phosphate-buffered saline (PBS). This assay required less lipid and DNA than the dynamic light scattering experiments, but the concentration of each component during complex formation was held constant. The cells were incubated with lipid/DNA complexes for 4 h before the medium was replaced with 3 ml DMEM containing serum and antibiotics as described above. The cells were allowed to grow for approximately 40 h before harvesting. Cells were harvested and washed as previously described [20,23]. Cell pellets were washed and resuspended in PBS for analysis of green fluorescent protein expression using a Coulter Epics XL flow cytometer (Hialeah, FL). Fluorescence at 525 nm was monitored for 5000 cells per sample, and triplicate transfections were measured per experimental condition [22]. Under these conditions, fresh complexes consistently exhibited transfection rates of 30%. Recovery is reported as the percent of cells expressing green fluorescent protein after transfection with thawed com-

plexes, relative to that obtained with fresh complexes.

2.5. Dynamic light scattering analysis

Triplicate suspensions containing 4 μg of plasmid, 12 μg of lipid, and stabilizer were subjected to freeze-thawing as described above. After rapid thawing, samples were diluted to 0.5 ml total volume with distilled water and transferred into a cuvette for dynamic light scattering analysis on a Nicomp 370 Submicron Particle Sizer (Particle Sizing Systems, Santa Barbara, CA). Channel width was set automatically based on the rate of fluctuation of scattered light intensity. The data were volume-weighted, and the analysis assumed that lipid/DNA complexes are solid particles. For very heterogeneous suspensions with X^2 values greater than 3 (e.g., after freezing at low excipient/DNA ratios), 10^6 running sums of intensity were compiled over 64 channels to ensure that sufficient data were incorporated into the autocorrelation function [22].

2.6. DSC

The glass transition temperature of the freeze-concentrated excipient solution (T_g') was quantified with a Perkin-Elmer DSC-7 differential scanning calorimeter (Norwalk, CT). Samples were carefully weighed, sealed in aluminum pans, and cooled at 2.5°C/min to simulate the freezing experiments. During warming (10°C/min), T_g' was calculated as the midpoint of the transition in heat capacity as previously described [30].

2.7. Surface tension measurements

The apparent surface tensions of concentrated solutions of HES, sucrose, and glucose were determined with a Surface Tensiometer (model 20, Fisher Scientific). The true surface tension of each solution was calculated as described by the manufacturer. Sterile solutions were transferred to 60-mm petri dishes, and triplicate determinations were made on each sample. We discovered that a 62.5% HES mixture forms a gel at room temperature, thereby prohibiting the assessment of surface tension at this concentration. However, lower concentrations of HES formed fluid solutions of which the surface tension could be accurately determined. Thus, measurements were conducted on a series of HES solutions (10, 20, 30, 40, 50%), and the data extrapolated to a concentration of 62.5%. To insure that thermal equilibrium had been achieved, all solutions and equipment were incubated overnight at each temperature (4, 24, 37°C) prior to the determination of surface tension.

2.8. Statistical analysis

Mean particle sizes of frozen complexes were compared to that of fresh preparations. Statistically significant differences were determined using a two-tailed Student's *t*-test with Graphpad Prism Software (San Diego, CA). Mean particle sizes having P values less than 0.05 were judged to be significantly different from fresh preparations, and these data points are indicated in each figure by closed symbols.

3. Results

As shown in previous studies, lipid/DNA complexes form large aggregates during slow freezing (Fig. 1). Aggregation can be attenuated by the presence of sucrose, and high excipient concentrations are capable of maintaining complex sizes comparable to unfrozen controls (Fig. 1A). However, if a similar experiment is conducted with a more dilute suspension of complexes, much lower concentrations of sucrose are needed to retain complex size (Fig. 1B). The observation that lower concentrations of complexes require less sucrose for protection indicates that the initial concentration of excipient is not the parameter determining maintenance of particle size. When data from these experiments are plotted against the sucrose/DNA ratio, it can be seen that attenuation of aggregation is achieved at ratios above 500 (Fig. 2). Furthermore, when data from similar experiments are plotted together, maintenance of particle size is observed in each case at sucrose/DNA ratios of 1000 (Fig. 2). It is interesting that the freezing of highly diluted suspensions of complexes resulted in markedly larger particle sizes than more concentrated suspensions at the same sucrose/DNA ratio (Fig. 2). Considering that a greater mass of ice will be formed in more dilute samples, we

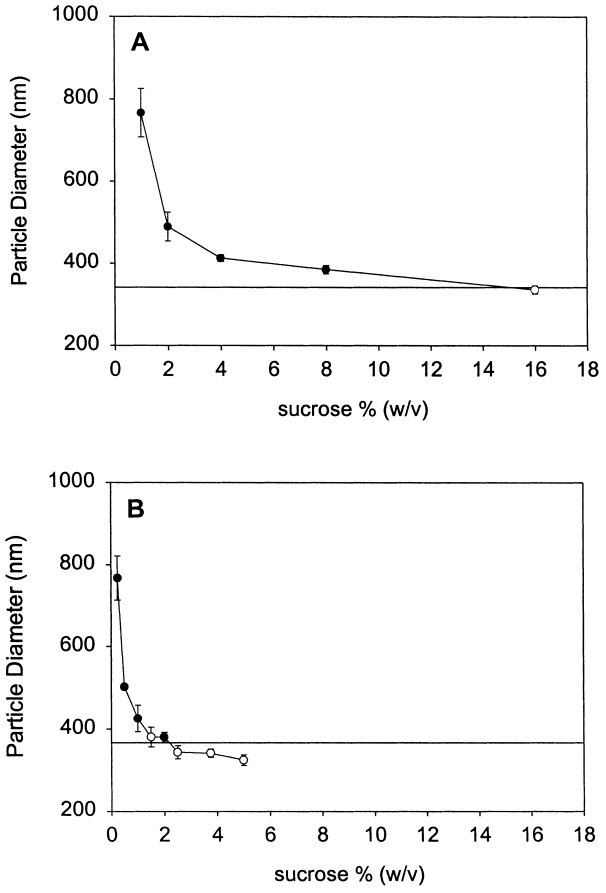

Fig. 1. Effect of sucrose concentration on the maintenance of particle size during freezing. DNA concentrations of 160 μg/ml (A) and 40 μg/ml (B) were employed in separate sets of samples subjected to freezing. The horizontal line at approximately 350 nm represents the size of complexes prior to freezing. Particle diameters that were significantly different ($P < 0.05$) from unfrozen controls are indicated by closed symbols. Each symbol represents the mean ± 1 S.D. of single measurements on triplicate samples.

suspect that increased interactions with the larger ice crystal surface could potentially explain this effect. Similar observations with proteins have been attributed to surface denaturation at the ice–water interface [31]. Regardless of the role of ice crystals in promoting damage to complexes, particle sizes comparable to fresh controls were observed at higher sucrose/DNA ratios.

As detailed in Section 2, samples in each of the experiments described above were frozen to −38°C and incubated overnight. Since the method of rapid warming results in practically instantaneous thawing, it is unlikely that sufficient time is available to allow

Table 1
Glass transition temperatures of different samples

Excipient	Excipient/DNA (w/w)	T_g' (°C)[a]
HES	300	−10.9 ± 02
HES	600	−10.2 ± 0.2
HES	∞	−9.4 ± 0.7
Sucrose	60	−37.2 ± 0.2
Sucrose	300	−32.4 ± 0.7
Sucrose	600	−31.6 ± 0.2
Sucrose	∞	−31.3 ± 0.5
Glucose	60	−47.2 ± 0.3
Glucose	300	−44.0 ± 0.4
Glucose	600	−43.1 ± 0.2
Glucose	∞	−42.6 ± 0.2

[a]Values represent the mean ± 1 S.D. of single calorimetric scans on triplicate samples.

aggregation during warming. Instead, our results suggest that aggregate formation occurred during the freezing process. We point out that frozen sucrose forms a glass at −32°C (Table 1) that immobilizes complexes, and could potentially explain the observed maintenance of particle size. However, even samples with low sucrose/DNA ratios that do not prevent aggregation are glassy under our experimen-

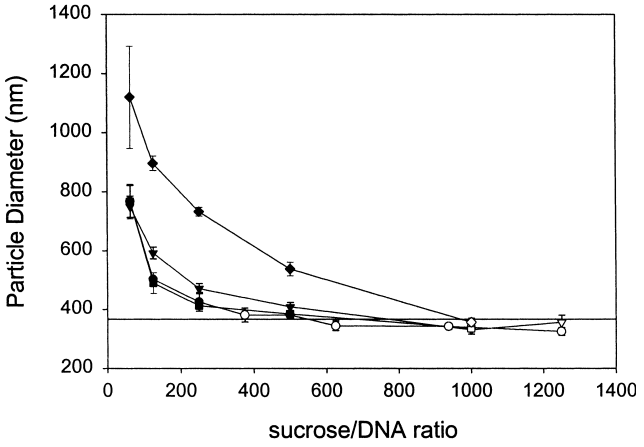

Fig. 2. Effect of sucrose/DNA weight ratio on the retention of particle size during freezing. Particle diameters of four different series of samples with constant DNA concentrations of 8 μg/ml (diamonds), 40 μg/ml (circles), and 160 μg/ml (squares) or constant sucrose concentration (1%, triangles) were assessed after freeze-thawing. The horizontal line at approximately 350 nm represents the size of complexes prior to freezing. Particle diameters that were significantly different ($P < 0.05$) from unfrozen controls are indicated by closed symbols. Each symbol represents the mean ± 1 S.D. of single measurements on triplicate samples.

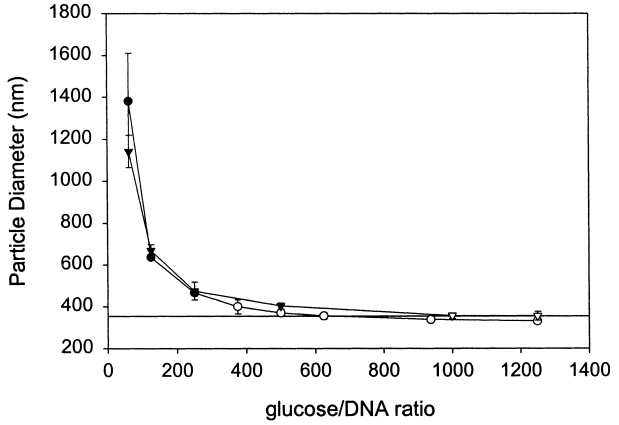

Fig. 3. Effect of glucose/DNA weight ratio on the retention of particle size during freezing. A constant concentration of glucose (1%, triangles) or DNA (40 μg/ml, circles) was employed in separate sets of samples subjected to freezing. The horizontal line at approximately 350 nm represents the size of complexes prior to freezing. Particle diameters that were significantly different ($P < 0.05$) from unfrozen controls are indicated by closed symbols. Each symbol represents the mean ± 1 S.D. of single measurements on triplicate samples.

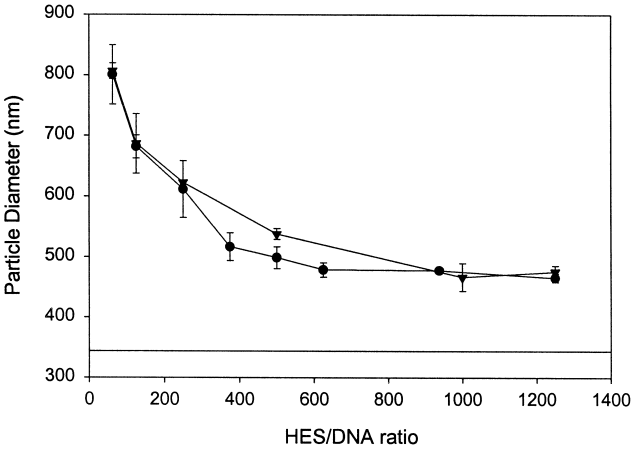

Fig. 4. Effect of HES/DNA weight ratio on the retention of particle size during freezing. A constant concentration of HES (1%, triangles) or DNA (40 μg/ml, circles) was employed in separate sets of samples subjected to freezing. The horizontal line at approximately 350 nm represents the size of complexes prior to freezing. The closed symbols at each ratio indicate that particle diameters were significantly different ($P < 0.05$) from unfrozen controls in every case. Each symbol represents the mean ± 1 S.D. of single measurements on triplicate samples.

tal conditions (compare Table 1 and Fig. 2). These data indicate that formation of a glassy state is not the mechanism by which sucrose prevents aggregation during freezing.

Similar protection was observed when glucose was used as a stabilizing excipient (Fig. 3). Again, particle sizes comparable to unfrozen controls were achieved at sugar/DNA ratios above 500. In contrast to the sucrose samples, complexes suspended in glucose did not vitrify under our conditions, and therefore immobilization of particles in a glassy matrix cannot explain the observed protection (Table 1). Consistent with this assertion, samples containing HES ($T_g' = -10$°C) vitrified at high subzero temperatures, yet particle size was not retained even at the highest concentrations investigated (Fig. 4). While an increase in HES/DNA ratio did attenuate aggregation to some degree, the ability of HES to maintain vector size was clearly inferior to that observed with sucrose and glucose (compare Fig. 4 with Figs. 2 and 3). Analysis of the calorimetric data shows that frozen mixtures of complexes and HES exhibited two distinct glass transitions, suggesting that the physical properties of these two components are not coupled (Fig. 5). This is not surprising considering that complexes form a suspension, and are therefore phase-separated from the excipient solution. It is worth noting that the presence of complexes clearly alters the T_g' of each of the excipients (Table 1). These findings indicate that although particles in suspension are phase-separated from the excipients, interactions with suspended complexes do influence the

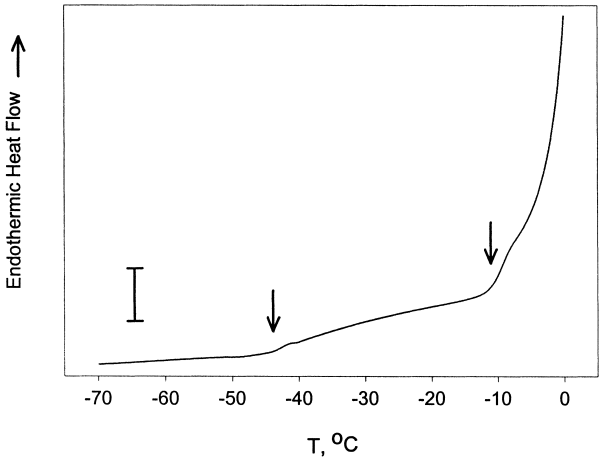

Fig. 5. DSC indicates that lipid/DNA complexes remain separated from the excipient solution. DSC thermogram shows the different glass transitions (arrows) in a frozen mixture of DMRIE-C/DNA complexes and HES. Vertical bar equals 0.1 W/g.

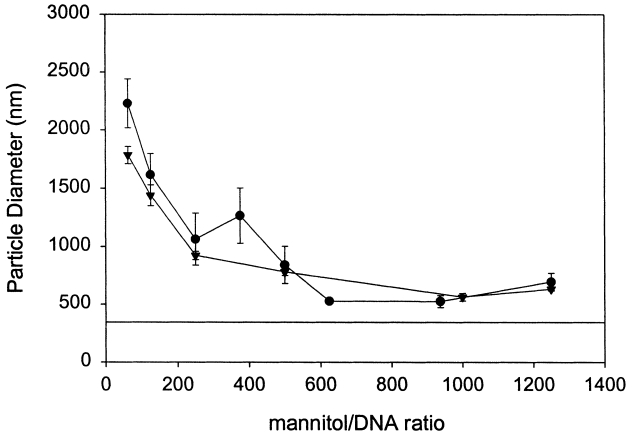

Fig. 6. Effect of mannitol/DNA weight ratio on the retention of particle size during freezing. A constant concentration of mannitol (1%, triangles) or DNA (40 μg/ml, circles) was employed in separate sets of samples subjected to freezing. The horizontal line at approximately 350 nm represents the size of complexes prior to freezing. The closed symbols at each ratio indicate that particle diameters were significantly different ($P < 0.05$) from unfrozen controls in every case. Each symbol represents the mean ± 1 S.D. of single measurements on triplicate samples.

physical properties of the excipient solution. Therefore, it is possible that the characteristics of the excipient may impact the stability of the suspended complexes.

Similar to protection by HES, increasing concentrations of mannitol helped maintain complex size, but diameters comparable to fresh samples were not recovered even at the highest mannitol/DNA ratios (Fig. 6). The fact that mannitol does not fully preserve complex size is consistent with our observation that it crystallizes during freezing [22,32]. However, these data show that mannitol does offer significant levels of protection (Fig. 6). We suggest that eutectic crystallization of mannitol during freezing may be capable of spatially separating complexes and attenuating particle interactions.

Another factor that may limit the dispersion of complexes within excipient solutions is surface tension. Considering that the surface tension of aqueous solutions progressively increases with greater excipient concentrations and lower temperatures, an excipient solution possessing high surface tension could potentially reduce energetically unfavorable surface area by expelling phase-separated particles. Accordingly, the rejection of particles from the excipient solution could cause complexes to coalesce and aggregate during freezing. Of course, this argument is not valid if the solution has vitrified. But, prior to glass formation, surface tension may partially determine whether complexes remain dispersed (isolated) in the unfrozen fraction. To investigate the potential role of surface tension, we first utilized DSC measurements to calculate the excipient concentrations in the unfrozen fraction. By analyzing the enthalpy of melting, we calculated the amount of water that

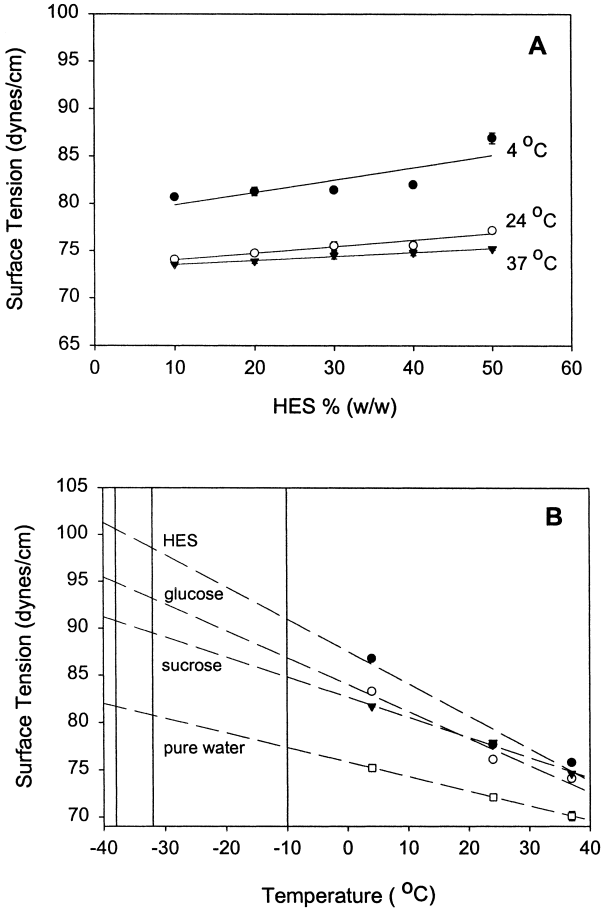

Fig. 7. Effect of excipients on surface tension in the unfrozen fraction. The surface tension of concentrated excipient solutions was assessed at 4°C, 24°C, and 37°C. Different HES concentrations (10–50%) are plotted with fitted regression lines (A). The regression analysis was used to extrapolate the surface tension of 62.5% HES at each temperature. The surface tensions of excipient solutions at their respective concentrations in the unfrozen fraction are plotted with regression lines extrapolating to subzero temperatures. Vertical lines indicate the sample temperature (−38°C) and measured T_g' values for sucrose (−32°C) and HES (−10°C). Each symbol represents the mean of triplicate measurements on a single solution. Standard deviations are within the size of the symbols.

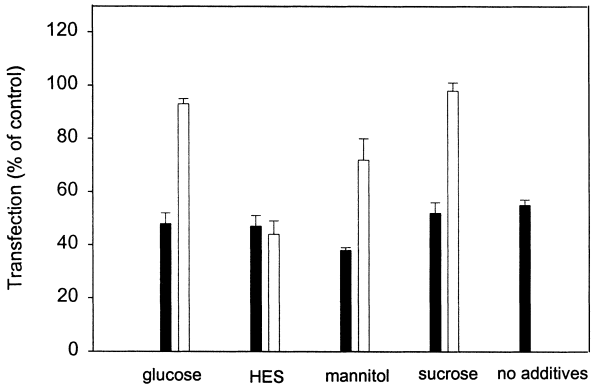

Fig. 8. Transfection rates after freezing at high and low excipient/DNA ratios. Transfection rates of complexes frozen at excipient/DNA ratios of 12.5 (closed bars) and 625 (open bars) were assessed after freezing. Each bar indicates the mean ± 1 S.D. of transfection rates in triplicate cell dishes.

remained unfrozen in different samples at −38°C. Our results indicate that HES, sucrose, and glucose have concentrations of 62.5%, 58% and 45.5% (w/w), respectively, in their unfrozen fractions. Thus, we measured the surface tension of these solutions at different temperatures and extrapolated these data to subzero temperatures. Our data show the surface tensions of HES solutions are markedly higher at low temperatures than comparable solutions of either glucose or sucrose (Fig. 7). However, if our measurements are extrapolated to temperatures at which complexes would have experienced a fluid (non-glassy) environment (i.e., −10°C, −32°C, −38°C for HES, sucrose, and glucose, respectively), the surface tension of unfrozen HES is comparable to that of sucrose or glucose. It should be noted that the extrapolation of surface tension measurements to subzero temperatures may not be linear as depicted in Fig. 7. Unfortunately, technical difficulties involved in determining the surface tension of partially frozen solutions prevent us from directly testing the accuracy of our extrapolation.

Additional experiments were performed to determine if the maintenance of particle size during freezing was sufficient to preserve transfection rates. For each additive investigated here, transfection rates were assessed after freeze-thawing at low and high excipient/DNA ratios (Fig. 8). Consistent with that observed for the retention of particle size, low excipient/DNA ratios resulted in significant reductions in transfection rates. At high excipient levels, only sucrose and glucose were capable of maintaining transfection levels comparable to fresh preparations (Fig. 8). Surprisingly, high mannitol levels offered significantly more protection than HES, again suggesting that mechanisms other than the formation of a glassy matrix contribute to the observed preservation of complexes during the freezing step of lyophilization.

4. Discussion

The data in Fig. 1 are consistent with previous reports showing that sucrose is capable of maintaining complex sizes during freezing [20,22,24,25]. In addition, our observations demonstrate that the initial sucrose concentration (i.e., prior to freezing) at which protection is observed is altered dramatically if more dilute suspensions of complexes are employed. These findings demonstrate that it is not the initial sucrose concentration that determines the level of protection during freezing. Instead, the amount of sucrose relative to the quantity of complexes (i.e., sucrose/DNA ratio) appears to dictate preservation of particle size (Fig. 2). Previous studies have suggested that immobilization in a glassy excipient matrix is sufficient to prevent aggregation [28,29,33]. According to this suggestion, formation of a glass should be sufficient to preserve particle size. To the contrary, our data demonstrate that vitrification occurs at sucrose/DNA ratios well below that at which protection is observed (Table 1). Furthermore, HES, which readily vitrified during freezing, was not able to maintain particle size (Fig. 4). Moreover, glucose effectively preserved particle sizes despite the lack of glass formation under our conditions (Fig. 3). Although these findings do not completely rule out vitrification as a contributing factor in cryoprotection, our results are clearly incompatible with glass formation as the major determinant of particle size retention. Consistent with the effects on complex size after freezing, only sucrose and glucose were capable of maintaining transfection rates comparable to fresh preparations (Fig. 8).

To investigate the effect of excipients that crystallize during freezing, complexes were suspended in solutions of mannitol (Fig. 6). Surprisingly, increasing mannitol/DNA ratios dramatically inhibited aggregation, even though eutectic crystallization was clearly

evident (data not shown). In fact, protection by mannitol was very similar to that observed with HES despite the distinct differences in the physical state of these two excipients, i.e., crystalline vs. glassy (compare Figs. 4 and 6). It is possible that a very small amount of mannitol (undetectable by DSC) remains associated with the complexes and attenuates aggregation by sterically inhibiting interactions between particles. However, we would expect that any association with complexes would be saturated at mannitol concentrations that crystallize. Our data show that increasing levels of mannitol progressively inhibit aggregation despite crystallization at low mannitol/DNA ratios during freezing (Fig. 6). Therefore, we feel it is unlikely that a direct association of mannitol with the complexes is responsible for the observed attenuation of aggregation.

The inability of glass-forming excipients to preserve macromolecular integrity is well known [34,35]. Crowe et al. [29,33–36] have long argued that vitrification is insufficient to preserve macromolecules during freeze-drying. These authors have proposed that sugars hydrogen bond directly to dehydrated lipids, and prevent membrane phase transitions that cause leakage of encapsulated solutes during lyophilization [34,35]. However, their work has also shown that stabilization of liposomes with high phase transition temperatures (e.g., DPPC) requires only that aggregation and fusion be prevented [29]. During freezing, this can be accomplished by excipients that form glasses, e.g., dextran. To illustrate this effect, Crowe et al. [29] demonstrated that frozen DPPC liposomes readily aggregate and fuse when incubated at temperatures above T_g'.

In contrast to our experiments, the reports described above utilized traditional liposomes, and are therefore not directly comparable. Although the physical properties of lipid/DNA complexes have yet to be fully characterized [37], these particles are too large to be dissolved in solution. Thus, like liposomes, lipid/DNA complexes form a suspension that remains phase-separated from the aqueous excipient solution (Fig. 5). Furthermore, the electrostatic interactions between cationic lipids and DNA are sufficiently strong to prevent dissociation (i.e., 'leakage') during lipid phase transitions. However, the particles in our study contain lipids that can spontaneously aggregate and fuse with other complexes if physical contact is achieved. Therefore, we feel it is reasonable to compare our data on the maintenance of complex size to results by Crowe et al. that utilized DPPC liposomes [29]. Regardless of the validity of this comparison, our findings are not consistent with the suggestion that vitrification is sufficient to prevent aggregation of suspended particles during freezing [28,29]. Instead, our data indicate that vitrification, alone, does not fully preserve particle size (Fig. 4). Furthermore, incubation of complexes frozen in glucose above T_g' is sufficient to prevent aggregation over the time course of our experiments ($\sim$18 h).

In an effort to explain the lack of aggregation observed in our experiments with glucose, it seems possible that the mobility of complexes is sufficiently retarded to prevent interactions between particles over the duration of our experiments. Although glucose was not vitrified under our experimental conditions, the viscous unfrozen fraction at −38°C may sufficiently slow the diffusion of complexes to prevent aggregation. According to this hypothesis, the time complexes spend in the freeze-concentrated state plays a critical role in determining the extent of aggregation. Obviously, any non-zero rate of diffusion will permit aggregation given sufficient time. For the purposes of our studies, we chose a timeframe consistent with primary drying in a typical lyophilization protocol.

It is important to note that suspended particles should not significantly alter the freezing point of the excipient solution. Accordingly, the concentration of glucose in the unfrozen fraction is determined solely by the sample temperature regardless of the initial sugar concentration prior to freezing. Thus, the glucose concentrations at −38°C are equivalent for every data point shown in Fig. 3. If we assume that phase-separated particles have little effect on the physical properties of the unfrozen excipient solution (especially at the high sugar/DNA ratios employed here), the viscosity in each of these glucose samples should also be comparable. As mentioned above, the high viscosity of the unfrozen fraction may serve to immobilize complexes and prevent aggregation. While our experiments did not monitor viscosity during freezing, complete protection was only observed at high glucose/DNA ratios (Fig. 3), indicating that viscosity, alone, cannot explain our findings.

In addition to incubation time and viscosity, the average distance separating complexes in the unfrozen fraction will also determine whether particles can physically interact. Thus, a more concentrated suspension of complexes in the unfrozen solution will have a greater probability of aggregation. Although the glucose concentration at −38°C is the same in all cases, the volume of the unfrozen fraction is directly dependent on the initial glucose concentration prior to freezing. It follows that the concentration of complexes in the unfrozen fraction is determined by the glucose/DNA ratio used in sample preparation, i.e., higher ratios result in lower concentrations of suspended particles in the unfrozen fraction. This is consistent with the progressive inhibition of aggregation observed at higher glucose/DNA ratios (Fig. 3).

Considering the different factors discussed above, our data are most consistent with the suggestion that aggregation during freezing is prevented at high glucose/DNA ratios by isolating complexes within a viscous unfrozen syrup. The same mechanism could apply during freezing in the presence of other excipients that have T_g' values below the sample temperature during primary drying. Although complexes in sucrose are immobilized in a glass at −38°C, we presume that particle isolation is responsible for the maintenance of complex size during freezing above T_g'. Furthermore, we suggest that a similar mechanism could potentially explain the partial protection by mannitol, provided that crystalline material could serve to spatially isolate particles (Fig. 6). This is consistent with our previous work with mannitol, and the general observation that other excipients which crystallize during lyophilization (e.g., polyethylene glycol) also offer partial protection to lipid/DNA complexes [22]. However, we have yet to observe complete maintenance of complex size in formulations that do not remain amorphous.

The inability of HES to preserve complex size during freezing is puzzling. As shown in Table 1, HES has a high T_g' (~ -10°C) consistent with earlier reports [32]. It would seem that complexes dissolved in HES would be separated in a viscous, starchy solution similar to that proposed for sugars. Once the sample temperatures decreased below T_g', complexes would be immobilized in a glass, and could not aggregate. However, it is possible that the freeze-induced concentration of HES involves a process by which complexes are expelled from the viscous HES solution. We propose that this process does not allow individual complexes to remain fully dispersed in the unfrozen HES solution, in contrast to the mechanism proposed for protection by glucose and sucrose.

Complexes suspended in a solution represent an emulsion of which the stability is governed by the surface tension of the dispersion medium, i.e., the excipient solution [38]. Therefore, complexes will tend to coalesce in response to a high surface tension, whereas a low surface tension will allow particles to remain dispersed in the excipient solution. This effect is enhanced during cooling because the surface tension of aqueous solutions is greater at lower temperatures. Furthermore, the surface tension is abruptly increased upon ice nucleation due to the instantaneous rise in excipient concentration. In order to explain the inability of HES to fully preserve particle size, the HES solution in the unfrozen fraction would have to possess a significantly higher surface tension than that of sucrose and glucose. Our measurements are consistent with this hypothesis and demonstrate a markedly higher surface tension in HES solutions that simulate the unfrozen fraction (Fig. 7). Based on these results, we suggest that the high surface tension of HES solutions might cause suspended complexes to coalesce during freezing, resulting in the observed aggregation. Conversely, the relatively low surface tensions of concentrated sucrose and glucose solutions may allow complexes to remain dispersed in the sugar solution, thereby isolating individual particles and preventing aggregation during freezing.

While the surface tension argument presented above is compelling, it should be pointed out that viscosity increases many orders of magnitude and translational motion is greatly retarded upon glass formation [32]. As a result, aggregation cannot occur after vitrification. Thus, it could be argued that the relevant surface tension is that exhibited by the solution just prior to vitrification, i.e., at T_g'. If we take into account the temperature difference between glass formation in sucrose and HES (i.e., −32°C vs. −10°C), our extrapolated data indicate that surface tensions would be approximately equivalent in these solutions (Fig. 7). However, the higher viscosity at lower temperatures would also contribute toward

particle isolation, and therefore additional studies would be required to fully address this question. In addition, the observation that 62.5% HES forms a gel might indicate that a gelation process that occurs during freezing could also contribute to our observations (see Section 2).

In conclusion, our data show that sucrose and glucose effectively prevent aggregation of lipid/DNA complexes during the freezing step of the lyophilization process. We propose that these sugars isolate individual particles in the unfrozen fraction, thereby preventing aggregation during freezing, i.e., the particle isolation hypothesis. We point out that vitrification is not required for this effect, and suggest that spatial separation of particles within the unfrozen fraction is sufficient to prevent aggregation. According to this hypothesis, sufficient quantities of virtually any excipient should offer similar protection during freezing, a supposition that is consistent with our previous findings [20,22,23]. Furthermore, the high excipient/DNA ratios required for protection indicate that the concentrated nonviral vector suspensions employed in clinical trials may be difficult to preserve by lyophilization. For example, a sugar/DNA ratio of 1000 (as required for consistent protection in this study) would be physically impossible to achieve at a DNA concentration of 1 mg/ml. Similarly, an isotonic sucrose solution would only be sufficient to protect a vector preparation with a DNA concentration of 0.1 mg/ml. Of course, hypertonic sugar solutions could be employed to improve protection during lyophilization, but this approach would significantly lengthen primary drying times and increase production costs. Therefore, we suggest that combinations of excipients with optimized physical properties might be engineered for more efficient cryoprotection. We also point out that the conditions needed to preserve the DMRIE-C/DNA complexes employed in this study may not be generally applicable to other nonviral vectors. It is possible that vectors that incorporate different cationic components (e.g., polyethylene imine) and/or polyethylene glycol may be more resistant to freezing-induced aggregation. Therefore, other vectors might prove to be more amenable to lyophilization at high DNA concentrations. Future experiments are designed to investigate the applicability of our findings to the lyophilization of other nonviral vectors.

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