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Research Article

Effect of Lyophilization and Freeze-thawing on the Stability of siRNA-liposome Complexes

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Abstract. The purpose of this research was to describe the application of lyophilization in the delivery of siRNA using cationic lipids by addressing the long-term formulation/stability issues associated with cationic lipids and to understand the mechanism of lyoprotection. siRNA liposomes complexes were formed in different potential cyro/lyoprotectants and subjected to either lyophilization or freeze thaw cycles. siRNA, liposomes and/or lipoplexes were tested for activity, SYBR Green I binding, cellular uptake and particle size. The lipoplexes when lyophilized in the presence of sugars as lyoprotectants could be lyophilized and reconstituted without loss of transfection efficacy but in ionic solutions they lost 65–75% of their functionality. The mechanism of this loss of activity was further investigated. The lyophilization process did not alter siRNA's intrinsic biological activity as was evident by the ability of lyophilized siRNA to retain functionality and SYBR green I binding ability. While the lipoplex size dramatically increased (~50–70 times) after lyophilization in the absence of non-ionic lyoprotectants. This increase in size correlated to the decrease in cellular accumulation of siRNA and a decrease in activity. In conclusion, siRNAs can be applied in cationic lipid lyophilized formulations and these complexes represent a potential method of increasing the stability of pre-formed complex.

KEY WORDS: DLS; FACS; freeze-thaw; liposomes; lyophilization; siRNA.

INTRODUCTION

The introduction of 'RNA interference' (RNAi) to the scientific world by Fire and Mello (1) opened the door for treatment of diseases resulting from the expression of defective/undesired protein. Other systems for gene down-regulation such as antisense oligonucleotides and ribozymes have been limited by the concerns related to their potency; specificity and delivery. Studies comparing antisense oligonucleotides with RNAi attest the superior potency and efficiency of the latter (2). RNAi may be defined as post-transcriptional gene silencing due to the cleavage of mRNA, triggered by double-stranded RNA (dsRNA) homologous in sequence to the target mRNA (3,4). It is mediated through small double-stranded RNA molecules (small interfering RNAs, siRNAs).

The inability of siRNA to be therapeutically active when administered naked calls for the development of effective delivery systems. Several vectors (viral and chemical) have been used to prevent nuclease degradation and improve uptake of exogenously administered nucleic acids (5). The chemical delivery vectors-liposomes and polymers are both reported to be effective siRNA delivery agents. Previous evaluation of polyplexes and lipoplexes has demonstrated that due to their release characteristics, favoring unloading in the cytoplasm; liposomes may exhibit certain advantages as siRNA delivery agents over polymers (6). siTox siRNA when successfully transfected into the cells leads to apoptosis and cell death within 24–48 h and the degree of cell death can be correlated to siRNA delivery (http://www.dharmacon.com/docs/siCONTROL%20Flier.pdf.).

Several studies have demonstrated lipoplexes are not stable in liquid suspension for long-term storage and aggregate (7,8). This unstable nature of lipoplexes necessitates their preparation immediately before administration (8,9). Lipoplex formulations prepared by the bedside are difficult to control and test before use. Strategies to improve lipoplex stability include the use of high pH diluents (9), sucrose gradients (10,11) and conjugation of poly-(ethylene glycol) (PEG) to the surface of lipoplexes (12). Such strategies although effective are limited by the cost involved.

Lyophilization is another method for addressing this problem and has been attested to be effective for long-term storage of nucleic acid formulations (7,13,14) and thus enables storage at room temperature. Besides cost reduction in transportation and storage, lyophilization is preferred over freezing due to better stability of biomolecules resulting from the removal of non freezable water associated with most biomolecules (15).

Lyophilization can cause liposome fusion and phase separation during drying and rehydration (16). To overcome these problems lyoprotectants, e.g. carbohydrates, are used. Most plasmid delivery systems including PEI, polysine, adenovirus and lipoplexes require lyo/cryoprotection to maintain transfection efficiency (7). Cryo/lyoprotectants limit mechanical damage and rupture of the lipid bilayer, caused by ice crystals, during the

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freeze-drying and the rehydration process by maintaining the membrane in a flexible state (13,17).

Dye accessibility assays are used to provide a measure of free siRNA not associated with the lipids and are based on the fact that SYBR Green I fluorescence is significantly increased upon binding to double stranded nucleic acids (14). Zipper et. al. demonstrated the presence of several salt-dependent quenching mechanisms and that salt concentration significantly influenced SYBR green fluorescence due to the cationic nature of the dye (18). Salts have been shown to exert multiple effects not only on dye binding but also on the structure of dsDNA (19,20). Although it is not known if salts affect siRNA structure it has been reported that the assembly of RISC (RNA induced silencing complex) is salt sensitive while assembled RISC is resistant to high salt concentrations (21).

By substituting the non freezable water with other molecules, e.g. sugars, secondary structure can be maintained during lyophilization (22). While carbohydrate lyoprotectants interact with the DNA other lyoprotectants such as polyols, amino acids and lyotropic salts (23) seem to work via non-interactive covering of the pDNA and inhibition of conformationally induced denaturation. Poxon et. al. reported that mono- and disaccharides could completely restore loss of transfection efficiency resulting from lyophilization (22). The polynucleic acid (siRNA) may itself be damaged during the lyophilization process in the absence of a lyoprotectant. Here the effect of freeze-thawing and lyophilization in the presence and absence of lyoprotectants on siRNA lipoplexes and siRNA alone was evaluated.

MATERIALS AND METHODS

siTox (apoptosis inducing siRNA) and fluorescein (FITC) labeled siRNA (fl-siRNA) were from Dharmacon (Chicago, IL). 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) chloride and dioleoylphosphatidylethanolamine (DOPE) were obtained from Avanti Polar Lipids (Alabaster, AL). Cell culture plates (96 well) were obtained from Corning (Corning, NY). SYBR green I was purchased from Invitrogen (Carlsbad, CA). Dimethylsulfoxide, dextran sulfate sodium salt (MW ~8000) (2.3 sulfates per dextran) and all other reagents were from Sigma (St. Louis, MO) and were analytical grade or better.

B16F10 cells (ATCC number CRL-6475) were cultured as a monolayer in Dulbecco's Modified Eagle Medium (DMEM) (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (FBS) (Mediatech, Herndon, VA) and 0.01% penicillin-streptomycin. Cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere.

LIPOSOME PREPARATION

DOTAP and DOPE were dissolved in chloroform in 2:1 molar ratio (final concentration 5 mg of cationic lipid per ml). The organic solvent was removed at 55°C using a Rotavap (Bűchi, Postfach, Switzerland) followed by hydration of the lipid layer in sterile water under agitation to form the liposomes. To reduce size, the liposomes were sonicated at 40 W for 30 s using an ultrasonicator (Sonics Vibra cell). The liposome size was determined by NICOMP 380 ZLS (Particle Sizing Systems, Santa Barbara, CA).

FRESH, FREEZE-THAWED AND LYOPHILIZED LIPOPLEXES PREPARATION

siRNA (siTox or Fl-siRNA) and transfection reagent (liposomes) were separately diluted with 278 mM glucose, 278 mM sucrose, 278 mM trehalose, 278 mM lactose, 139 mM NaCl, 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES) buffered saline (HBS, 20 mM HEPES, 150 mM NaCl, pH=7.4), sterile water or serum free DMEM (Mediatech, Herndon, VA) (final volume 25 μl). The two dilutions were then vortexed and centrifuged briefly and incubated at room temperature for 5 min. The amount of transfection reagent added was based on the nitrogen:phosphate ratio (N/P) (nmol of nitrogen in the lipid or polymer ÷ nmol of phosphate in RNA) being evaluated. The siRNA solution was then added to the transfection reagent, vortexed immediately and incubated at room temperature for 20 min. After which the complexes were either used for transfection, freeze-thawed (liquid nitrogen −37°C water bath) 10 times or frozen (−80°C) and freeze dried (collect: −52°C; shell: 28°C; Vacuum: 0.31 mBar) (Freezone 6, Labconco Corporation, Kansas City, MO). The lyophilized complexes were reconstituted in 50 μl sterile water and used for transfection. The freeze-thawed or lyophilized lipoplexes and their respective fresh complexes were evaluated at the same time and on the same plate.

siRNA FREEZE-THAWING AND LYOPHILIZATION

To test if the freeze-thawing or lyophilization process causes physical damage to the siRNA itself, siRNA was freeze-thawed or lyophilized in the presence and absence of potential cryo/lyoprotectants. siTox was diluted with 278 mM glucose, 278 mM sucrose, 278 mM trehalose, 278 mM lactose, 139 mM NaCl, HBS, sterile water or serum free media (final volume 25 μl), vortexed and centrifuged briefly. After which the siTox was complexed with liposomes and used for transfection, freeze-thawed 10 times or frozen and freeze dried. The lyophilized siTox was reconstituted in sterile water, complexed with liposomes and then used for transfection.

TRANSFECTION

B16F10 cells (10,000 cells/well) were subcultured into 96 well plates, 24 h prior to transfection. Immediately before transfection the culture medium was removed from each well. Fresh serum free medium (50 μl) (containing 0.01% penicillin-streptomycin) was added to each well. Transfection reagent-siTox complex (50 μl) was then slowly added to each well. The solution was then mixed by gentle shaking. The plate was incubated at 37°C for 3 h. Fresh media (50 μl) containing 30% fetal bovine serum (FBS) and 0.01% penicillin-streptomycin was added to each well. The plate was then incubated for 48 h after which MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (24) was performed. Briefly, the media in each well was replaced by 200 μl of fresh DMEM containing 10% fetal bovine serum (FBS) and 0.01% penicillin-streptomycin and 50 μl MTT solution [5 mg/ml in phosphate-buffered saline (PBS)]. The cells were incubated in dark in a humidified atmosphere at 37°C for at least 4 h after which the media was replaced with 200 μl dimethyl sulfoxide and 25 μl Sorensen's

glycine buffer (0.1 M glycine, 0.1 M NaCl adjusted to pH 10.5 with 1 M NaOH). The absorbance was read immediately at 570 nm using a MRX revelation absorbance reader (Dynex Technologies Inc., Chantilly, VA). Delivery efficiency was expressed as 'percent cell death' with respect to the controls (cells treated with the same amount of transfection reagent only).

SYBR GREEN I BINDING ASSAY

Fresh, freeze-thawed and lyophilized siRNA or siRNA-liposome complexes were prepared similar to that in tissue culture experiments at increasing N/P ratios. SYBR green I nucleic acid gel stain (Invitrogen, Carlsbad, CA) (100 μl of 3× solution in PBS) was added to the complexes. The fluorescence intensity was measured at the excitation wavelength of 497 nm and emission wavelength of 520 nm using a *Synergy HT Multi-Detection Microplate Reader* (BioTek Instruments, Inc. Winooski, VT). To release siRNA from the lipoplexes, the polyanion, dextran sulfate (350 times excess of the positive charge) was added to the complexes, mixed, incubated for 1 min and fluorescence was quantified.

CELLULAR UPTAKE

Cellular uptake of the lyophilized and fresh transfection complexes was determined by fluorescence activated cell scanning (FACS). B16F10 cells were transfected with lyophilized and fresh complexes of fluorescein labeled siRNA (fl-siRNA) and liposomes at N/P ratio 8. Three hours later, the cells were washed and suspended in ice cold PBS. The samples were analyzed by flow cytometry with a FACScalibur flow cytometer (BD Biosciences, San Jose, CA), and data were acquired and analyzed with CellQuest software (BD Biosciences). Geometric means of fluorescence intensities were determined using software WinMDI 2.8 (Joseph Trotter, Scripps) to measure the central tendency of the fluorescent flow cytometric data. Propidium iodide (PI) (~0.1 μg/ml) was used to stain dead cells. The data was gated to include only live cells for fluorescence measurement and analysis.

LIPOPLEX SIZE ANALYSIS

The size of lipoplexes (fresh, lyophilized or freeze thawed) (volume weighted) was measured by NICOMP 380 ZLS (Particle Sizing Systems, Santa Barbara, CA). Depending on the χ^2 (chi square) value either NICOMP or Gaussian distribution algorithms were used ($\chi^2>3$, Gaussian analysis is inappropriate). Lower χ^2 result when sufficient statistics have been collected in the autocorrelation function which indicates that the Gaussian representation of the particle size distribution is a good assumption (25).

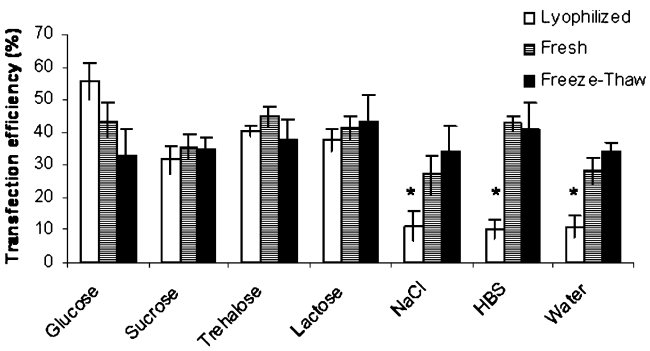

Fig. 1. Effect of additives on transfection efficiency of fresh, freeze-thawed or lyophilized lipoplexes in B16F10 cells. *Asterisks* Significantly different from respective controls ($p<0.05$) using two way ANOVA with Tukey's post test (mean±SD; $n=4$)

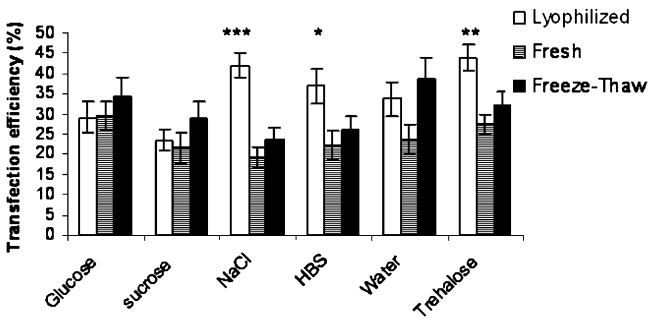

Fig. 2. Transfection efficiency of fresh, freeze-thawed or lyophilized lipoplexes in B16F10 cells (mean±SD; $n=4$; *single asterisk*, $p<0.05$; *double asterisks*, $p<0.001$; *triple asterisks*, $p<0.0001$ significantly different from corresponding fresh treatment by unpaired t-test)

ELECTRON MICROSCOPY

A small amount (few mg) of the powder obtained after lyophilization of lipoplexes in the presence and absence of a lyoprotectant was placed onto a glass wafer and coated with carbon. Afterward, SEM measurements were conducted at a FEG-SEM JEOL JSM-6335F instrument at operation voltage of 2 or 5 KV.

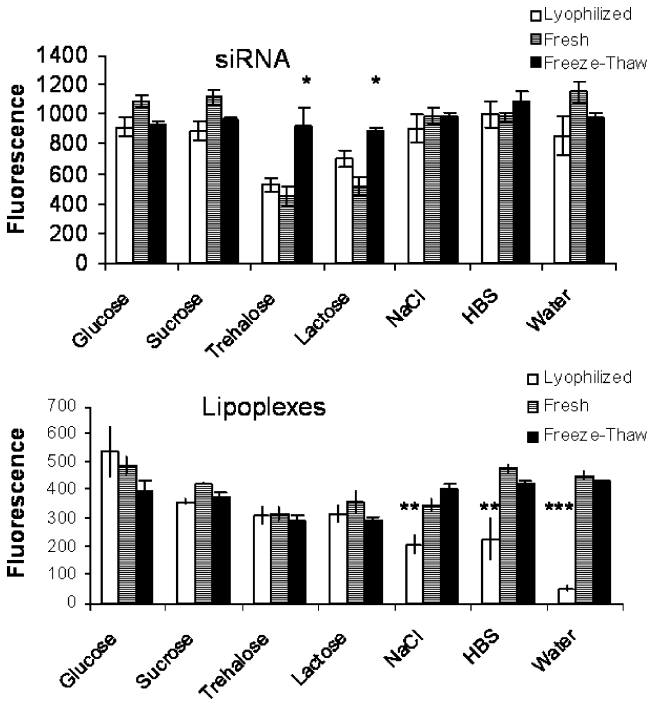

Fig. 3. siRNA: Fluorescence due to siRNA-SYBR green binding. Lipoplexes: Recovered fluorescence due to lipoplexes-SYBR green binding in the presence of dextran sulfate. *Double asterisks* $p<0.001$ and *triple asterisks* $p<0.0001$ by unpaired t- test (mean±SD; $n=4$)

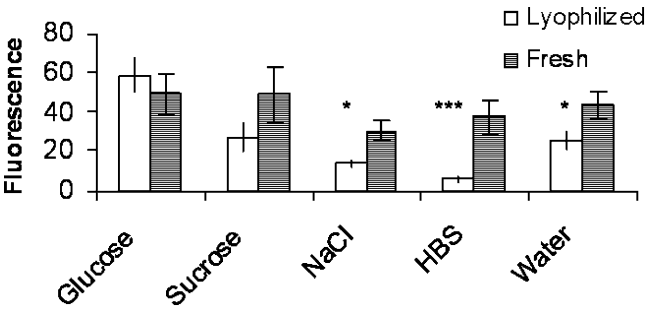

Fig. 4. Cellular uptake of lyophilized and fresh FITC-siRNA liposomes complexes in the presence of lyoprotectants. *Single asterisks* $p<0.05$ and *double asterisks* $p<0.001$ significantly different from corresponding fresh treatment using unpaired t-test (mean±SD; $n=4$)

ELECTROPHORETIC GEL ANALYSIS

Fresh or lyophilized lipoplexes in the presence or absence of dextran sulfate were loaded on a 15% Tris-borate-EDTA (TBE)-urea PAGE gel (Bio-Rad, Hercules, CA). Blue/orange 6× loading dye (Promega, Madison WI) was used to track gel migration. The Page gel was run at 90 V for 30 min followed by 20 mA for 30 min in 1× TBE. SYBR green I was used to stain the siRNA and a picture of the gel was taken using a digital camera (Kodak DC120, Eastman Kodak Company, Rochester, NY).

STATISTICAL ANALYSIS

All statistical analyses, unpaired t-test, one way ANOVA with Tukey's post test (when comparing all treatments to each other) or with Dunnet's post test (when comparing all treatments to a single control treatment) were performed using GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA).

RESULTS AND DISCUSSION

siRNA DOTAP:DOPE liposome (liposomes size=~100± 75 nm) complexes when lyophilized in the absence of lyoprotectant lose their activity (Fig. 1), represented as percent transfection efficiency or cell death induced by 50 nM siTox (complexed with liposomes at N/P ratio 8) 48 h post-transfection in B16F10 cells. Fig. 1 shows no significant difference between the activity of lyophilized and freshly prepared lipoplexes in the presence of glucose ($p=0.20$), sucrose ($p=0.46$), trehalose ($p=0.74$) and lactose ($p=0.86$). Thus, the lipoplexes when lyophilized in the presence of lyoprotectants (glucose, sucrose, trehalose and lactose) retain their functionality.

It was reported that siRNAs can be applied in lyophilized formulations and that nucleic acid and PEI complexes formed in 278 mM glucose, but not in 139 mM NaCl, can be lyophilized and reconstituted without loss of transfection efficacy (26). Polyplexes in glucose lead to the formation of smaller sized complexes but not in 139 mM NaCl (27). Supporting these reports, results here show that electrolytes, e.g. sodium chloride and HBS, do not exert a protective effect on siRNA liposome complexes during lyophilization. Fig. 1 also shows that under the conditions tested freeze thawing did not result in any loss in lipoplex activity.

Each treatment was compared to its respective control (fresh lipoplex) using an unpaired t-test. In HBS, saline and in the absence of any additives (sterile water) significant loss in activity (65–75% with respect to fresh complexes) was observed. The additives (glucose, sucrose, trehalose, lactose, sodium chloride, HBS and sterile water) or their absence did not significantly affect the transfection efficiency of the freshly prepared lipoplexes ($p>0.05$) as compared to lipoplexes made in serum free media (SFM) by one way ANOVA with Dunnet's post test.

EFFECT OF LYOPHILIZATION ON UNCOMPLEXED siRNA

To test whether the loss in activity of lyophilized complexes is due to the destabilization of lipoplexes or physical damage to the siRNA itself, siRNA was lyophilized in the presence and absence of potential lyoprotectants. Experiments with freshly prepared complexes between liposomes and lyophilized siRNA favored the hypothesis that the loss in activity of lyophilized complexes is due to the destabilization of lipoplexes and not due to siRNA physical damage (Fig. 2). While the presence of the ionic substances did not protect lipoplexes against loss of activity, no damage to the siRNA was seen when lyophilized in the absence of liposomes but in the presence of the ionic substances. In some cases an increase in siRNA activity after lyophilization was observed which may be a result of better annealing due to the temperature variations during the freeze-dry process or a larger fraction of cells being transfected. With current results, it is not possible to distinguish whether increased transfection efficiency or increased siRNA activity is most responsible for this phenomenon. Under the tested conditions no loss in siTox activity was seen after freeze-thawing of siRNA in the absence of liposomes.

Table I. Mean Diameter of the Lipoplexes in the Absence and Presence of Various Additives Before (Fresh Complexes) and After Lyophilization or Freeze-thawing

	Before Lyophilization[a]		After Lyophilization		After Freeze-thawing	
Additive	Diameter (nm)	Standard Deviation	Diameter (nm)	Standard Deviation	Diameter (nm)	Standard Deviation
Glucose	41.58	3.91	59.65	20.43	33.50	0.42
Sucrose	44.93	13.67	189.2	196.5	37.60	3.11
NaCl	41.93	16.06	3022[b]	1402	92.65	15.60
HBS	59.91	21.90	2893[b]	1345	48.65	6.29
No additive	31.97	0.52	1566[b]	1633	36.25	5.87

[a] Fresh complexes showed bimodal size distribution: size for ~65% presented, remaining 35% were ~200 nm
[b] Significantly different from respective controls ($p<0.001$) by unpaired t-test (mean±SD; $n=4$)

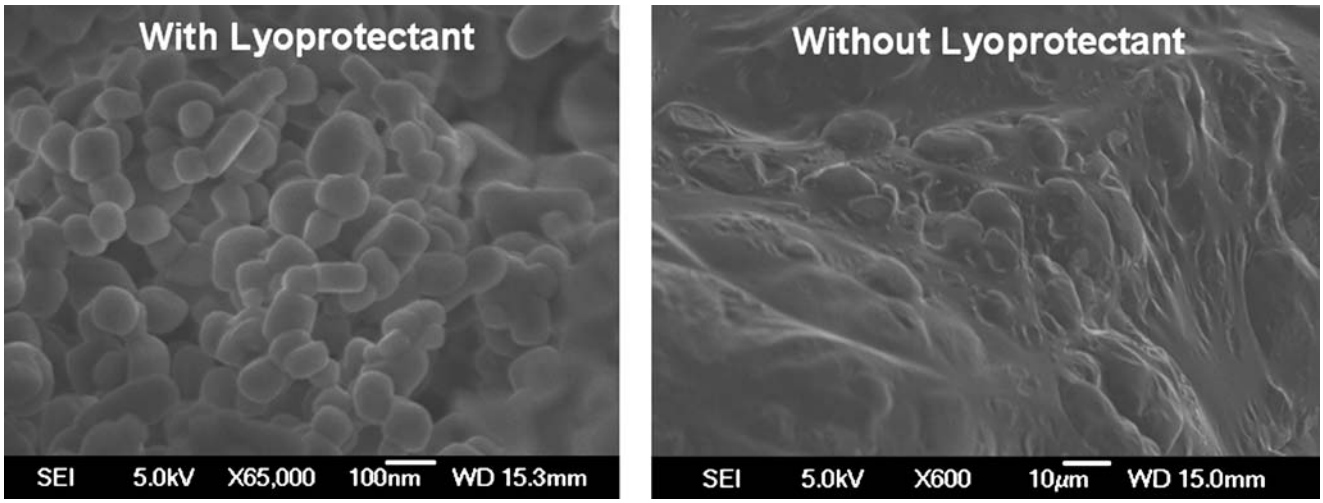

Fig. 5. Scanning Electron Microscopy Images: Representative SEM image of **a** lipoplexes lyophilized in the presence of lyoprotectant, glucose; **b** lipoplexes lyophilized in the absence of any lyoprotectant

EFFECT OF LYOPROTECTANTS ON SYBR GREEN BINDING

The results from SYBR green I binding assay correlated with the activity experiment showing that lipoplexes when lyophilized in the absence of a lyoprotectant show a loss (~88% with respect to fresh lipoplexes) in recovered fluorescence. The lipoplexes when lyophilized in the presence of lyoprotectants (glucose, sucrose, trehalose and lactose) retain their ability to release siRNA in the presence of dextran sulfate (Fig. 3). Lyophilization in the presence of ionic substances, e.g. salt and HBS, leads to significant ($p=0.002$ and 0.003 respectively) loss of recovered fluorescence (~ 40–50%) compared to fresh complexes. The treatments were compared using unpaired t-test. Freeze thawing of lipoplexes did not have any significant effect on the recovered fluorescence.

SYBR green I fluorescence assay with siRNA lyophilized in the presence and absence of lyoprotectants and then complexed with liposomes also supported the activity data. After lyophilization in the presence of ionic substances lipolplexes lost SYBR green I binding ability but when siRNA was lyophilized in the absence of liposome no change in fluorescence was seen. This favored the hypothesis that the loss in activity of lyophilized complexes is due to the destabilization of lipoplexes (Fig. 3a). SYBR green binding of siRNA freeze-thawed in the absence of liposomes was not significantly different from that of the fresh complexes.

CELLULAR UPTAKE

Cellular uptake studies with fluorescently labeled siRNA demonstrated lipoplexes lyophilized in the presence of salt ($p=0.004$) and HBS ($p=0.002$) were taken up by the cells significantly less efficiently (lower percent cell transfected as well as lower fluorescence) than those lyophilized in the presence of glucose ($p=0.48$) and sucrose ($p=0.19$) as compared to their respective controls (fresh complexes) (Fig. 4). The treatments were compared using unpaired t-test. The cellular uptake of lipoplexes lyophilized in the absence of any additive was similar to that of lipoplexes lyophilized in the presence of sucrose although the former complexes were incapable of showing siTox induced cell death (Fig. 1). This may be explained by the uptake of fluorophore alone. Fresh complexes made in isoosmotic solutions of the lyoprotectants (glucose, sucrose, NaCl and HBS) did not show a significant difference ($p>0.05$) in their

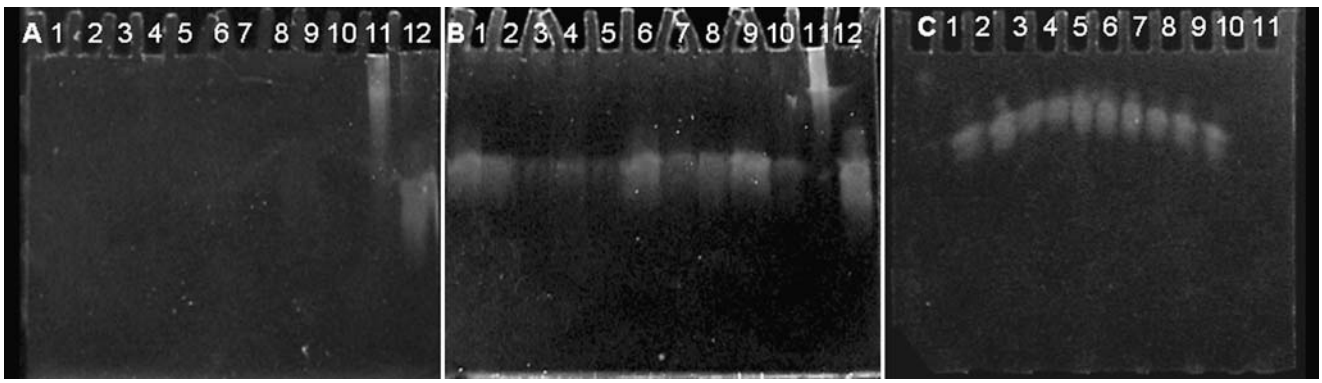

Fig. 6. Gel electrophoresis: PAGE urea gel. Lipoplexes in the absence (**a**) and presence (**b**) of dextran sulfate (*Lane 1–5* lyophilized lipoplexes, *lane 6–10* fresh lipoplexes, *lane 11* RNA marker, *lane 12* siRNA control); **c** siRNA alone (no lipids). (*Lane 1–5* fresh siRNA, *lane 5–10* lyophilized siRNA, *lane 11* liposomes) (*Lane 1–5* and *lane 5–10* samples in glucose, trehalose, NaCl, HBS and water respectively)

uptake suggesting that the additives themselves do not affect the cellular uptake process. The treatments were compared using one way ANOVA with Tukey's post test.

LIPOPLEX SIZE

Maintenance of particle size was demonstrated by several researchers to be correlated to transfection efficiency (28). Particle size may affect biodistribution and hence the efficacy of a system without affecting the transfection efficiency. Thus changes in particle size due to freeze-thawing or lyophilization must be prevented.

Table I shows the mean diameter of the lipoplexes in the absence and presence of various additives before (fresh complexes) and after lyophilization or freeze-thawing. The diameter of the lipoplexes lyophilized in the presence of sugars was significantly (p value<0.001, by unpaired t-test) lower than that of lipoplexes lyophilized in the absence of additives or in the presence of salt or HBS. No significant difference was observed in the diameter of fresh lipoplexes in all groups suggesting that in the absence of additives and in the presence of ionic compounds lipoplexes tend to aggregate upon lyophilization leading to poor SYBR green I binding ability, poor transfection and inactivity. Freeze-thawing, under the conditions tested, did not have significant affect on the lipoplex size. This could be because of rapid freezing and thawing of sample ten times which may be inadequate to result in significant aggregation of the lipoplexes. Lipid bilayers are damaged most when frozen quickly and thawed slowly. Even when freeze thawing does not result in damage of the delivery, the transportation and storage cost associated with it are discouraging.

ELECTRON MICROSCOPY

SEM images (Fig. 5) of lipoplexes lyophilized in the presence of lyoprotectant (glucose) show intact liposome structures while lipoplexes lyophilized in the absence of any lyoprotectant are amorphous and much larger in size. The lyoprotected lipoplexes were ~100 nm in diameter while when lyophilized in the absence of any lyoprotectant lipoplexes aggregated and observation under high magnification did not show any intact liposomes.

GEL ELECTROPHORESIS

Fifteen percent TBE PAGE urea gel supported the hypothesis that lyophilization does not lead to siRNA damage/degradation (Fig. 6c). Lipoplexes lyophilized in the presence of ionic substances or in the absence of any lyoprotective agent lose their ability to efficiently release the complexed siRNA upon the addition of the polyanion dextran sulfate (Fig. 6b).

The data also supports, the previously made conclusion, that the presence of sugars (glucose and trehalose) during lyophilization was observed to exert a lyoprotective effect. This lyoprotective effect may be explained by several mechanisms. These include prevention of mechanical damage and rupture of the lipid bilayer, maintenance of membrane flexibility (13,17), water replacement, prevention of aggregation due to diluting of complexes within the freeze-concentrated solutions or dried cake (17) and prevention of aggregation by formation of a steric barrier between particles by polymer such as polyethylene glycol (29,30).

CONCLUSION

Lyophilization allows controlled preparation and pretesting of liposome-siRNA formulation but leads to loss in functionality that can be prevented by the use of carbohydrates as lyoprotectants. The mechanism behind the loss of activity does not appear to be due to siRNA damage/degradation as lyophilized siRNA when rehydrated and complexed with liposomes were active. The change in activity correlates to the change in lipoplex size, SYBR green I binding ability and cellular uptake. Thus damage is mediated by aggregation of lipoplexes upon lyophilization and lyoprotectants are able to prevent loss in activity of siRNA-liposome complexes caused by the lyophilization process mainly by preventing liposome fusion/aggregation.

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