

Effects of disaccharide and cationic lipid types on reverse transfection with lyophilized mRNA lipoplexes

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Abstract. Recently, the development of messenger RNA (mRNA) therapeutics has received increased attention, and mRNA/cationic liposome complexes (mRNA lipoplexes) have been studied as effective mRNA delivery carriers. However, owing to the vast diversity of the mRNA and carrier components employed, establishing a comprehensive analysis of their efficacy remains a critical issue. Reverse transfection using lyophilized mRNA lipoplexes has the potential to simplify and automate the efficacy evaluation processes. The present study investigated the effects of cationic lipids in cationic liposomes and disaccharides as cryoprotectants during reverse transfection with lyophilized mRNA lipoplexes. Overall, five types of dialkyl or trialkyl cationic lipids were used to prepare cationic liposomes. The mRNA lipoplexes were lyophilized in the presence of trehalose or sucrose in multi-well plates. An increase in the concentration of the disaccharide solution during the lyophilization of mRNA lipoplexes enhanced the transfection activity. Furthermore, mRNA lipoplexes lyophilized in 150 mM sucrose solution exhibited long-term stability for up to 1 month. The transfection activity of mRNA lipoplexes composed of dialkyl cationic lipids was largely unaffected by lyophilization, whereas a significant reduction in transfection activity was observed for mRNA lipoplexes composed of trialkyl cationic lipids. These findings suggest that although dependent on the lipid type used for the preparation of cationic liposomes, the reverse transfection method using lyophilized mRNA lipoplexes has the potential to be applied for screening the transfection efficiency of mRNA lipoplexes and the function of proteins translated from mRNA.

Introduction

In recent years, RNA-based therapeutics, such as small interfering RNA (siRNA) and messenger RNA (mRNA), have garnered significant attention, offering new therapeutic approaches that specifically modulate gene expression associated with various diseases (1,2). The success of mRNA vaccines against coronavirus disease 2019 (COVID-19) has accelerated the development of mRNA-based pharmaceuticals. Consequently, mRNA therapeutics are expected to have broad applications in fields such as cancer immunotherapy, genome editing, genetic disorder treatments, and regenerative medicine (3,4).

RNA therapeutics, including mRNA vaccines, require the carriers for delivery to the targeted tissue. Various mRNA carriers have been investigated, including cationic polymers, lipoplexes, lipid-polymer hybrid nanoparticles, and lipid nanoparticles (5,6). Among these, mRNA/cationic liposome complexes (mRNA lipoplexes) have been extensively studied as efficient delivery systems (7). Lipoplexes are nanoparticles formed by complexation of mRNA and cationic liposomes (8). Although mRNA lipoplex-based products have been investigated for clinical applications such as cancer immunotherapy (7,9), successful cases remain limited, and further fundamental research is needed on the lipid compositions and mRNA used in mRNA lipoplexes.

To comprehensively investigate the efficacy of mRNA lipoplexes, establishing analytical technologies is essential to evaluate how differences in lipid composition influence the efficiency of cellular delivery and gene expression. Achieving this goal requires a reproducible, high-throughput mRNA transfection method using multi-well plates *in vitro*. There are two major approaches for *in vitro* mRNA transfection: forward and reverse. For forward transfection, target cells are first seeded and cultured, after which a suspension of pre-formed mRNA lipoplexes is added. Notably, mRNA lipoplexes are typically prepared immediately before use because mRNA and mRNA lipoplexes can be unstable in aqueous suspensions and are unsuitable for long-term storage (10,11). However, this method involves multiple handling steps and is time-consuming. To address these limitations, reverse transfection can be utilized to reduce the labor and time required. In this method, mRNA lipoplexes are directly mixed with the cell suspension, enabling simultaneous cell seeding and mRNA transfection without prior cell culture. This approach

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(commonly referred to as liquid-phase reverse transfection) simplifies workflow. However, freshly prepared mRNA lipoplexes are required, making them unsuitable for comprehensively evaluating the effects of a wide variety of lipoplexes and mRNA. To overcome these challenges, we propose the use of solid-phase reverse transfection. In this method, mRNA lipoplexes are pre-applied and lyophilized onto the surface of the culture plate prior to cell seeding. The cell suspension is then directly added to the dried mRNA lipoplexes, allowing for mRNA transfection upon contact. The advantages of this approach include reduced manual handling, compatibility with automation, and the ability to prepare large batches of transfection-ready plates. Consequently, this method facilitates the efficient and scalable screening of lipid components for mRNA transfection into cells.

We previously reported a method for solid-phase reverse transfection using lyophilized siRNA lipoplexes (12). Additionally, several studies have investigated lyophilization conditions for plasmid DNA or siRNA lipoplexes (13,14). However, little is known about the effects of lyophilization on mRNA lipoplexes. In our previous study, we demonstrated that the gene-silencing activity of siRNA lipoplexes could be preserved regardless of the type of cationic lipid used by employing disaccharides such as sucrose or trehalose as cryoprotectants during lyophilization (15). However, whether the same strategy can be applicable to mRNA lipoplexes remains unclear. To investigate the impact of disaccharides and cationic lipid types on the transfection efficiency of lyophilized mRNA lipoplexes, cationic liposomes were prepared using five types of dialkyl or trialkyl cationic lipids. Lyophilized mRNA lipoplexes were then prepared in multi-well plates in the presence of trehalose or sucrose solutions, and their transfection efficiency was evaluated, in addition to the retention of transfection activity after long-term storage.

Materials and methods

Materials. 11-((1,3-Bis(dodecanoyloxy)-2-((dodecanoyloxy)methyl)propan-2-yl)amino)-*N,N,N*-trimethyl-11-oxodecan-1-aminium bromide (cat. no. TC-1-12), 2-(bis(2-(tetradecanoyloxy)ethyl)amino)-*N,N,N*-trimethyl-2-oxoethan-1-aminium chloride (cat. no. DC-6-14), *N*-hexadecyl-*N,N*-dimethylhexadecan-1-aminium bromide (cat. no. DC-1-16) and dimethyldioctadecylammonium bromide (DDAB, cat. no. DC-1-18) were purchased from Sogo Pharmaceutical Co., Ltd. (Tokyo, Japan). 1,2-Dioleoyl-3-trimethylammonium-propane methyl sulfate salt (DOTAP) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE, COATSOME® ME-8181) and polyethylene glycol-cholesterol ether (PEG-Chol, mean MW of PEG: 1600) were purchased from NOF Co., Ltd. (Tokyo, Japan).

mRNAs. CleanCap® firefly luciferase mRNA (FLuc mRNA, 1922 nucleotides, cat. no. L-7602) and CleanCap® enhanced green fluorescent protein mRNA (EGFP mRNA, 997 nucleotides, cat. no. L-7601) were purchased from TriLink Biotechnologies (San Diego, CA, USA). EZ Cap™ Cy5 firefly luciferase mRNA (5-moUTP) (Cy5-mRNA, 1921 nucleotides,

cat. no. R1010) was purchased from APEX BIO Technology, LLC (Boston, MA, USA).

Preparation of cationic liposomes and mRNA lipoplexes. Liposomes were prepared using a mixture of cationic lipid (DDAB, DOTAP, DC-1-16, DC-6-14, and TC-1-12), DOPE, and PEG-Chol at a molar ratio of 49.5:49.5:1 via the thin-film hydration method. Briefly, the lipids were dissolved in chloroform and, subsequently, chloroform was removed using a rotary evaporator set at 60°C to obtain a thin lipid film. The lipid film was hydrated with sterile water at 60°C and sonicated to reduce the particle size for 10 min at 100 W, 42 kHz, and room temperature in a bath-type sonicator (Branson 2510J-MTH; Branson Ultrasonics Corporation, Danbury, CT, USA). To prepare mRNA lipoplexes, each mRNA was mixed with cationic liposomes at a charge ratio (+/-) of 4:1 and incubated at room temperature for 10 min.

Size measurement of liposomes and lipoplexes. The particle size distribution and polydispersity index (PDI) were measured using an ELS-Z2 light-scattering photometer (Otsuka Electronics Co., Ltd., Osaka, Japan) at 25°C after diluting the particle dispersion to an appropriate concentration with sterile water. The ζ -potential was measured using electrophoretic light scattering with the same instrument at 25°C after diluting the sample in the same manner.

Lyophilization process of mRNA lipoplexes. The mRNA lipoplexes containing 0.5 μ g mRNA were dissolved in 125 μ l of 50, 100 or 150 mM trehalose or sucrose solution and placed in a 12-well plate for transfection assay. For cytotoxicity assay, those containing 0.05 μ g mRNA were dissolved in 12.5 μ l of 150 mM trehalose or sucrose solution and placed in a 96-well plate. The mixture was first frozen at -80°C and then dried under high vacuum (10-20 Pa) using a freeze dryer (FDU-540, Tokyo Rikakikai Co., Ltd. (EYELA), Tokyo, Japan) equipped with a drying chamber (DRC-2L, EYELA). For characterization after long-term storage, the freeze-dried mRNA lipoplexes were stored under vacuum in a desiccator at room temperature before use.

Cell culture and reverse transfection. HeLa cells (cat. no. 93021013; Cellosaurus; CVCL_0030) were obtained from the European Collection of Authenticated Cell Cultures. The cells were cultured under the standard condition in Eagle's Minimum Essential Medium (EMEM; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 100 μ g/ml kanamycin in humidified atmosphere with 5% CO₂ at 37°C. For liquid-phase reverse transfection, mRNA lipoplex solution containing 0.5 μ g mRNA was mixed with 1x10⁵ HeLa cells seeded in 1 ml medium and transferred into the wells of a 12-well plate. Then it was incubated at 37°C with 5% CO₂ for 24 h. For solid-phase reverse transfection, HeLa cells were seeded at a density of 1x10⁵ cells per well in a 12-well plate or 1x10⁴ cells per well in a 96-well plate, both containing freeze-dried mRNA lipoplexes, and incubated at 37°C with 5% CO₂ for 24 h.

Luciferase activity quantification. HeLa cells in 12-well plate were lysed 24 h post-transfection by adding 125 μ l of cell lysis buffer (Pierce™ Luciferase Cell Lysis Buffer, ThermoFisher Scientific Inc.). The lysate was frozen at -80°C , followed by thawing at 37°C , and then centrifuged at $13,000 \times g$ for 10 sec. A 10 μ l aliquot of the supernatants were mixed with 50 μ l of PicaGene MelioraStar-LT Luminescence Reagent (Toyo Ink Mfg. Co. Ltd., Tokyo, Japan) and the luminescence was measured as counts per sec (cps) with a chemoluminometer (ARVO X2, PerkinElmer, inc., Waltham, MA, USA). The protein concentration in the supernatant was measured using the BCA reagent (Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific, Inc.), with bovine serum albumin (BSA) as the standard and then luciferase activity was calculated as cps/ μ g protein.

Cytotoxicity of lyophilized mRNA lipoplexes on HeLa cells. In a 96-well plate containing freeze-dried mRNA lipoplexes, HeLa cells were seeded at a density of 1×10^4 cells per well, and incubated at 37°C with 5% CO_2 for 24 h. After transfection, cell viability was evaluated using the WST-8 assay (Cell Counting Kit-8, Dojindo Laboratories, Tokyo, Japan), according to the manufacturer's instructions.

Flow cytometry analysis for expression analysis and cellular uptake evaluation. To evaluate the cellular uptake of mRNA lipoplexes, HeLa cells were seeded at a density of 1×10^5 cells per well in a 12-well plate containing freeze-dried Cy5-mRNA lipoplexes (Cy5-mRNA: 0.5 μ g/well), and incubated at 37°C with 5% CO_2 for 3 h. On the other hand, for expression analysis, HeLa cells were seeded at a density of 1×10^5 cells per well in a 12-well plate containing freeze-dried EGFP mRNA lipoplexes (EGFP mRNA: 0.5 μ g/well), and incubated at 37°C with 5% CO_2 for 24 h. After transfection, the cells were harvested using TrypLE™ Express Enzyme (Gibco; Thermo Fisher Scientific, Inc.) and resuspended in phosphate-buffered saline containing with 0.1% BSA and 1 mM ethylenediaminetetraacetic acid. To evaluate the Cy5 fluorescence intensity or EGFP expression in the cells, 10,000 events per sample were processed using BD FACSVerser™ (BD Biosciences, Franklin Lakes, NJ, USA) and analyzed using BD FACSuite software ver. 1.0.3 (BD Biosciences).

Intracellular co-localization of the Cy5-mRNA and lysosome observed by confocal microscopy. HeLa cells were seeded at a density of 1×10^5 cells per well in a 35 mm dish containing freeze-dried Cy5-mRNA lipoplexes (Cy5-mRNA: 0.5 μ g/dish), and incubated at 37°C with 5% CO_2 for 3 h. The cells were stained with 75 nM LysoTracker® Red DND-99 (Life Technologies, Carlsbad, CA, USA) for 30 min. After washing with PBS, the cells were fixed with Mildform® 10N (FUJIFILM Wako Pure Chemical Corporation) and then stained with 5 μ g/ml Hoechst 33342 (Thermo Fisher Scientific, Inc.) for 10 min. Fluorescence images were acquired using a FLUOVIEW FV3000 confocal laser scanning microscope (Olympus, Tokyo, Japan).

Statistical analysis. Data were collected from at least three independent experiments. All results are presented as mean + standard deviation (S.D.). Statistical analyses were

performed using unpaired Student's t-test or one-way ANOVA followed by Tukey's Multiple Comparison Test. Statistical analyses were performed using GraphPad Prism software (version 4.0; GraphPad Software Inc., San Diego, CA, USA). Statistical significance was set at $P < 0.05$.

Results

Characterization of mRNA lipoplexes after lyophilization. When liposomes or lipoplexes are lyophilized, cryoprotectants such as disaccharides are commonly used to enhance their stability. Previously, we reported that freeze-drying siRNA lipoplexes in trehalose or sucrose solution resulted in long-term stability (1 month) without a significant loss of gene-silencing activity, regardless of the type of cationic lipid used in the cationic liposomes (15). In this study, we investigated whether trehalose or sucrose solutions could have similar effect as cryoprotectants for mRNA lipoplexes during lyophilization and examined the effect of these disaccharides on transfection activity after lyophilization. DOTAP, DDAB, DC-6-14, DC-1-16, and TC-1-12 were used as cationic lipids (Fig. 1). Based on our previous report (16), liposomes were prepared with cationic lipids, DOPE, and PEG-Chol at a molar ratio of 49.5:49.5:1, using the thin-film hydration method. The sizes of the prepared cationic liposomes were approximately 85-106 nm, and the ζ -potentials were approximately 49-58 mV (Table I).

Based on our previous report that the optimal charge ratio (+/-) for mRNA lipoplexes composed of dialkyl or trialkyl cationic lipids is 4:1 (17), we used the same charge ratio for preparation of mRNA lipoplexes in subsequent experiments. The mRNA lipoplexes composed of DDAB, DOTAP, and DC-1-16 formed relatively large particles with diameters of approximately 310-370 nm. In contrast, the mRNA lipoplexes containing DC-6-14 were approximately 200 nm in size, whereas those containing TC-1-12 were approximately 280 nm in size, forming relatively smaller particles than the aforementioned lipoplexes. All lipoplexes exhibited a monodisperse distribution (PDI: 0.14-0.18) and the ζ -potentials were approximately 14-40 mV (Table I).

Effect of lyophilization on the transfection activity of the mRNA lipoplexes. To investigate the effect of lyophilization on the transfection activity of mRNA lipoplexes, lyophilized FLuc mRNA lipoplexes containing 0.5 μ g of mRNA per well in 12-well plate, were reverse-transfected into HeLa cells and luciferase activity was measured 24 h after transfection, because our previous results showed that a dose of 0.5 μ g mRNA with an incubation time of 24 h resulted in the highest transfection activity in HeLa cells after forward transfection with mRNA lipoplexes composed of cationic lipid, neutral lipid and, PEG-Chol at a molar ratio of 49.5:49.5:1 (16). Initially, liquid-phase reverse transfection was performed using mRNA lipoplexes containing DDAB, DOTAP, DC-1-16, DC-6-14, or TC-1-12 without lyophilization. As previously reported (16), the transfection of mRNA lipoplexes showed that those containing DC-1-16 or TC-1-12 exhibited high luciferase activity (Fig. 2A). FLuc mRNA lipoplexes were lyophilized in 50, 100, or 150 mM trehalose or sucrose solutions, followed by reverse transfection into HeLa cells (Fig. 2B). Regardless of

Table I. Particle size of cationic liposomes and mRNA lipoplexes.

Cationic lipid	Liposome			Lipoplex		
	Size, nm	PDI	ζ -potential, mV	Size, nm	PDI	ζ -potential, mV
DDAB	96.0 $\pm$ 0.6	0.22 $\pm$ 0.00	50.8 $\pm$ 0.9	358.8 $\pm$ 12.2	0.17 $\pm$ 0.00	34.7 $\pm$ 2.1
DOTAP	85.4 $\pm$ 0.5	0.22 $\pm$ 0.00	53.4 $\pm$ 1.9	367.6 $\pm$ 13.4	0.17 $\pm$ 0.01	14.3 $\pm$ 0.4
DC-1-16	103.5 $\pm$ 3.6	0.24 $\pm$ 0.02	58.4 $\pm$ 1.4	306.6 $\pm$ 40.0	0.18 $\pm$ 0.04	20.8 $\pm$ 0.4
DC-6-14	106.4 $\pm$ 0.8	0.25 $\pm$ 0.01	48.9 $\pm$ 2.2	203.2 $\pm$ 14.5	0.15 $\pm$ 0.07	39.9 $\pm$ 2.0
TC-1-12	85.8 $\pm$ 1.5	0.22 $\pm$ 0.00	50.9 $\pm$ 1.0	278.0 $\pm$ 6.4	0.14 $\pm$ 0.01	29.6 $\pm$ 0.8

Data are presented as mean $\pm$ standard deviation (n=3 in each group). DDAB, dimethyldioctadecylammonium bromide; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane methyl sulfate salt; DC-1-16, *N*-hexadecyl-*N,N*-dimethylhexadecan-1-aminium bromide; DC-6-14, 2-(bis(2-(tetradecanoyloxy)ethyl)amino)-*N,N,N*-trimethyl-2-oxoethan-1-aminium chloride; TC-1-12, 11-((1,3-bis(dodecanoyloxy)-2-((dodecanoyloxy)methyl)propan-2-yl)amino)-*N,N,N*-trimethyl-11-oxoundecan-1-aminium bromide; PDI, polydispersity index.

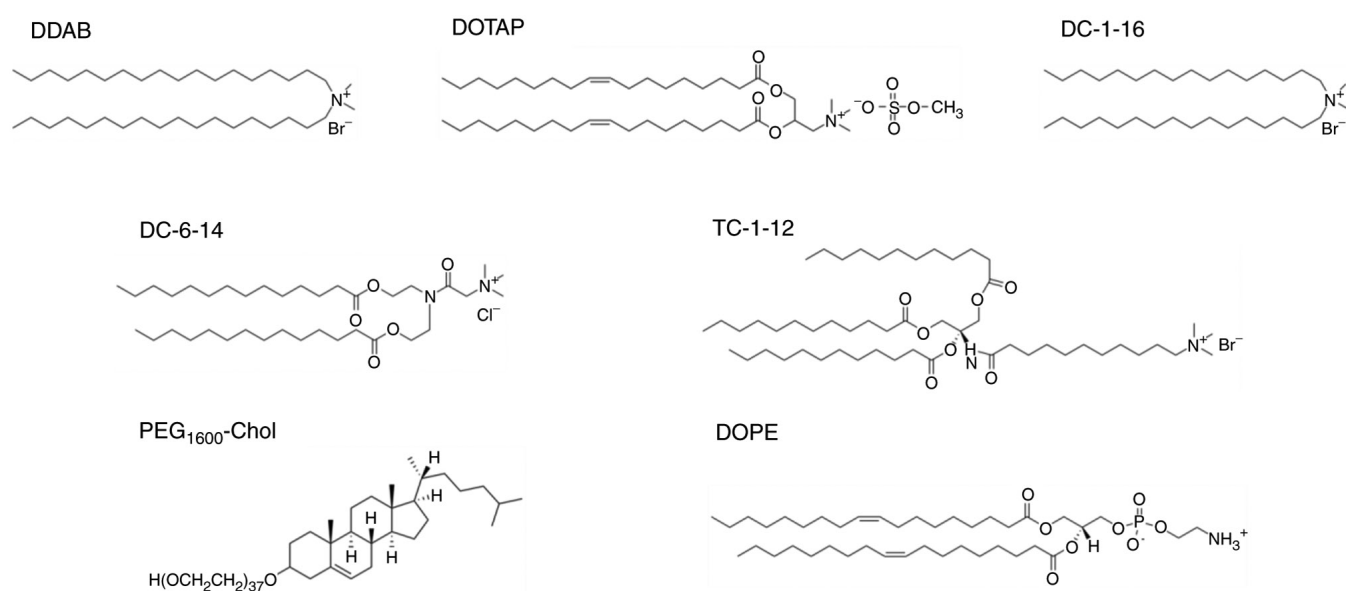


Figure 1. Structures of the lipid components of liposomes used for mRNA lipoplexes. DDAB, dimethyldioctadecylammonium bromide; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane methyl sulfate salt; DC-1-16, *N*-hexadecyl-*N,N*-dimethylhexadecan-1-aminium bromide; DC-6-14, 2-(bis(2-(tetradecanoyloxy)ethyl)amino)-*N,N,N*-trimethyl-2-oxoethan-1-aminium chloride; TC-1-12, 11-((1,3-bis(dodecanoyloxy)-2-((dodecanoyloxy)methyl)propan-2-yl)amino)-*N,N,N*-trimethyl-11-oxoundecan-1-aminium bromide; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; PEG₁₆₀₀-Chol, polyethylene glycol-cholesteryl ether.

the cationic lipid type used to prepare the lipoplexes, higher disaccharide concentrations tended to correlate with increased luciferase activity. Furthermore, in mRNA lipoplexes containing DDAB, DC-6-14, or TC-1-12, the use of sucrose as a cryoprotectant resulted in significantly higher activity than trehalose. Although lyophilization tended to reduce luciferase activity compared to non-lyophilized conditions, this reduction was minimized when lipoplexes were lyophilized in 150 mM sucrose solution. Therefore, in subsequent experiments, a disaccharide solution with a concentration of 150 mM was used as the cryoprotectant.

Effect of lyophilization on the particle size and cytotoxicity of the mRNA lipoplexes. To investigate whether lyophilization affects the physical properties of mRNA lipoplexes, mRNA lipoplexes were lyophilized on a plate using 150 mM trehalose

or sucrose solution as a cryoprotectant. Particle size measurements after rehydration of lyophilized mRNA lipoplexes revealed that regardless of the type of cryoprotectant, mRNA lipoplexes containing DDAB, DOTAP, or DC-1-16, which exhibited a tendency for larger particle sizes before lyophilization, showed a reduction in size to approximately 250–280 nm (Table II). In contrast, the particle size of the mRNA lipoplexes containing DC-6-14, which were relatively small in diameter before lyophilization, tended to increase slightly upon lyophilization, reaching approximately 230 nm. In addition, the particle size of the mRNA lipoplexes containing TC-1-12 increased upon lyophilization, reaching approximately 350–380 nm. The ζ -potentials after lyophilization were approximately 23–40 mV (Table II).

Furthermore, HeLa cell viability was assessed 24 h after reverse transfection with lyophilized mRNA lipoplexes (Fig. 3).

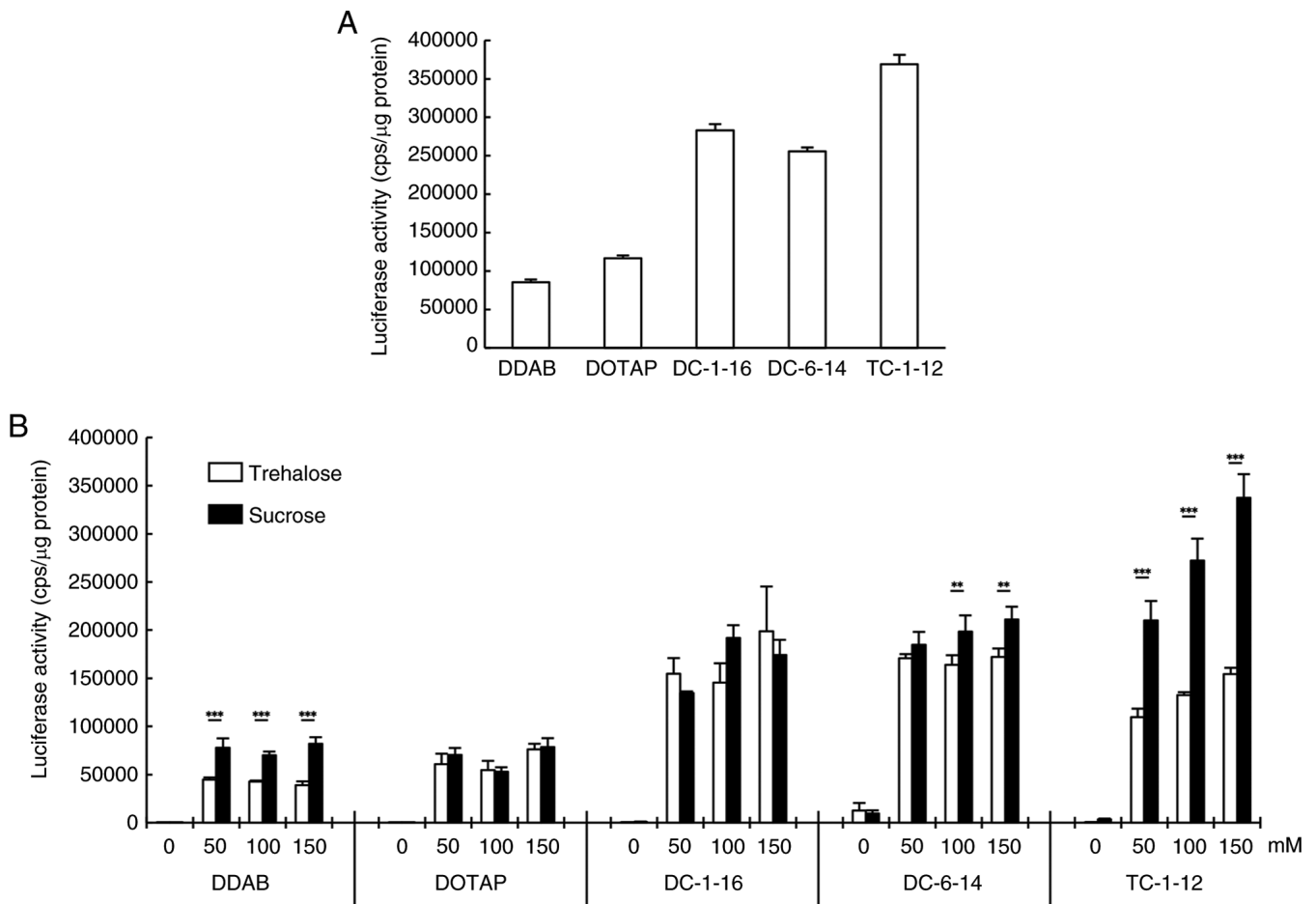


Figure 2. Luciferase activity following reverse transfection with FLuc mRNA lipoplexes. (A) non-lyophilized FLuc mRNA lipoplexes containing each cationic lipid were reverse transfected into HeLa cells and luciferase activity was measured 24 h after the transfection. (B) Lyophilized FLuc mRNA lipoplexes with 0-150 mM trehalose or sucrose were reverse transfected into HeLa cells. Luciferase activity was measured 24 h after the transfection. ** $P < 0.01$ and *** $P < 0.001$ (trehalose vs. sucrose). Data are presented as mean $\pm$ standard deviation ($n=3$ in each group). DDAB, dimethyldioctadecylammonium bromide; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane methyl sulfate salt; DC-1-16, *N*-hexadecyl-*N,N*-dimethylhexadecan-1-aminium bromide; DC-6-14, 2-(bis(2-(tetradecanoyloxy)ethyl)amino)-*N,N,N*-trimethyl-2-oxoethan-1-aminium chloride; TC-1-12, 11-((1,3-bis(dodecanoyloxy)-2-((dodecanoyloxy)methyl)propan-2-yl) amino)-*N,N,N*-trimethyl-11-oxoundecan-1-aminium bromide; cps, count per sec.

When lyophilized in the presence of a 150 mM trehalose or sucrose solution, the mRNA lipoplexes containing DC-1-16 exhibited a slightly lower cell viability of approximately 60%. In contrast, the mRNA lipoplexes containing DOTAP showed cell viability of approximately 90%. For mRNA lipoplexes containing other cationic lipids, cell viability was approximately 70%. No significant effect on cell viability was owing due to the differences in disaccharides.

Effect of lyophilization on the transfection efficiency and cellular uptake. To further investigate the effects of lyophilization on the transfection activity of mRNA lipoplexes, we examined the transfection efficiency using mRNA lipoplexes containing DC-1-16, DC-6-14 or TC-1-12, which exhibited high transfection activity in the experiments using Luc mRNA. EGFP mRNA lipoplexes were lyophilized and reverse transfected into HeLa cells, and 24 h after transfection, flow cytometry showed the appearance of a new peak on the higher fluorescence intensity side of the histogram compared to untreated cells, indicating the presence of EGFP-expressing cells (Fig. S1). Based on these results, the percentage of EGFP-positive cells was calculated (Fig. 4A).

When mRNA lipoplexes containing DC-6-14 were lyophilized in a trehalose solution, the proportion of EGFP-expressed cells decreased compared to that in the non-lyophilized lipoplexes. However, unexpectedly, when the mRNA lipoplexes containing DC-1-16 were lyophilized in a sucrose solution, the proportion of them was increased. In other mRNA lipoplexes lyophilized in sucrose or trehalose solution, no significant difference in percentage was observed between lyophilized and non-lyophilized lipoplexes.

These differences in transfection efficiency could be influenced by the efficiency of the intracellular uptake of mRNA lipoplexes. Therefore, to investigate this question, we examined cellular uptake using the same liposome as in the aforementioned experiment. Lyophilized Cy5-mRNA lipoplexes were reverse transfected into HeLa cells and the fluorescence intensity of the internalized Cy5-mRNA was measured using flow cytometry 3 h after transfection. Compared to untreated cells, the cells transfected with Cy5-mRNA lipoplexes showed a rightward shift in the histogram peak, indicating successful uptake of Cy5-mRNA (Fig. S2). The mean fluorescence intensity of Cy5-mRNA internalized cells was measured, lyophilization

Table II. Particle size of lyophilized and rehydrated mRNA lipoplexes.

Cationic lipid	Trehalose			Sucrose		
	Size, nm	PDI	ζ -potential, mV	Size, nm	PDI	ζ -potential, mV
DDAB	250.6 $\pm$ 38.0	0.17 $\pm$ 0.08	28.1 $\pm$ 2.5	253.1 $\pm$ 7.0	0.12 $\pm$ 0.00	26.6 $\pm$ 1.6
DOTAP	251.2 $\pm$ 30.1	0.18 $\pm$ 0.05	24.2 $\pm$ 2.7	282.3 $\pm$ 15.5	0.13 $\pm$ 0.01	25.4 $\pm$ 0.2
DC-1-16	279.1 $\pm$ 9.1	0.13 $\pm$ 0.01	30.4 $\pm$ 2.1	283.0 $\pm$ 17.3	0.13 $\pm$ 0.01	29.8 $\pm$ 0.6
DC-6-14	230.3 $\pm$ 16.0	0.19 $\pm$ 0.07	22.8 $\pm$ 0.4	234.4 $\pm$ 7.1	0.18 $\pm$ 0.05	24.7 $\pm$ 0.6
TC-1-12	350.7 $\pm$ 16.3	0.16 $\pm$ 0.01	40.0 $\pm$ 0.4	379.1 $\pm$ 37.1	0.17 $\pm$ 0.02	36.3 $\pm$ 1.3

Data are presented as mean $\pm$ standard deviation (n=3 in each group). DDAB, dimethyldioctadecylammonium bromide; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane methyl sulfate salt; DC-1-16, *N*-hexadecyl-*N,N*-dimethylhexadecan-1-aminium bromide; DC-6-14, 2-(bis(2-(tetradecanoyloxy)ethyl)amino)-*N,N,N*-trimethyl-2-oxoethan-1-aminium chloride; TC-1-12, 11-((1,3-bis(dodecanoyloxy)-2-((dodecanoyloxy)methyl) propan-2-yl) amino)-*N,N,N*-trimethyl-11-oxoundecan-1-aminium bromide; PDI, polydispersity index.

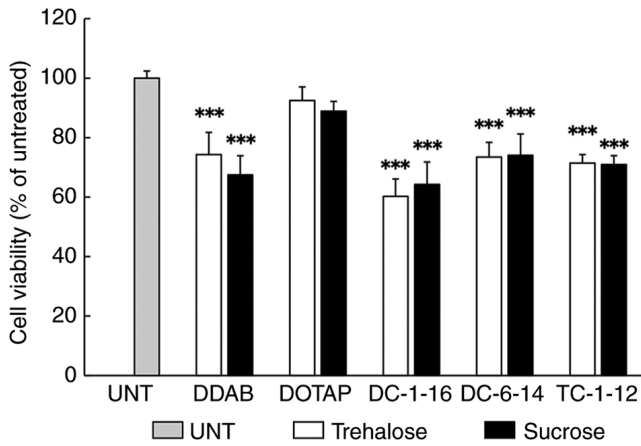


Figure 3. Toxicity test of lyophilized mRNA lipoplexes. FLuc mRNA lipoplexes containing each cationic lipid were lyophilized with 150 mM trehalose or sucrose solution. The lipoplexes were reverse transfected into HeLa cells and the cell viability was measured using WST-8 assay 24 h after the transfection. *** P <0.001 vs. UNT. Data are presented as mean $\pm$ standard deviation (n=5 in each group). UNT, untreated group; DDAB, dimethyldioctadecylammonium bromide; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane methyl sulfate salt; DC-1-16, *N*-hexadecyl-*N,N*-dimethylhexadecan-1-aminium bromide; DC-6-14, 2-(bis(2-(tetradecanoyloxy)ethyl)amino)-*N,N,N*-trimethyl-2-oxoethan-1-aminium chloride; TC-1-12, 11-((1,3-bis(dodecanoyloxy)-2-((dodecanoyloxy)methyl) propan-2-yl) amino)-*N,N,N*-trimethyl-11-oxoundecan-1-aminium bromide.

led to a reduction in the Cy5 fluorescence intensity in the cells, regardless of the type of cationic lipid used (Fig. 4B). Focusing on the differences in cationic lipids within the lipoplexes, mRNA lipoplexes containing DC-6-14 exhibited the highest cellular uptake of mRNA regardless of whether lyophilization was performed with trehalose or sucrose as the cryoprotectant. Moreover, a similar tendency was observed in liquid-phase reverse transfection without lyophilization (Fig. 4B). In contrast, focusing on the differences in the disaccharides used as cryoprotectants, mRNA lipoplexes containing DC-6-14 lyophilized in sucrose exhibited higher cellular uptake than those lyophilized in trehalose. In contrast, those containing DC-1-16 lyophilized in sucrose showed lower fluorescence intensity than those in trehalose.

However, for those containing TC-1-12, no significant difference in cellular uptake was observed between lyophilization with sucrose and trehalose solutions (Fig. 4B). These results indicate that the cellular uptake of mRNA lipoplexes may have a limited impact on transfection efficiency.

On the other hand, endosomal escape is also an important factor for protein expression from transfected mRNA. Therefore, we additionally examined the intracellular localization of mRNA and lysosomes. As a result, although partial colocalization of mRNA with lysosomes was detected regardless of the type of lipoplex used (Fig. S3), no noticeable differences in colocalization were observed among the types of cationic lipid contained or between the disaccharides used during lyophilization.

Assessment of long-term stability of lyophilized mRNA lipoplexes. Since we confirmed that lyophilization does not necessarily lead to a reduction in the transfection activity of mRNA lipoplexes, depending on the cationic lipid of the liposome and the type of cryoprotectant, we evaluated the long-term stability of mRNA lipoplexes after lyophilization. mRNA lipoplexes containing DC-1-16, DC-6-14, or TC-1-12 were lyophilized under the same conditions as in the previous experiments and stored in a desiccator under vacuum at room temperature for 1 month.

The particle size, PDI, and ζ -potential of mRNA lipoplexes rehydrated after one month of storage are shown in Table III. The particle size of the mRNA lipoplexes containing DC-1-16 or TC-1-12 remained unchanged. However, those containing DC-6-14 exhibited an increase in particle size to approximately 300–320 nm after one month of storage. For the ζ -potential, mRNA lipoplexes containing DC-1-16 exhibited values of approximately 25–26 mV, while those containing DC-6-14 showed approximately 27–35 mV, and those with TC-1-12 had approximately 40–44 mV.

To investigate whether these changes also affected transfection activity, we performed reverse transfection of HeLa cells with lyophilized Luc mRNA lipoplexes after one month of storage. Compared with the results of reverse transfection performed immediately after lyophilization (Fig. 2B), the

Table III. Particle size of rehydrated mRNA lipoplexes after 1 month of storage.

Cationic lipid	Trehalose			Sucrose		
	Size, nm	PDI	ζ -potential, mV	Size, nm	PDI	ζ -potential, mV
DC-1-16	264.4 $\pm$ 14.4	0.13 $\pm$ 0.01	26.0 $\pm$ 1.3	270.1 $\pm$ 4.2	0.13 $\pm$ 0.00	24.6 $\pm$ 2.5
DC-6-14	321.0 $\pm$ 8.3	0.24 $\pm$ 0.08	35.4 $\pm$ 0.3	308.0 $\pm$ 4.2	0.18 $\pm$ 0.07	27.3 $\pm$ 1.4
TC-1-12	342.6 $\pm$ 18.2	0.15 $\pm$ 0.01	43.5 $\pm$ 1.5	351.0 $\pm$ 13.1	0.16 $\pm$ 0.01	40.2 $\pm$ 1.5

Data are presented as mean $\pm$ standard deviation (n=3 in each group). DC-1-16, *N*-hexadecyl-*N,N*-dimethylhexadecan-1-aminium bromide; DC-6-14, 2-(bis(2-(tetradecanoyloxy)ethyl)amino)-*N,N,N*-trimethyl-2-oxoethan-1-aminium chloride; TC-1-12, 11-((1,3-bis(dodecanoyloxy)-2-((dodecanoyloxy)methyl) propan-2-yl) amino)-*N,N,N*-trimethyl-11-oxoundecan-1-aminium bromide.

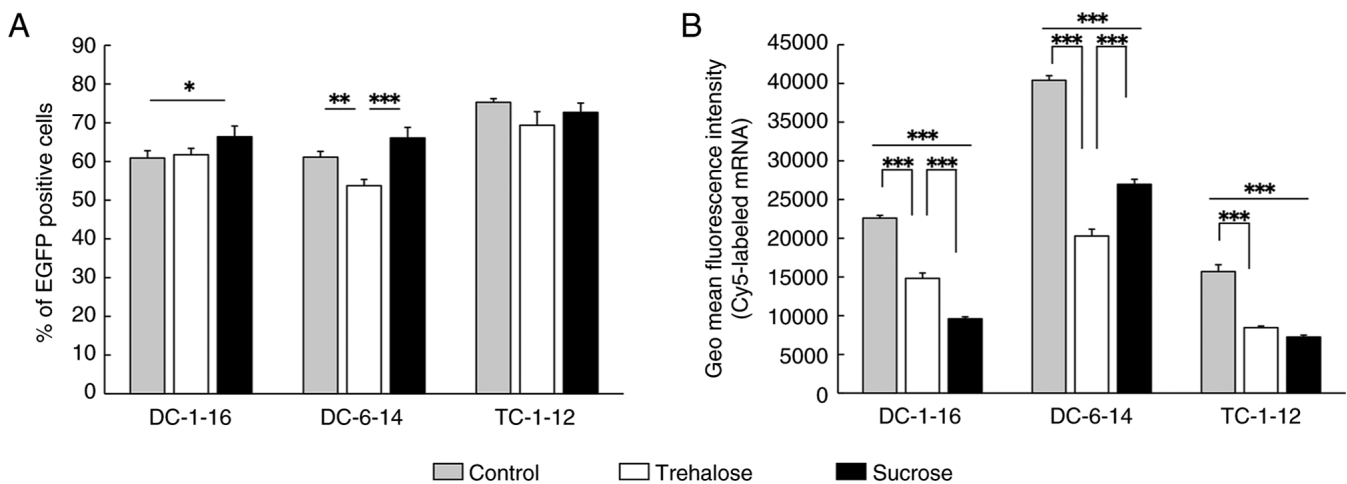


Figure 4. Analysis of transfection efficiency and cellular uptake of the lyophilized mRNA lipoplexes. (A) EGFP mRNA lipoplexes containing DC-1-16, DC-6-14 or TC-1-12 were reverse transfected into HeLa cells. The percentage of EGFP positive cells was measured 24 h after transfection. (B) Cy5-mRNA lipoplexes containing DC-1-16, DC-6-14 or TC-1-12 were reverse transfected into HeLa cells. The fluorescence intensity was measured 3 h after transfection. Control, Group subjected to reverse transfection with non-lyophilized mRNA lipoplexes. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$. Data are presented as mean $\pm$ standard deviation (n=3 in each group). EGFP, enhanced green fluorescent protein; DC-1-16, *N*-hexadecyl-*N,N*-dimethylhexadecan-1-aminium bromide; DC-6-14, 2-(bis(2-(tetradecanoyloxy)ethyl)amino)-*N,N,N*-trimethyl-2-oxoethan-1-aminium chloride; TC-1-12, 11-((1,3-bis(dodecanoyloxy)-2-((dodecanoyloxy)methyl) propan-2-yl) amino)-*N,N,N*-trimethyl-11-oxoundecan-1-aminium bromide.

luciferase activity of mRNA lipoplexes containing DC-1-16 or DC-6-14 showed little to no decrease (Fig. 5). In contrast, mRNA lipoplexes containing TC-1-12 exhibited a substantial reduction in luciferase activity after one month of storage (Fig. 5). Consistent with the trend observed immediately after lyophilization (Fig. 2B), the use of sucrose solution as a cryoprotectant resulted in significantly higher luciferase activity than trehalose solution for all lipoplexes (Fig. 5). From the above results, although dependent on the type of lipid, it was suggested that the long-term storage of mRNA lipoplexes is feasible through lyophilization in a disaccharide solution.

Discussion

Lyophilization removes water via sublimation under low temperature and vacuum conditions, and is considered an effective method for maintaining the long-term stability of lipoplexes (18,19). However, lyophilization imposes significant stress on the liposomes. Therefore, in the absence of appropriate cryoprotectants such as sugars, liposomes

may be damaged (13,20). Several studies have reported that, with certain modifications or optimizations, the lyophilization of various lipid nanoparticles complexed with nucleic acids, such as mRNA, can enhance their long-term stability (21,22). In this study, cationic liposomes were prepared using five types of cationic lipids in combination with DOPE and PEG-Chol and used as mRNA carriers for solid-phase reverse transfection. First, we investigated the effect of lyophilization in the presence of disaccharide solutions on the particle size of lipoplexes prepared using these liposomes. The results showed that the mRNA lipoplexes formulated with cationic lipids with relatively long carbon chains exhibited a decrease in particle size after lyophilization and rehydration. In contrast, lipoplexes formulated with cationic lipids having relatively short carbon chains showed a slight increase in particle size after lyophilization and rehydration. Furthermore, the particle size of lyophilized plasmid DNA-based lipoplexes containing DOTAP decreased after rehydration (23). In contrast, it has also been reported that the particle size of siRNA lipoplexes containing DDAB or DOTAP can increase depending on

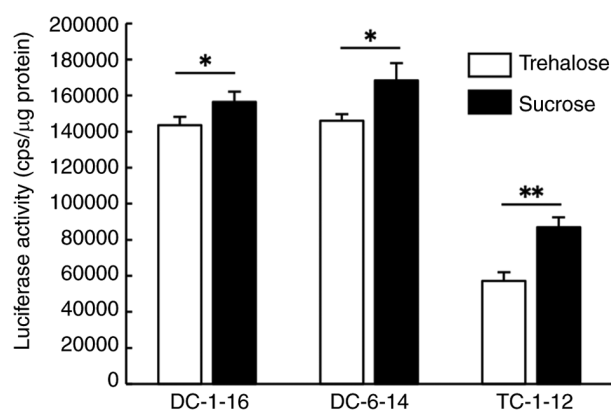


Figure 5. Luciferase activity following reverse transfection with lyophilized FLuc mRNA lipoplexes after 1 month of storage. FLuc mRNA lipoplexes containing each cationic lipid were lyophilized with 150 mM trehalose or sucrose and stored for 1 month. The lipoplexes were reverse transfected into HeLa cells and luciferase activity was measured 24 h after transfection. * $P < 0.05$ and ** $P < 0.01$. Data are presented as mean $\pm$ standard deviation ($n = 3$ in each group). DC-1-16, *N*-hexadecyl-*N,N*-dimethylhexadecan-1-aminium bromide; DC-6-14, 2-(bis(2-(tetradecanoyloxy)ethyl)amino)-*N,N,N*-trimethyl-2-oxoethan-1-aminium chloride; TC-1-12, 11-((1,3-bis(dodecanoyloxy)-2-((dodecanoyloxy)methyl) propan-2-yl) amino)-*N,N,N*-trimethyl-11-oxoundecan-1-aminium bromide; cps, count per sec.

the type of disaccharide solution used during lyophilization (12). These findings, together with our results, suggest that both the type of cationic lipid and the disaccharide solution influence particle size during the lyophilization process of mRNA lipoplexes.

To evaluate the effect of lyophilization on the transfection activity, transfection experiments using Luc mRNA were performed on cultured cells. Initially, we conducted transfection experiments using the liquid-phase reverse transfection. As previously reported (16), mRNA lipoplexes containing cationic lipids with relatively short carbon chains exhibited higher luciferase activity than those with longer carbon chains. To examine whether the activity trend due to lipid differences changes upon lyophilization, solid-phase reverse transfection was performed, and similar trends were observed. These results suggest that the lyophilization process does not significantly affect the variation in luciferase activity owing to the type of cationic lipid.

Furthermore, the effects of the type and concentration of disaccharides used during lyophilization were investigated. When Luc mRNA lipoplexes were lyophilized in trehalose or sucrose solutions at concentrations ranging from 0 to 150 mM, luciferase activity increased in a concentration-dependent manner. Additionally, lipoplexes lyophilized in sucrose solution retained luciferase activity better than those lyophilized in trehalose solution. These results suggest that, at least for the liposomes containing cationic lipids used in this study, sucrose is more suitable for maintaining transfection activity during lyophilization. In recent years, various saccharides, including monosaccharides such as glucose and mannitol, and disaccharides such as sucrose and trehalose, have been used as cryoprotectants for mRNA lipid nanoparticles (24–26); however, limited knowledge regarding mRNA lipoplexes exist. Further research is required to clarify the effects of other saccharide solutions on mRNA lipoplexes.

To investigate whether the differences in luciferase activity due to the lipid formulation were attributable to differences in transfection efficiency, the percentage of EGFP-expressing cells after reverse transfection with lyophilized EGFP mRNA lipoplexes was evaluated by flow cytometry. The results showed that although there were slight increases and decreases depending on the lipid, similar to the luciferase activity results, transfection efficiency with EGFP mRNA lipoplexes did not show a significant difference between liquid- and solid-phase reverse transfection methods. However, the lipid-dependent differences in activity observed in the luciferase assay were not observed in the transfection efficiency of EGFP mRNA lipoplexes. Therefore, to investigate whether the differences in luciferase activity due to the lipid formulation resulted from differences in cellular uptake, the intracellular uptake of Cy5-mRNA was examined. As a result, unexpectedly, both in the lyophilized and non-lyophilized conditions, luciferase activity followed the order TC-1-12 > DC-1-16 $\geq$ DC-6-14, whereas intracellular uptake followed the reverse order DC-6-14 > DC-1-16 > TC-1-12, although the differences were not large. Protein expression following mRNA transfection may not always correlate with cellular uptake (27,28). Considering that there were no remarkable differences observed in endosomal escape, the differences in transfection activity caused by the lipid composition may be attributed to changes in mRNA stability or translation efficiency and so on. While these factors were not fully explored in this study, identifying the underlying causes is a key challenge for future studies.

Finally, to evaluate the long-term stability of these lipoplexes, the lyophilized mRNA lipoplexes were stored under vacuum at room temperature in a desiccator for one month. No significant changes in particle size were observed in the mRNA lipoplexes containing DC-1-16 or TC-1-12. However, those containing DC-6-14 exhibited an increase in particle size. This suggests that long-term storage after lyophilization may cause structural changes in mRNA lipoplexes, depending on the type of cationic lipid used. However, the changes in luciferase activity after long-term storage did not necessarily correlate with the changes in particle size. Although no significant change in particle size was observed in mRNA lipoplexes containing TC-1-12, luciferase activity was significantly decreased; the underlying causes remain unclear. However, we previously reported that siRNA lipoplexes containing TC-1-12 were unstable following lyophilization (15). Although no clear signs of aggregation were observed in this study, mRNA lipoplexes containing TC-1-12 are likely to become unstable over time, which may ultimately affect the transfection efficiency and luciferase activity.

In this study, we investigated the effects of cationic lipids in liposomes and disaccharides used as cryoprotectants during lyophilization on the transfection efficiency of lyophilized mRNA lipoplexes. These results suggested that lyophilization in 150 mM sucrose solution could stabilize various types of mRNA lipoplexes for at least one month while minimizing the impact on particle size and transfection activity. However, certain cationic lipids may not be suitable for long-term storage, highlighting the need for further investigations into the relationship between the type of disaccharide cryoprotectant, selection of cationic lipids, and the long-term stability of lyophilized mRNA lipoplexes. Furthermore, it remains to be investigated whether this approach is applicable not only

to HeLa cells but also to other cell types, including primary cultured cells. This will be an important subject for future studies. Among the cationic lipids and disaccharide solutions examined, it was confirmed that lyophilization of mRNA lipoplexes containing DC-6-14 in sucrose solution provided optimal conditions for long-term storage without significant loss of transfection activity. Based on the findings of this study, the use of lyophilized mRNA lipoplexes in reverse transfection strategies suggests their potential for the development of mRNA-based therapies and gene function screening methods for various diseases. Specifically, in this platform, by fixing candidate therapeutic mRNA lipoplexes in multi-well plates, we can simultaneously test them against multiple types of cancer cells to identify which types are effective. Additionally, the preloading of multiple types of therapeutic mRNA as lipoplexes into multi-well plates enables determination of the most effective therapeutic mRNA for cancer cells. These approaches enable rapid identification of therapeutic mRNA for effective cancer therapy, thereby informing the design of personalized therapies.

In this study, all experiments were conducted in HeLa cells. Future studies will need to confirm the transfection efficacy of mRNA lipoplexes across various cancer types. In particular, to advance toward clinical application, it will be essential to assess the feasibility of implementing this platform with patient-derived cancer cells.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

YH conceived the study and designed the project. RS conducted the experiments. Both YH and RS confirm the authenticity of the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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