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Effect of sugars on storage stability of lyophilized liposome/DNA complexes with high transfection efficiency

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Abstract

Cationic lipid-based gene delivery systems have shown promise in transfecting cells in vitro and in vivo. However, liposome/DNA complexes tend to form aggregates after preparation. Lyophilization of these systems, therefore, has become of increasing interest. In this study, we investigated the feasibility of preserving complexes as a dried preparation using a modified dehydration rehydration vesicle (DRV) method as a convenient and reliable procedure. We also studied storage stability of a lyophilized novel cationic gene delivery system incorporating sucrose, isomaltose and isomaltotriose. Liposomes were composed of 3β-[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) and L-dioleoylphosphatidylethanolamine (DOPE), plus sucrose, isomaltose or isomaltotriose. Lyophilized liposome/DNA complexes were stored at −20, 25, 40 and 50 °C and their stability was followed for 50 days. Liposome/DNA complexes with sucrose could be stored even at 50 °C without large loss of transfection efficiency. The transfection efficiency of formulations stored at various temperatures indicated that the stabilizing effect of sugars on plasmid DNA was higher in the following order: isomaltotriose < isomaltose < sucrose, which was inverse to the order of their glass transition temperature (T_g) values. It was concluded that we could prepare novel lyophilized liposome/DNA complexes with high transfection efficiency and stability, which might be concerned that sucrose stabilized plasmid DNA in liposomes by directly interacting with plasmid DNA rather than by vitrifying to a high T_g solid.

Keywords: Cationic liposome; Sucrose; Dehydration rehydration vesicle; Transfection; Storage stability; Lyophilization

1. Introduction

Cationic liposome-mediated transfer of DNA is a promising approach, because of low immunogenicity and toxicity, ease of preparation, and potential applications for active targeting. The disadvantages include poor efficiency of transfection in vivo. Therefore, many cationic lipid-based transfection reagents have been developed for the efficient delivery of DNA into cells (Gao and Huang, 1991; Vigneron et al., 1996). Commercially available cationic liposomes or particles are mixed with plasmid DNA, and tend to form large liposome/DNA aggregates in solution, especially at high DNA concentrations. They form as a result of electrostatic binding between cationic liposomes and negatively charged DNA, and are inherently difficult to manipulate, resulting in a decrease of transfection (Sternberg et al., 1994; Lai and van Zanten, 2002). Because of this problem, cationic liposome/DNA complexes have to be freshly prepared when they are used. This would make it demanding to prepare them, and make quality control very difficult due to the fact that preparation of cationic liposome/DNA complexes is a process that is poorly defined and difficult to control.

To produce stable gene delivery systems that avoid these problems, lyophilization is suitable for long-term storage. There are many studies about lyophilization of liposome vectors using sugars (Anchordoquy et al., 1997; Li et al., 2000; Molina et al., 2004). Disaccharides were used in most studies. Especially, sucrose, which has a high glass transition temperature (T_g), is known to be effective to maintain the stability of liposomes, presumably by forming glasses under the typical freezing conditions used for lyophilization (Molina et al., 2001). To develop lyophilized liposome complexes with plasmid DNA vector, we used a modified dehydration rehydration vesicle (DRV) method as a convenient and reliable procedure (Perrie and Gregoriadis, 2000). The technique of the DRV method, employing sucrose at

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the lyophilization stage, has been evaluated for a range of solutes (Zadi and Gregoriadis, 2000; Kawano et al., 2003) and plasmid DNA (Perrie et al., 2004). The effects of sugars on the stability of lyophilized liposomes, sizes of liposomes and entrapment efficiency of solutes using DRV methods have been reported (Zadi and Gregoriadis, 2000; Kawano et al., 2003), but there have been a few reports about the effect of sugars on the stability of plasmid DNA in liposomes during storage (Li et al., 2000).

In this study, we examined stability of lyophilized liposome/DNA complexes with sucrose, isomaltose or isomaltotriose at different temperatures over 50 days, and determined which sugars could inhibit aggregation and maintain the transfection activity of plasmid DNA during preservation at temperatures above T_g. We found that DRV/DNA complexes with sucrose could be stored even at 50 °C without a large loss of transfection activity. Isomaltose and isomaltotriose were selected as excipients because their T_g values were higher than that of sucrose and therefore, they were expected to exhibit a greater stabilizing effect.

2. Materials and methods

2.1. Materials

3β-[*N*-(*N*′,*N*′-Dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) was purchased from Sigma Chemical Co. (St. Louis, MO, USA), and L-dioleoylphosphatidylethanolamine (DOPE) was obtained from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Lipofectamine 2000 was purchased from Invitrogen Corp. (Carlsbad, CA, USA). The Pica gene luciferase assay kit was purchased from Toyo Ink Mfg. Co. Ltd. (Tokyo, Japan). BCA protein assay reagent was purchased from Pierce (Rockford, IL, USA). All other chemicals used were of reagent grade. The plasmid DNA encoding the luciferase marker gene (pAAV-CMV-Luc) was supplied by Dr. S. Tanaka in Mt. Sinai School of Medicine (NY, USA). All reagents were of analytical grade. RPMI1640 medium and fetal bovine serum (FBS) were purchased from Life Technologies, Inc. (Grand Island, NY, USA).

2.2. Preparation of DRV

The preparation method has been reported previously (Perrie and Gregoriadis, 2000). Briefly, lipids (e.g., DC-Chol:DOPE = 3:2 and 1:2 mol/mol) were dissolved in chloroform and a dried film was formed by rotary-evaporation. The preparation was hydrated with filtered water and vortexed at room temperature. The resulting multilamellar vesicle (MLV) suspension was extruded through a series of polycarbonate membranes with pore sizes of 0.6 and 0.2 μm (Millipore, Billerica, MA) to yield about 200-nm-sized vesicles. A sugar/total lipid (w/w) of 5, and 12.5–100 μg of plasmid DNA at a charge ratio of (+/−) of 2 and 16 were carefully added to the vesicle suspension, and the mixture was transferred to polypropylene tubes (10 mm in diameter and 40 mm in length), frozen by immersing in liquid nitrogen for 10 min, and lyophilized (DRVs) using a Freezevac C-1 lyophilizer (Tozai Tsusho Co., Tokyo, Japan) at a vacuum level below 5 Pa. Shelf temperature was controlled at −40 °C for 12 h, at −20 °C for 12 h, at 0 °C for 8 h, at 20 °C for 4 h, and at 30 °C for 4 h. After lyophilization, dry nitrogen was introduced in the drying chamber, and vials of DRVs were sealed with screw caps in a nitrogen atmosphere. Water contents of formulations obtained were less than 0.5%, as determined by the Karl Fischer method.

Prior to the measurement of transfection efficiency, the dry cake of DRVs was rehydrated with milli-Q water (1 ml of water per vial) and ultracentrifuged at 45,000 rpm for 45 min to partition sugars from the liposome suspension. The supernatant was collected and then milli-Q water was added to the DRV pellets to achieve 100 μg DNA/ml (DRV pellet suspension).

2.3. Measurement of size

The mean particle size of the DRVs suspended in water was determined using a light scattering instrument (DLS-7000, Otsuka Electronics Co. Ltd., Osaka, Japan) by a dynamic laser light scattering method at 25 ± 1 °C. The reported particle size was the average value of two measurements.

2.4. Stability test

Vials of DRVs were transferred to vessels containing P_2O_5 and were stored at −20, 25, 40 and 50 °C for 50 days.

2.5. Measurement of T_g

A T_g of DRV formulation was measured by using a model 2920 differential scanning calorimeter (DSC) with a refrigerator cooling system (TA Instruments, Newcastle, DE, USA). Approximately 3 mg of DRV cake was put in an aluminum sample pan, dried in vacuum at 25 °C for 16 h and sealed hermetically in a nitrogen atmosphere in order to prevent water sorption during sample preparation. DSC traces were measured at a heating rate of 20 °C/min. An empty pan was used as a reference sample. Temperature calibration of the instrument was carried out using indium. T_g values reported were obtained for first heating scan. The T_g values and changes in the heat capacity at T_g of stored samples were similar to those before storage, indicating that crystallization of amorphous excipient in the formulations did not occur during stability studies.

2.6. Entrapment efficiency of plasmid DNA in DRV

The plasmid DNA in the supernatant after ultracentrifugation of the rehydrated DRV suspension at 45,000 rpm for 45 min was measured as free plasmid DNA using a PicoGreen dsDNA Quantitation Kit *200-2000 assays* (Molecular Probes, Inc., OR, USA).

2.7. Cell culture

Human cervical carcinoma HeLa cells were kindly provided by Toyobo Co. Ltd. (Osaka, Japan) and grown in DMEM supplemented with 10% FBS at 37 °C in a humidified 5% CO_2

atmosphere. Cell cultures were prepared by plating cells in 35-mm culture dishes 24 h prior to each experiment.

2.8. Transfection of cells

The DRV pellet suspension prepared as described was diluted with medium without FBS to a final concentration of 2 μg plasmid DNA per well. After transfection in the medium without FBS for 3 h, 1 ml of the growth medium containing 10% FBS was added to the wells and culturing was continued for an additional 21 h.

2.9. Expression assays

Luciferase expression was measured using the luciferase assay system. Incubation was terminated by washing the plates three times with cold phosphate-buffered saline (pH 7.4, PBS). Cell lysis solution (Pica gene) was added to the cell monolayers, which were then subjected to freezing (−80 °C) and thawing at 37 °C, followed by centrifugation at 13,000 rpm for 10 min. The supernatants were frozen and stored at −80 °C until the assays. Aliquots of 10 μl of the supernatants were mixed with 100 μl of luciferin solution (Pica gene) and counts per second (cps) were measured with a chemoluminometer (Wallac ARVO SX 1420 Multilabel Counter, Perkin Elmer Life Science, Japan, Co. Ltd., Kanagawa, Japan). The protein concentration of the supernatants was determined with BCA reagent, using bovine serum albumin as a standard, and cps/μg protein was calculated.

2.10. Statistical analysis

Statistical significance of the data was evaluated by Student's *t*-test. A p value of 0.05 or less was considered significant. All experiments were repeated at least two times. Duplicate determinations of luciferase expression values typically differed by less than 10%.

3. Results and discussion

Notably, liposomes composed of DC-Chol together with DOPE (DC-Chol/DOPE liposomes) have been classified as one of the most efficient vectors for the transfection of plasmid DNA into cells (Zhou and Huang, 1994; Farhood et al., 1994, 1995) and in clinical trials (Nabel et al., 1993, 1994). It has been demonstrated that a 3:2 or 1:1 molar ratio of DC-Chol/DOPE in liposomes results in high transfection efficiency (Farhood et al., 1995). Recently, we reported that DC-Chol/DOPE liposomes with molar ratio 1:2 showed more efficient transfection than those with molar ratio 3:2 or 1:1 in medium with FBS, having transfection efficiency comparable to that of Lipofectamine 2000, a commercial transfection reagent. Also, these lipoplexes showed a maximum at (+/−) 2:1 of transfection efficiency (Maitani et al., 2007). Therefore, we selected two kinds of DRV formulations: the conventional one (DC-Chol/DOPE = 3:2, A1–A3) and the novel one (DC-Chol/DOPE = 1:2, B1–B6), and prepared DRV/DNA complexes at sugar/total lipid (w/w) of 5, and charge ratio (+/−) of cationic lipid (DC-Chol) to DNA of 2 or 16 (Table 1).

3.1. DRVs without sugars

In preliminary experiments of the preparation of DRVs without sugars, the size was increased to over 1 μm. For the process of freeze-drying, fast freezing and addition of sugars in the freezing state resulted in less aggregation. This finding agreed with that reported by Molina et al. (2001). Moreover, in this study, since some preparations of dry cakes of DRVs after lyophilization were needed to perform the subsequent procedures, sugars were added to the suspension of liposomes and plasmid DNA at a weight ratio of 5:1 (sugar:total lipids) and mixed before freezing.

3.2. T_g measurement of DRVs

Fig. 1 shows representative DSC traces of DRV formulations containing plasmid DNA (formulations B1–B3) and lyophilized sugar. The formulations were considered to be amorphous because they exhibited base line shifts due to the glass transition. The T_g values of the lyophilized sugar (T_g^S) were higher in the order: sucrose < isomaltose < isomaltotriose. The T_g values of DRV formulations increased in the order: B1 < B2 < B3, indicating that the molecular mobility of the formulation containing sucrose was higher than that of the formulations containing isomaltose or isomaltotriose. The T_g values of DRV formulations and lyophilized sugars with and without liposomes are summarized in Table 2. The T_g values of lyophilized sugars with cationic liposomes (DRV formulation without DNA) were slightly lower than those of the corresponding lyophilized sugars. In contrast, a

Table 1
Composition of DRV formulation

Formulation	DC-Chol:DOPE (mol:mol)	Charge ratio, lipid:DNA (+/−)	Sugar
A1	3:2	16:1	Sucrose
A2	3:2	16:1	Isomaltose
A3	3:2	16:1	Isomaltotriose
B1	1:2	2:1	Sucrose
B2	1:2	2:1	Isomaltose
B3	1:2	2:1	Isomaltotriose
B4	1:2	16:1	Sucrose
B5	1:2	16:1	Isomaltose
B6	1:2	16:1	Isomaltotriose

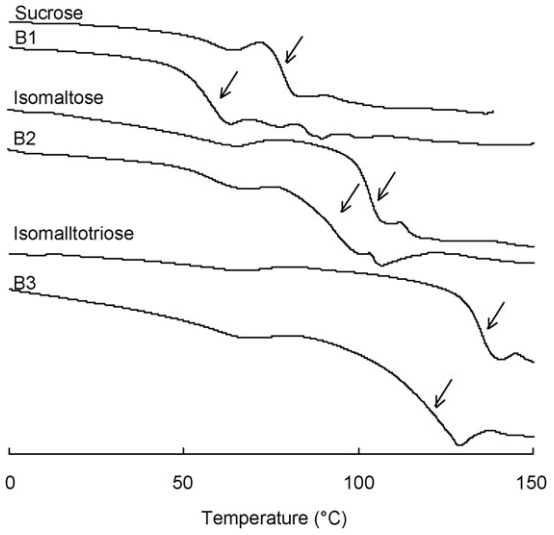

Fig. 1. Representative DSC traces of DRV formulations and sugars for first heating scan. Arrows in the figure represent T_g.

large decrease in T_g of the DRV/DNA complexes was observed, as indicated by the T_g values of formulations B1–B3, as shown in Fig. 2. The T_g values of the formulations can be expressed by the Gordon–Taylor equation (Eq. (1)) assuming that miscibility of the sugars and the liposome/plasmid DNA complex is complete:

$$T_g = \frac{kW_c T_g^c + W_s T_g^s}{W_s + kW_c} \quad (1)$$

where W_s, W_c, T_g^s, and T_g^c are the weight fraction and T_g of sugar and complex, respectively, and k is a constant. Differences in the

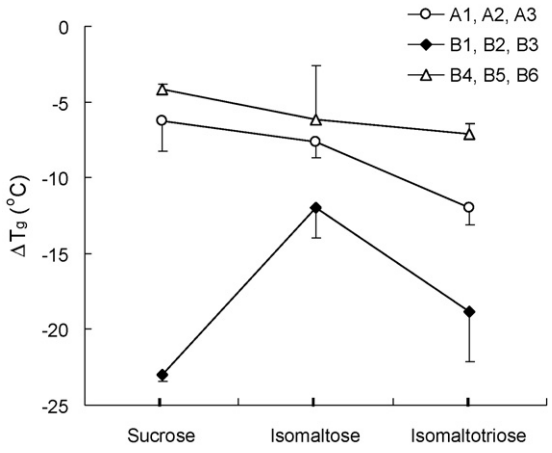

Fig. 2. Difference in $T_g^{midpoint}$ between DRV formulations and corresponding lyophilized sugars in Table 2. Error bars represent standard deviation ($n = 3$).

T_g between the formulations and the corresponding sugars (ΔT_g) are expressed by the following equation:

$$\Delta T_g = T_g - T_g^s = -\frac{kW_c(T_g^s - T_g^c)}{W_s + kW_c} \quad (2)$$

T_g^c (not determined) is expected to be lower than T_g^s, since T_g values of the formulations studied were lower than those of corresponding lyophilized sugar. For formulations B1–B3, the T_g^c value is considered to be the same, since the formulations contain the same amount of plasmid DNA in liposomes. A smaller $T_g^s - T_g^c$ value is consequently expected for the formulation containing the sugar with lower T_g. Assuming that k is not largely different between sugars, the smallest difference in T_g between a formulation and the corresponding lyophilized sugar should

Table 2
T_g of DRV formulations and lyophilized sugars

	$T_g^{midpoint}$ (°C)	S.D.	T_g^{onset} (°C)	S.D.
DRV formulation[a]				
A1 (sucrose (DC/DOPE = 3/2))	72.0	2.1	68.8	2.6
A2 (isomaltose (DC/DOPE = 3/2))	94.4	1.1	87.3	4.9
A3 (isomaltotriose (DC/DOPE = 3/2))	121.1	1.2	108.2	1.6
B1 (sucrose (DC/DOPE = 1/2))	55.2	0.5	50.4	0.5
B2 (isomaltose (DC/DOPE = 1/2))	90.7	0.5	83.1	1.6
B3 (isomaltotriose (DC/DOPE = 1/2))	114.2	3.3	104.6	2.7
B4 (sucrose (DC/DOPE = 1/2))	74.0	0.4	71.1	0.5
B5 (isomaltose (DC/DOPE = 1/2))	96.6	3.6	86.9	1.4
B6 (isomaltotriose (DC/DOPE = 1/2))	125.9	0.6	118.3	0.9
Lyophilized sugar with cationic liposomes				
Sucrose + (DC/DOPE = 3/2)	75.1	0.1	73.7	0.8
Isomaltose + (DC/DOPE = 3/2)	98.1	0.8	97.5	1.0
Isomaltotriose + (DC/DOPE = 3/2)	129.5	2.0	123.3	2.7
Sucrose + (DC/DOPE = 1/2)	77.8	0.8	72.5	0.5
Isomaltose + (DC/DOPE = 1/2)	101.8	0.1	94.2	3.1
Isomaltotriose + (DC/DOPE = 1/2)	129.1	0.1	123.9	0.5
Lyophilized sugar				
Sucrose	78.2	0.2	74.5	0.2
Isomaltose	102.7	0.6	98.6	0.2
Isomaltotriose	133.0	0.8	128.8	0.2

T_g values reported were obtained for first heating scan ($n = 3$).
[a] Sugar, liposome and plasmid DNA used in Table 1.

Table 3
Particle size of liposomes after rehydration of DRVs stored at −20 °C for 50 days

Formulation	Diameter (nm)	Dispersion (%)	Diameter (nm)	Dispersion (%)
A1	179	62	1274	38
A2	178	69	1269	31
A3	191	70	1558	30
B1	448	10	3495	90
B2	364	8	3826	92
B3	487	11	3161	88
B4	238	30	1451	70
B5	256	32	1591	68
B6	205	32	1130	68

be observed for sucrose-based formulations. Formulation B1, however, exhibited a larger difference in T_g than formulation B2 or B3 (Fig. 2), suggesting that the Gordon–Taylor equation was not applicable to formulation B1. This deviation from the Gordon–Taylor equation suggests that plasmid DNA may interact with sucrose more strongly than with isomaltose or isomaltotriose.

3.3. Size distribution of DRV suspension after rehydration and entrapment efficiency

DRVs stored at −20 °C for 50 days were rehydrated. The size distribution of DRV/DNA complex suspensions was heterogeneous, depending on the charge ratio (+/−), but not depending on sugars (Table 3). The DC-Chol/DOPE = 3/2 complex (formulations A1–A3) showed the smallest size. In the DC-Chol/DOPE = 1/2 complex, formulations B1–B3 with low cationic charge (+/−) of 2 showed larger size than formulations B4–B6 with that of 16. It seemed that the charge of DRVs was critical in determining sizes because the electric repulsion of the complex opposed aggregation. Also sugar amount might affect size of DRV. Molina et al. (2001) reported that formulations with high sucrose/DNA ratios are capable of maintaining particle size during the freezing step, and suggested that the separation of individual particles within sugar matrices is responsible for the protection of cationic lipid DOTAP/DOPE liposome vectors during the freezing step of a typical lyophilization protocol.

DRV methods in which lyophilization is performed after the addition of sucrose to the liposome suspension increase the entrapment efficiency of DNA in DRVs. Crowe and Crowe (1993) reported that trehalose outside and inside liposomes prevented the aggregation of liposomes and stabilized liposomes to entrap solutes. Zadi and Gregoriadis (2000) reported that a small amount of sugar outside liposomes disturbs the liposomal membrane and makes solute outside liposomes enter them. In our case, free plasmid DNA was not detected in the supernatant of any DRV suspensions using a PicoGreen Kit. The cationic charge of DRVs was so high that whether plasmid DNA was entrapped and/or adsorbed on DRVs was not clear.

3.4. Effect of sugars on transfection efficiency (TE)

In preliminary experiments, when lyophilized formulations B1–B3 of DRV/DNA complexes were preserved at room temperature for 24 h and then rehydrated, they showed similar TE values with nonlyophilized ones. To investigate the effect of sugars on lyophilization of the DRV/DNA complexes, the stability of plasmid DNA was evaluated by measuring TE after preservation at various temperatures.

At −20 and 25 °C, formulations B1–B3 showed the highest TE, and then formulations B4–B6 showed intermediate TE higher than those of formulations A1–A3 (Fig. 3). This finding indicated that TE was affected by the cationic lipid ratio in liposomes, and by the charge ratio (+/−) of cationic lipid to plasmid DNA, more than by the sugar. Molina et al. (2004) reported that progressive degradation of DOTAP lipoplex in terms of TE was observed during storage in the dried state at −20 °C, and the presence of DOPE enhanced degradation under these conditions. To the contrary, formulations B1–B3 with rich DOPE and the low charge ratio (+/−), exhibited high TE. This difference may be due to difference of cationic lipids.

The TE of formulations at various temperatures indicated that the stabilizing effect of sugars on DNA was higher in the following order: isomaltotriose < isomaltose < sucrose, except for formulation B1. This order of the stabilizing effects of sugars was inverse to the order of their T_g values. About the effects of the glassy state on liposome in the freeze-drying state, it was reported that the solute retention in dry liposomes may be prolonged by increasing the T_g of the dry liposome preparation (Sun et al., 1996; Crowe et al., 1997; van Winden and Crommelin, 1999). On the other hand, in cationic lipid/DNA complexes during lyophilization, sample vitrification did not correlate with maintenance of transfection efficiency (Allison and Anchordoquy, 2000). It was likely that sucrose might stabilize plasmid DNA by directly interacting with plasmid DNA rather than by vitrifying to a high T_g solid. Supporting our inference, it was reported that the efficacy of transfection of lipoplexes was enhanced by mixing medium and disaccharides (Tseng et al., 2007).

Formulation B1 maintained a high TE value even during storage at 50 °C for 50 days, having about one-fourth of the TE of a commercially available transfection reagent, Lipofectamine 2000. We examined the cytotoxicity of the DRV/DNA complex, as indicated by the protein concentration after transfection (data not shown). All formulations showed low cytotoxicity compared with Lipofectamine 2000.

Entrapment of plasmid DNA inside DRVs, rather than a greater association at the surface of liposomes, may offer a more

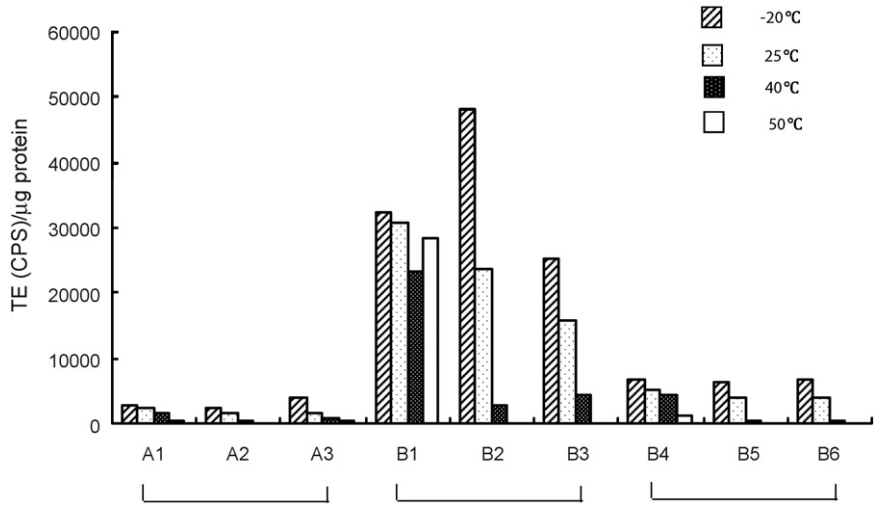

Fig. 3. Transfection efficiency in HeLa cells transfected with DRV/DNA complex with sugars after storage at various temperatures for 50 days. Each DRV/DNA complex was diluted with medium without FBS to a final concentration of 2 μg of plasmid DNA in 1 ml of medium per well. Each column represents the mean ($n = 2$). TE of B5 and B6 at 50 °C was not detected.

controlled approach to vesicle formation. This cryo-protective effect may be desirable when preparing DRV/DNA complexes without aggregation and with entrapment of DNA.

4. Conclusion

We developed lyophilized formulations of liposome (DRV)/plasmid DNA complex vectors prepared with the DRV method. DRV/DNA complexes lyophilized with sucrose could be stored even at 50 °C for 50 days without a large loss of transfection efficiency. This finding suggests that sucrose might stabilize plasmid DNA by directly interacting with plasmid DNA rather than by vitrifying to a high T_g solid. Further long-term stability studies will be required to determine the shelf life of lyophilized liposome/DNA complexes at room temperature. These findings provide new information about the effects of physicochemical changes of nonviral vectors during lyophilization.

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References

Allison, S.D., Anchordoquy, T.J., 2000. Mechanisms of protection of cationic lipid–DNA complexes during lyophilization. J. Pharm. Sci. 89, 682–691.

Anchordoquy, T.J., Carpenter, J.F., Kroll, D.J., 1997. Maintenance of transfection rates and physical characterization of lipid/DNA complexes after freeze-drying and rehydration. Arch. Biochem. Biophys. 348, 199–206.

Crowe, J.H., Crowe, L.M., 1993. In: Gregoriadis, G. (Ed.), Liposome Technology, 2nd ed. CRC Press, Boca Raton, FL, p. 232.

Crowe, J.H., Oliver, A.E., Hoekstra, F.A., Crowe, L.M., 1997. Stabilization of dry membranes by mixtures of hydroxyethyl starch and glucose: the role of vitrification. Cryobiology 35, 20–30.

Farhood, H., Gao, X., Son, K., Yang, Y.Y., Lazo, J.S., Huang, L., Barsoum, J., Bottega, R., Epand, R.M., 1994. Cationic liposomes for direct gene transfer in therapy of cancer and other diseases. Ann. N.Y. Acad. Sci. 716, 23–34.

Farhood, H., Serbina, N., Huang, L., 1995. The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer. Biochim. Biophys. Acta 1235, 289–295.

Gao, X., Huang, L., 1991. A novel cationic liposome reagent for efficient transfection of mammalian cells. Biochem. Biophys. Res. Commun. 179, 280–285.

Kawano, K., Takayama, K., Nagai, T., Maitani, Y., 2003. Preparation and pharmacokinetics of pirarubicin loaded dehydration–rehydration vesicles. Int. J. Pharm. 252, 73–79.

Lai, E., van Zanten, J.H., 2002. Real time monitoring of lipoplex molar mass, size and density. J. Control. Release 82, 149–158.

Li, B., Li, S., Tan, Y., Stolz, D.B., Watkins, S.C., Block, L.H., Huang, L., 2000. Lyophilization of cationic lipid–protamine–DNA (LPD) complexes. J. Pharm. Sci. 89, 355–364.

Maitani, Y., Igarashi, S., Sato, M., Hattori, Y., 2007. Cationic liposome (DC-Chol/DOPE = 1:2) and a modified ethanol injection method to prepare liposomes, increased gene expression. Int. J. Pharm. 342, 33–39.

Molina, M.C., Allison, S.D., Anchordoquy, T.J., 2001. Maintenance of nonviral vector particle size during the freezing step of the lyophilization process is insufficient for preservation of activity: insight from other structural indicators. J. Pharm. Sci. 90, 1445–1455.

Molina, M.C., Armstrong, T.K., Zhang, Y., Patel, M.M., Lentz, Y.K., Anchordoquy, T.J., 2004. The stability of lyophilized lipid/DNA complexes during prolonged storage. J. Pharm. Sci. 93, 2259–2273.

Nabel, G.J., Nabel, E.G., Yang, Z.Y., Fox, B.A., Plautz, G.E., Gao, X., Huang, L., Shu, S., Gordon, D., Chang, A.E., 1993. Direct gene transfer with DNA–liposome complexes in melanoma: expression, biologic activity, and lack of toxicity in humans. Proc. Natl. Acad. Sci. U.S.A. 90, 11307–11311.

Nabel, E.G., Yang, Z., Muller, D., Chang, A.E., Gao, X., Huang, L., Cho, K.J., Nabel, G.J., 1994. Safety and toxicity of catheter gene delivery to the pulmonary vasculature in a patient with metastatic melanoma. Hum. Gene Ther. 5, 1089–1094.

Perrie, Y., Gregoriadis, G., 2000. Liposome-entrapped plasmid DNA: characterisation studies. Biochim. Biophys. Acta 1475, 125–132.

Perrie, Y., Barralet, J.E., McNeil, S., Vangal, A.A., 2004. Surfactant vesicle-mediated delivery of DNA vaccines via the subcutaneous route. Int. J. Pharm. 284, 31–41.

Sternberg, B., Sorgi, F.L., Huang, L., 1994. New structures in complex formation between DNA and cationic liposomes visualized by freeze-fracture electron microscopy. FEBS Lett. 356, 361–366.

Sun, W.Q., Leopold, A.C., Crowe, L.M., Crowe, J.H., 1996. Stability of dry liposomes in sugar glasses. Biophys. J. 70, 1769–1776.

Tseng, W.C., Tang, C.H., Fang, T.Y., Su, L.Y., 2007. Using disaccharides to enhance in vitro and in vivo transgene expression mediated by a lipid-based gene delivery system. J. Gene Med. 9, 659–667.

van Winden, E.C., Crommelin, D.J., 1999. Short term stability of freeze-dried, lyoprotected liposomes. J. Control. Release 58, 69–86.

Vigneron, J.P., Oudrhiri, N., Fauquet, M., Vergely, L., Bradley, J.C., Basseville, M., Lehn, P., Lehn, J.M., 1996. Guanidinium–cholesterol cationic lipids: efficient vectors for the transfection of eukaryotic cells. Proc. Natl. Acad. Sci. U.S.A. 93, 9682–9686.

Zadi, B., Gregoriadis, G., 2000. A novel method for high-yield entrapment of solute into small liposomes. J. Liposome Res. 10, 73–80.

Zhou, X., Huang, L., 1994. DNA transfection mediated by cationic liposomes containing lipopolylysine: characterization and mechanism of action. Biochim. Biophys. Acta 1189, 195–203.