Development of Lyophilized Gemini Surfactant-Based Gene Delivery Systems: Influence of Lyophilization on the Structure, Activity and Stability of the Lipoplexes

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ABSTRACT – Purpose. Cationic gemini surfactants have been studied as non-viral vectors for gene therapy. Clinical applications of cationic lipid/DNA lipoplexes are restricted by their instability in aqueous formulations. In this work, we investigated the influence of lyophilization on the essential physiochemical properties and *in vitro* transfection of gemini surfactant-lipoplexes. Additionally, we evaluated the feasibility of lyophilization as a technique for preparing lipoplexes with long term stability. **Methods.** A gemini surfactant [12-7NH-12] and plasmid DNA encoding for interferon-γ were used to prepare gemini surfactant/pDNA [P/G] lipoplexes. Helper lipid DOPE [L] was incorporated in all formulation producing a [P/G/L] system. Sucrose and trehalose were utilized as stabilizing agents. To evaluate the ability of lyophilization to improve the stability of gemini surfactant-based lipoplexes, four lyophilized formulations were stored at 25˚C for three months. The formulations were analyzed at different time-points for physiochemical properties and *in vitro* transfection. **Results.** The results showed that both sucrose and trehalose provided anticipated stabilizing effect. The transfection efficiency of the lipoplexes increased 2-3 fold compared to fresh formulations upon lyophilization. This effect can be attributed to the improvement of DNA compaction and changes in the lipoplex morphology due to the lyophilization/rehydration cycles. The physiochemical properties of the lyophilized formulations were maintained throughout the stability study. All lyophilized formulations showed a significant loss of gene transfection activity after three months of storage. Nevertheless, no significant losses of transfection efficiency were observed for three formulations after two months storage at 25 ˚C. **Conclusion.** Lyophilization significantly improved the physical stability of gemini surfactant-based lipoplexes compared to liquid formulations. As well, lyophilization improved the transfection efficiency of the lipoplexes. The loss of transfection activity upon storage is most probably due to the conformational changes in the supramolecular structure of the lipoplexes as a function of time and temperature rather than to DNA degradation.

INTRODUCTION

Gene therapy is a promising therapeutic approach that has the potential to improve, significantly, human health (1). Successful gene therapy depends on the design of efficient, safe and stable gene delivery systems. Chemically mediated non-viral vectors, such as cationic lipids, exhibit low immunogenicity compared to viral vectors (2, 3). One specific group of cationic lipids that has demonstrated efficient transfection activity is the gemini surfactants [Figure 1] (4, 5). They are dimeric surfactants primarily used in material sciences because of their characteristic low surface tension (6, 7). In recent years, gemini surfactants have been investigated extensively as a non-viral gene delivery carriers for both *in vitro* and *in vivo* applications.

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These agents have versatile chemical structure, can be produced easily on a laboratory scale, are able to compact DNA to nano-sized lipoplexes, and show relatively low toxicity compared to monomeric surfactants (4, 8, 9). Several classes of gemini surfactant-based cationic lipids have been synthesized and characterized for the purpose of gene delivery (10-12). Similarly to other cationic lipids, the transfection activity of gemini surfactants is influenced by the chemical nature of the head groups, length and saturation of the hydrophobic chains, and the chemical composition and length of the spacer (13, 14).

The low transfection efficiency and the instability of lipid-based gene delivery vectors in liquid pharmaceutical dosage forms are two major deficiencies that limit their wide clinical application (15, 16). Over the last decade, a large number of cationic lipids have been synthesized and modified to overcome their low transfection activity, but little concern has been given to the stability of lipoplexes from a pharmaceutical perspective. The transfection efficiency of lipoplexes depends not only on the stability and integrity of all components of the delivery system but also on the maintenance of their related physiochemical properties (particle size and surface charge ratio) (17-19). The stability of a non-viral gene delivery system depends upon the conformational integrity of the genetic material, the chemical stability of the carrier and the physiochemical stability of the DNA-carrier complexes (20-22). In aqueous formulations, lipoplexes tend to aggregate and form large particles. This phenomenon can also lead to the dissociation of DNA from the lipoplexes and loss of the biological activity due to enzymatic degradation of the unprotected genetic material (23). To evade the stability issue, most of the studies that employ cationic lipids as non-viral carrier for gene delivery use freshly prepared lipoplexes. Three different formulation methods have been explored to optimize the physical stability of cationic lipid/DNA complexes: liquid, frozen, and dehydrated (24). To maintain the stability of the liquid and frozen formulations, special storage conditions and formulation strategies are needed that limit large scale production of the lipoplexes using good manufacturing procedures (25-28). Lyophilized (freeze-dried) formulations demonstrated the most efficient stability among these three formulation techniques (29).

Lyophilization has been employed widely for the production of highly stable protein-based pharmaceutical products (30, 31). Recently, lyophilization was also investigated as a practical technique to produce non-viral vectors with long-term stability (32-34). However, lyophilization is a complicated process that includes freezing and drying stresses which can damage the DNA structure and cause aggregation of lipoplexes (29). The damage to DNA integrity and lipoplex structure during the freezing step can result from the increased concentration of the suspended materials (cryoconcentration effect) as the liquid freezes, leading to formation of larger aggregates in the unfrozen part. In addition, the formation of ice crystals or the crystallization of solutes in the formulation have been reported to damage the lipoplex integrity (27, 35). The removal of the unbound water and ice from the frozen formulations during the drying step can affect the lipoplexes as the condition shifts from a fully hydrated environment to a drier state (29, 35). In addition, phase transition of lipid membrane in lyophilized liposomal formulations during dehydration-rehydration has been reported (29). The optimization of the freeze-drying protocol and incorporation of certain stabilizers, known as cryo- or lyo-protectant agents, have been shown to improve the stability of the lipid-based DNA formulations (29, 36). Different classes of stabilizing agent have been used for the preparation of lyophilized non-viral gene delivery systems: monosaccharaides (glucose), disaccharides (sucrose, trehalose), oligosaccharides (inulin) and polymers (dextran, povidone, polyethylene glycol) (28, 37, 38). It has been reported that several aspects govern the ability of the lyophilization process to stabilize and preserve the activity of cationic lipoplexes: lyophilization protocol, type and amount of stabilizing agent, nature of the cationic lipid, DNA to cationic lipid charge ratio and incorporation of helper lipid (24, 33, 34).

Although several studies have investigated the influence of lyophilization on the cationic lipid-DNA vectors, most of these studies utilized singly charged cationic lipids (e.g., DOTAP, DC-Chol, DMRIE) (32, 39, 40). To the best of our knowledge, the effect of lyophilization on lipoplexes formed with multiply charged cationic lipids and high concentration of helper lipid DOPE has not been addressed. The aim of this work was to

evaluate the feasibility of lyophilization to stabilize gemini surfactant-based lipoplexes over long periods of storage at room temperature. The influence of the lyophilization process and stabilizing agents on the physiochemical properties, DNA compaction and *in vivo* transfection activity were investigated and the results are reported here.

MATERIALS AND METHODS

Materials

The construction of the plasmid (pGThCMV.IFN-GFP), encoding for murine interferon gamma (IFN-γ) and green fluorescent protein (GFP) with total size of 5588 bps was described previously (4). Plasmid DNA was isolated and purified using QIAGEN Plasmid Giga Kit (Mississauga, ON, Canada) as prescribed in the manufacturer's protocols. The synthesis and characterization of the gemini surfactants used in this study have been previously described (41). Aqueous solutions of 3 mM gemini surfactant were used to prepare plasmid DNA/gemini surfactant lipoplexes. Helper lipid 1,2 dioleyl-*sn*-glycero-phosphatidylethanolamine (DOPE) (Avanti Polar Lipids, Alabaster, AL) was co-formulated in all formulations. Stabilizer agents (analytical grade) sucrose and trehalose were obtained from Sigma Aldrich (Oakville, ON, Canada). All excipients were used without further purification.

Preparation of lipoplexes

Two preparation methods were evaluated in this work [Table 1]. In method A, the plasmid/gemini surfactant [P/G] complexes were prepared by mixing an aqueous solution of pDNA with an appropriate amount of 3 mM gemini surfactant solution at 1:10 charge ratio and incubated at room temperature for 20 minutes. Lipoplexes were formulated in the presence of DOPE as helper lipid creating plasmid/gemini surfactant/lipid lipoplexes [P/G/L]. These [P/G/L] lipoplex systems were prepared by mixing [P/G] complexes with the DOPE vesicles at gemini surfactant to DOPE molar ratio of 1:10 and incubated at room temperature for 20 minutes. The lipoplexes were then lyophilized.

The stabilizing solutions (S: sucrose, T: trehalose) were prepared by dissolving the sugar in nuclease-free ultrapure water (Gibco, Invitrogen Corporation, Grand Island, NY, USA) on weight/weight (w/w) percentage basis and the pH was adjusted with NaOH solution to 9. These solutions were used to redisperse DOPE film, as described previously (4), at a final DOPE concentration of 1 mM and filtered through Acrodisc® 0.45 µm syringe filters (Pall Gelman, Ann Arbor, MI).

In the second preparation method [Method B, Table 1], gemini surfactant/DOPE [G/L] vesicles were prepared at a gemini surfactant to DOPE molar ratio 1:10. A stock solution of 12-7NH-12 gemini surfactant and DOPE was prepared in anhydrous ethanol and used to prepare a [G/L] film. Stabilizing solutions were used to re-disperse the [G/L] film and then filtered through Acrodisc® 0.45 µm syringe filters. An aliquot of plasmid solution and the G/L dispersion were mixed to obtain lipoplexes at a plasmid to gemini surfactant charge ratio of 1:10. Only the [G/L] component was lyophilized. Then fresh DNA solution was added to the reconstituted formulations for all assays.

Table 1 summarizes the preparation methods of these formulations. In all analyses, triplicate batches of each formulation were evaluated.

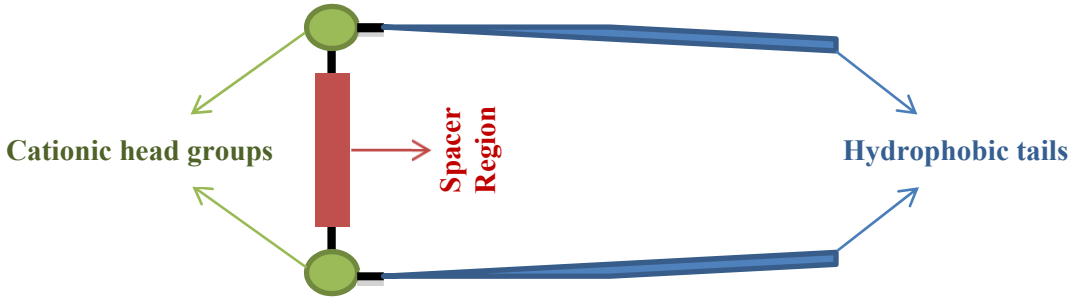

Figure 1. General structure of cationic gemini surfactants

Table 1. Preparation methods for the formulations used in this study

Formulation	Preparation method	Description
$[P/G/L\text{-}S]_{lyp}$	A	P/G/L lipoplexes prepared with DOPE vesicles dispersed in 9.25% sucrose solution were lyophilized for 48h
$[P/G/L\text{-}T]_{lyp}$		P/G/L lipoplexes prepared with DOPE vesicles dispersed in 10% trehalose solution were lyophilized for 48h
$P\text{-}[G/L\text{-}S]_{lyp}$	B	G/L vesicles prepared in 9.25% sucrose were lyophilized for 48h and the plasmid solution was added to the lipid vesicles after reconstitution to prepare the lipoplexes
$P\text{-}[G/L\text{-}T]_{lyp}$		G/L vesicles prepared in 10% trehalose were lyophilized for 48h and the plasmid solution was added to the lipid vesicles after reconstitution to prepare the lipoplexes

Lyophilization of the formulations

A volume of 2 mL of freshly prepared formulations, P/G/L-S and P/G/L-T containing a total of 7.4 µg/mL pDNA and G/L-S and G/L-T without pDNA were transferred to 5-mL flat bottom low extractable borosilicate USP Type I lyophilization serum vials (Wheaton Industries Inc, Millville, NJ, USA). The vials were partially closed with three-legged lyophilization stoppers and stored at -80 ˚C for 2 h. After freezing, the formulation vials were transferred to a Labconco® Freezone Plus 6 L cascade freeze dryer (Labconco, Kansas City, MO, USA) at -80 ˚C and 0.03 mBar pressure and lyophilized for 48 h. The vials were removed from the freeze dryer, flushed with nitrogen gas, and the vial stoppers were fully closed and sealed with a crimp aluminum cap. All formulations were prepared under aseptic conditions.

Stability study

For the stability study, the lyophilized formulations were stored in a stability chamber at 25 ºC and 75% relative humidity (RH) (Sanyo growth cabinet MLR-350, Sanyo, Osaka, Japan) for three months. Samples were tested at one, two and three month storage periods. Formulations were prepared and analyzed in triplicate (n=3).

Rehydration of the lyophilized formulations

Lyophilized formulations containing the pDNA, $[P/G/L\text{-}S]_{lyp}$ and $[P/G/L\text{-}T]_{lyp}$, were rehydrated to a final volume of 2 mL with ultrapure water (Gibco, Invitrogen Corporation, Grand Island, NY, USA). Formulations $[G/L\text{-}S]_{lyp}$ and $[G/L\text{-}T]_{lyp}$, without pDNA, were rehydrated using pDNA solution in UltraPure water to a final pDNA concentration of 7.4 µg/mL (1:10 plasmid to gemini charge ratio) and incubated for 30 minutes at room temperature, generating the $P\text{-}[G/L\text{-}S]_{lyp}$ and $P\text{-}[G/L\text{-}T]_{lyp}$ formulations. All rehydrated formulations, containing 0.2 µg pDNA, were used for *in vitro* transfection evaluation.

Determination of moisture content

Lyophilized formulations at the time of preparation and after 3 months of storage were evaluated for moisture content using a Karl Fisher Titrator (Automat Model 633; Metrohm, Herisau, Switzerland). The lyophilized formulations (approximately 45±5 mg of lyophilized cake) were dissolved in HPLC grade methanol (Fisher Scientific, Edmonton, AB, Canada) previously blanked with pyridine-free Karl Fischer reagent (BDH, Edmonton, AB, Canada) and titrated with the same reagent. Ten microliters of purified water (Milli-Q™ Water System, Milford, MA, USA) was used to standardize the Karl Fischer reagent. A 20 second delay was used to ensure end point stabilization. Formulations were analyzed in triplicate (n=3).

Size and zeta potential measurements

Fresh and rehydrated formulations were transferred into a cuvette (DTS1061, Malvern Instruments, Worcestershire, UK) for size distribution and zeta-potential measurements using a Zetasizer Nano ZS instrument (Malvern Instruments, Worcestershire, UK). Each sample was measured four times, and the results were expressed as the average ± standard deviation (SD) of three samples (n=3) with a corresponding polydispersity index (PDI) value.

Ethidium bromide binding

Fresh and rehydrated samples containing 0.5 µg pDNA were tested in 1% agarose gel stained with ethidium bromide (EtBr) (0.01%) using Bio-Rad

PowerPac HC electrophoresis apparatus (Biorad, Mississagua, ON, Canada) in tris-acetate-EDTA (TAE) buffer at 100 V for 45 minutes. EtBr was visualized by UV fluorescence using an AlphaImager system™ (Alpha Innotech, San Leandro, CA, USA).

Circular dichroism spectroscopy

Fresh, lyophilized, and stored formulations (3 months), prepared/reconstituted to a 15 µg/mL pDNA concentration, were evaluated by using circular dichroism (CD) spectroscopy. CD spectra were obtained by using a Pi-star-180 instrument (Applied Photophysics, Leatherehead, UK) with 2 nm slit at 37 ˚C under a N_2 atmosphere. The CD plots were stacked for the purpose of comparison.

Cell culture and *in vitro* transfection

COS-7 African green monkey kidney fibroblasts cell line (ATCC, CRL-1651) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% Antibiotic Antimycotic Solution (Sigma, Oakville, ON), and incubated at 37 ˚C with 5% CO_2. On the day before transfection, the cells were seeded in 96-well tissue culture plates (Falcon, BD Mississauga, ON, Canada) at a density of 1.5×10^4 cells/well. One hour prior to transfection, the supplemented DMEM was replaced with DMEM. The cells were transfected with 0.2 µg pGThCMV.IFN-GFP plasmid/well in quadruplicate. Lipofectamine Plus reagent (Invitrogen Life Technologies) was used as a positive control according to the manufacturer's protocol with 0.2 µg pDNA/well in quadruplicate. The 96-well tissue culture plates were then incubated at 37 °C in CO_2 for five hours. The transfection agents were removed and replaced with supplemented DMEM. Supernatants containing the secreted IFN-γ were collected at 24, 48 and 72 h and replaced with fresh supplemented DMEM. The collected supernatants were stored at – 80 ˚C.

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was performed using flat bottom 96-well plates (Immulon 2, Greiner Labortechnik, Frickenhausen, Germany) following the BD Pharmingen protocol and as described earlier (4). The concentration of expressed IFNγ was calculated from a standard IFNγ curve using recombinant mouse IFN-γ standard (BD Pharmingen, BD Biosciences).

STATISTICAL ANALYSIS

Statistical analyses were performed using SPSS software (Version 17.0). Results expressed as the average of $n \geq 3 \pm$ SD. One way analysis of variance (ANOVA, Dunnett's test) and Pearson's correlation were used for statistical analyses. Significant differences were considered at $p<0.05$ level.

RESULTS

Characterization of fresh formulations

Sugars are widely used as cryoprotectants and stabilizing agents for lyophilized lipid-based gene delivery systems (34, 37, 42). Based on preliminary formulation studies (results not shown), sucrose and trehalose were selected for this work. In addition, two different formulation approaches were developed in an aim to maintain the essential physiochemical properties of the P/G/L delivery system (i.e., particle size and zeta potential) upon lyophilization. For the first method [Method A, Table1], the pDNA was complexed with the gemini surfactant to form P/G lipoplexes first, and the DOPE vesicles were incorporated afterward. [P/G/L-S] lipoplexes prepared by this method, formulated in 9.25% sucrose, were comprehensively characterized in our previous work (41, 43). The lipoplexes had particle size of 124±1.9 nm and zeta potential of +29±4.8 mV [Table 2-A]. However, when 10% trehalose was used in the formulation [P/G/L-T] instead of sucrose, a major drop in both size and zeta potential (average size of 81.7±0.6 nm, zeta potential of +21.4±2.5) were observed [Table 2-A].

In addition to the previously established lipoplex preparation method [Method A], another formulation method was described in this work [Method B, Table 1]. In the P-[G/L-S] formulation, sucrose solution (9.25%) was used to prepare the G/L vesicles. While the composition of this formulation was the same as [P/G/L-S], this preparation method caused a significant increase in both particle size (by approximately 60 nm) and zeta potential (16 mV) [Table 2-A]. Similarly, trehalose was used as cryoprotectant to prepare formulation P-[G/L-T] following Method B [Table 1]. Both particle size and zeta potential showed in excess of 65% increase compared to the chemically

identical formulation prepared by Method A [P/G/L-T].

Circular dichroism (CD) measurements showed that all fresh formulations induced changes in the native structure of the DNA, as observed in the alterations in the CD spectra [Figure-2]. The spectrum of free pDNA showed two positive peaks at 255 nm and 290 nm and a negative tail in the region of 240-250 nm [Figure 2-A]. Upon complexation of the pDNA with the fresh formulation [P/G/L-S], a blue-shift was observed for the positive peak at 290 nm and a depression of the 255 nm peak [Figure 2-B]. Conversely, the [P/G/L-T] formulation caused a red-shift of the positive peak at 290 nm and a flattening of the 255 nm peak [Figure 2-C]. The complexation of pDNA with the [G/L] system caused a red-shift of the 290 nm peak with a negative tail for the area below 270 nm in both P-[G/L-S] and P-[G/L-T] formulations.

The influence of different sugars and preparation methods on the *in vitro* transfection activity of fresh formulations was also investigated [Figure 3, white bars]. All fresh formulations showed significant levels of gene expression compared to non-transfected COS-7 cells and cells treated with [G/L] system. The [P/G/L-S] formulation showed the highest gene expression activity with 8.2±2.6 ng of IFNγ/1.5×10^4 COS-7 cells after 72 hour of the transfection. The lowest gene expression among all fresh formulations was observed for the [P/G/L-T] formulation, which showed 2.3±1.9 ng IFNγ/1.5×10^4 COS-7 cells. Formulation P-[G/L-S] [Method B] showed significantly lower gene expression activity compared with the standard [P/G/L-S] formulation. However, no significant difference between the transfection efficiency of the P-[G/L-T] and standard formulation [P/G/L-S] was observed.

Influence of lyophilization/rehydration processes

Particle size and zeta potential

The lyophilized formulations $[P/G/L-S]_{lyp}$ and $[P/G/L-T]_{lyp}$ showed particle sizes less than 130 nm whereas the particle size values of the formulations P-$[G/L-S]_{lyp}$ and P-$[G/L-T]_{lyp}$ were approximately 200 nm [Table 2-B]. The lyophilization process caused a significant increase ($p < 0.05$) in the particle size of all formulations (11 to 41 nm increase) except for the [P/G/L-S] formulation (2 nm increase was observed). The zeta potential [Table 2-B] showed a trend of increase in the positive value in all formulations upon lyophilization (2 to 13 mV, $p < 0.05$).

DNA compaction

Similar to fresh formulations, lyophilized formulations altered the CD spectra of pDNA. The CD spectra of $[P/G/L-S]_{lyp}$ and $[P/G/L-T]_{lyp}$ showed an increase in the positive ellipticity of the 290 nm peak and a flat positive area above 290 nm. In addition, a blue-shift of the peak at 255 nm with a depression to negative values was observed [Figure 2-D and E, solid line].

Table 2. The influence of lyophilization process on the physiochemical properties (particle size, zeta potential and pH of lipoplexes

Formulation	[A] Fresh (time zero)			[B] After lyophilization		
	Size (nm) (PDI)	Zeta potential (mV)	pH	Size (PDI)	Zeta potential (mV)	pH
[P/G/L-S]	124.3±1.9 (0.220±0.007)	29.0±4.8	5.8±0.08	126.8±1.8 (0.237±0.023)	36.4±5.9	5.7±0.15
[P/G/L-T]	81.7±0.6 (0.221±0.007)	21.4±2.5	7.4±0.17	100.8±1.3 (0.298±0.009)	23.3±2.3	7.0±0.07
P-[G/L-S]	183.6±2.7 (0.393±0.012)	45.3±1.8	6.0±0.10	194.3±5.6 (0.377±0.012)	47.7±5.2	5.8±0.08
P-[G/L-T]	158.7±2.7 (0.289±0.016)	35.8±1.5	6.8±0.17	199.7±4.0 (0.250±0.016)	49.7±4.0	6.8±0.17

Values are shown as the average of three measurements of each formulation at [A] zero time (fresh) and [B] just after lyophilization cycle ± standard deviation.

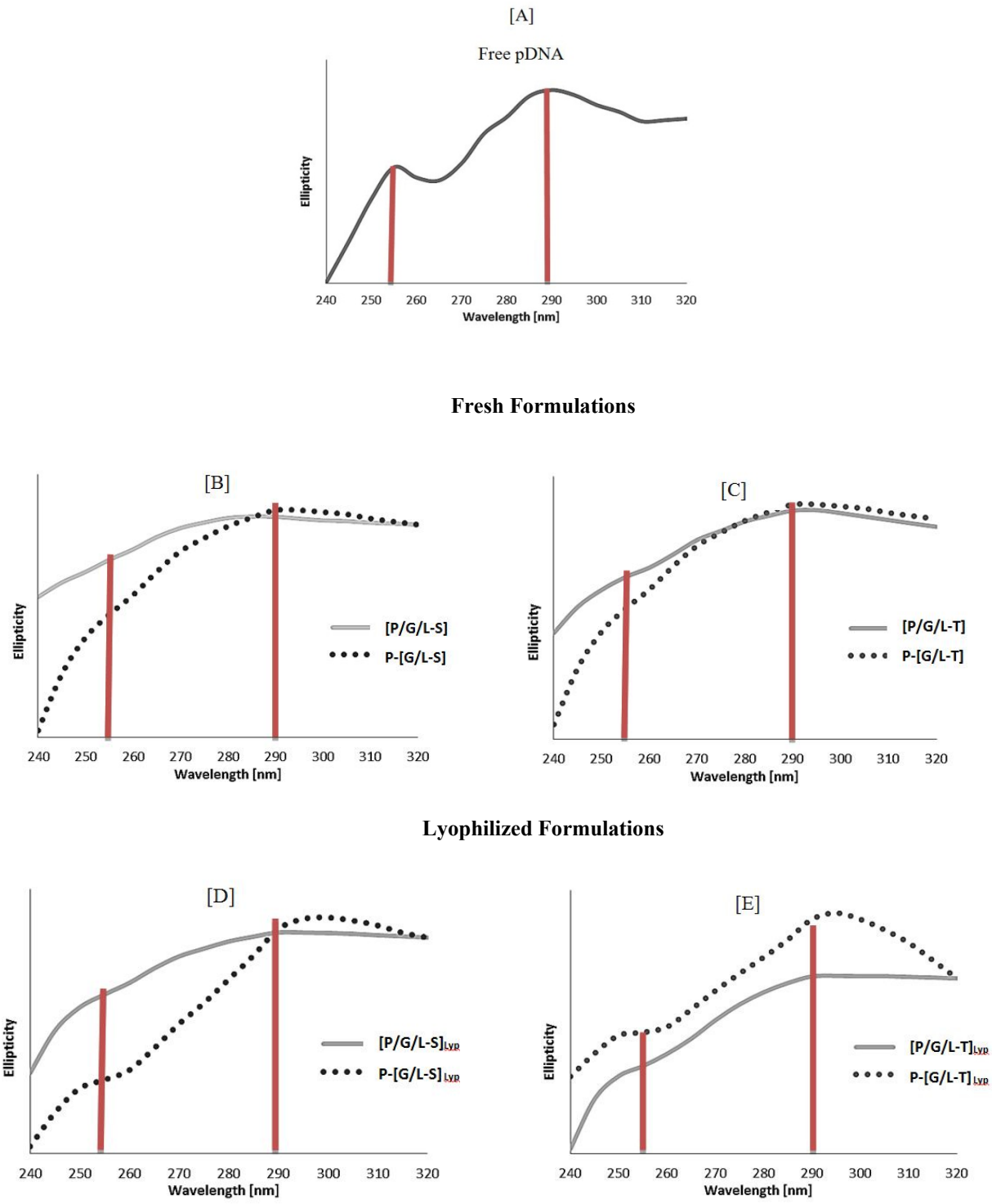

Figure 2. Circular dichroism [CD] of [A] free pDNA, [B,C] fresh formulations and [D,E] the lyophilized formulations. Values are average of three measurements [n=3].

Ethidium bromide binding

The influence of lyophilization on the interaction between the pDNA and gemini surfactant 12-7NH-12 was assessed by using ethidium bromide binding assay and gel electrophoresis [Figure 4]. The gel image shows that the pDNA was completely retarded in all freshly prepared [P/G/L] and P-[G/L] systems incorporating both sucrose and trehalose cryoprotectants, indicating that it was totally shielded by the gemini surfactant [Figure 4-B]. The lyophilization process had no effect on the pDNA/gemini surfactant interaction as no pDNA migration was observed in any of the lyophilized formulations [Figure 4-C].

In vitro transfection activity

Lyophilized formulations were evaluated for their *in vitro* transfection in COS-7 cell line to investigate the influence of the lyophilization process on the gene expression activity [Figure 3, gray bars]. Interestingly, freeze-drying significantly improved the transfection activity of three formulations ($p < 0.05$) in comparison to corresponding fresh formulations. The P-$[G/L-S]_{lyp}$ formulation showed the most significant improvement in transfection activity (approximately 3.5 fold). Similarly, the $[P/G/L-T]_{lyp}$ and P-$[G/L-T]_{lyp}$ formulations exhibited a significant increase (2.5 fold). The $[P/G/L-S]_{lyp}$ formulation showed no significant change in transfection activity after lyophilization.

Stability study

Particle size and zeta potential

The lyophilized cake of all formulations retained a free powdery appearance throughout the stability study [Figure 5]. The rehydrated formulations at all sampling times were clear dispersions with no visible particles. Particle size and zeta potential values of the lyophilized formulations were measured during the stability study and compared to the corresponding fresh non-lyophilized formulations (time zero) [Figure 6].

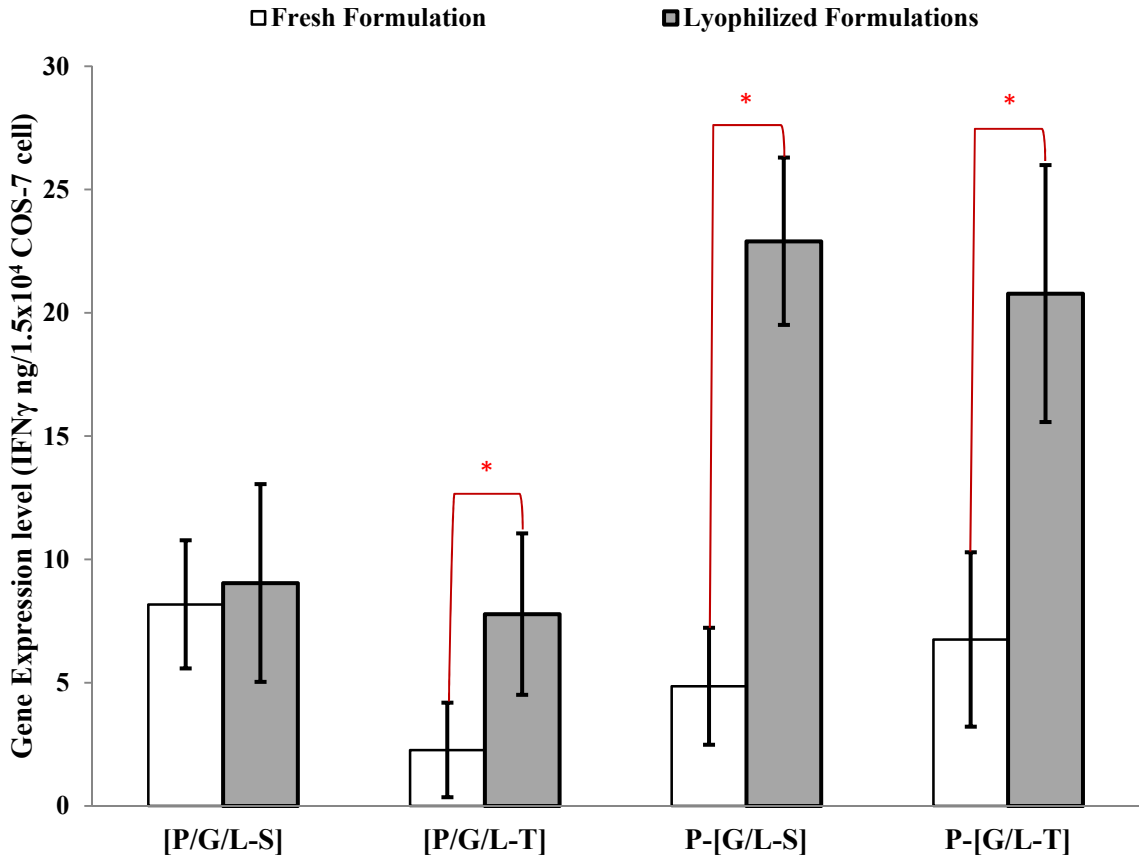

Figure 3. Gene expression activity of lipoplex (ELISA-IFNγ) after 72 h. White columns represent fresh formulations. Grey columns represent the influence of lyophilization [lyophilized formulations just upon lyophilization cycle]. Results are average of three samples of each formulation (n=3), error bars ± SD. * Indicates significant at $p < 0.05$.

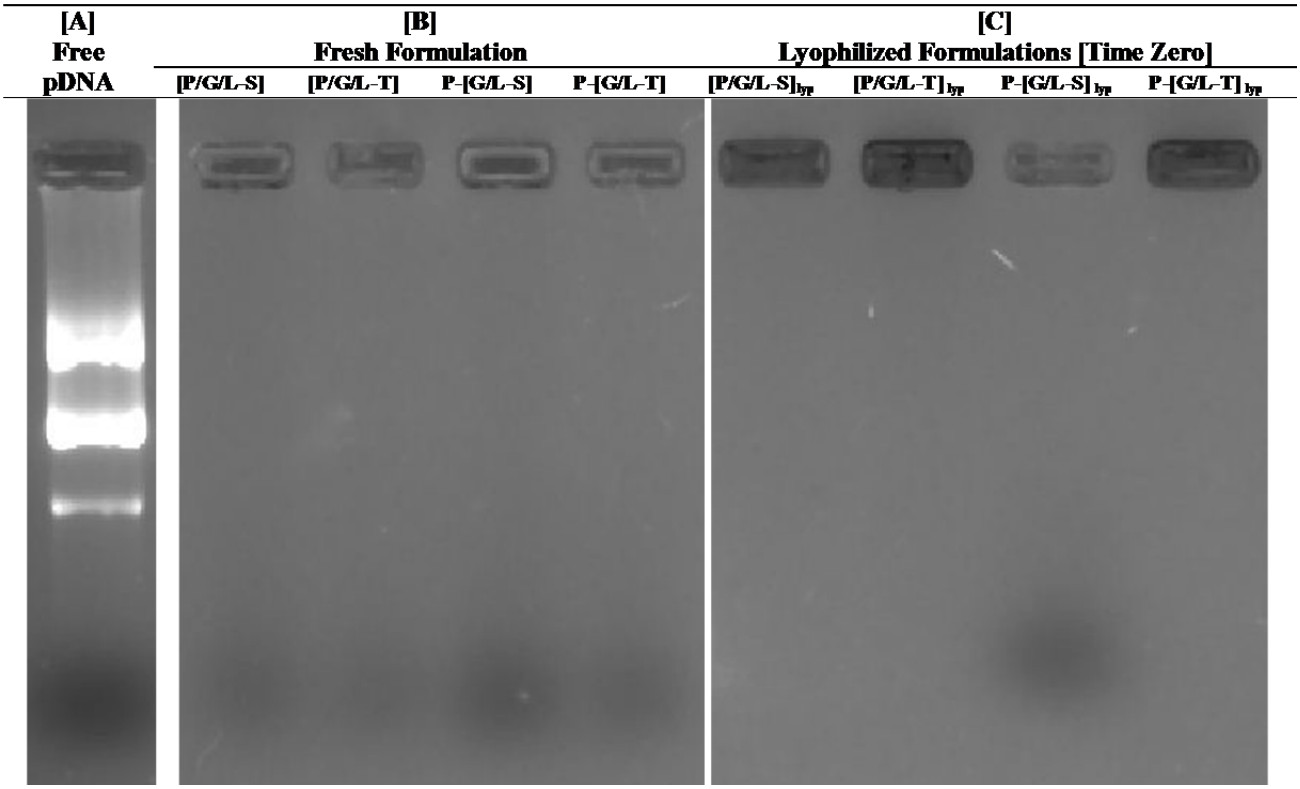

Figure 4. Ethidium bromide binding assay using agarose gel electrophoresis [A] free pDNA 0.5 µg, [B] fresh formulations showed total binding of the pDNA to the gemini surfactant with no pDNA band being observed in all four formulations, [C] lyophilized formulations, no pDNA band was observed in all formulations upon lyophilization proving that the lyophilization process did not affect the pDNA-gemini surfactant binding.

The particle size and PDI of the formulations preserved with sucrose, $[P/G/L\text{-}S]_{lyp}$, revealed no significant changes during the three-month study compared with the fresh lipoplexes [P/G/L-S] at time zero. Conversely, the particle size and PDI values of the lyophilized $[P/G/L\text{-}T]_{lyp}$ formulation, stabilized with trehalose, increased with time and displayed a significant size increase of approximately 20% within the first month of storage ($p < 0.05$).

The values of the particle size of formulations $P\text{-}[G/L\text{-}S]_{lyp}$ and $P\text{-}[G/L\text{-}T]_{lyp}$ displayed some fluctuation during the study. For instance, in comparison to the fresh P-[G/L-S] at time zero, the particle size of lyophilized $P\text{-}[G/L\text{-}S]_{lyp}$ showed a significant decrease within the first two months of storage ($p < 0.05$). The particle size decreased significantly ($p < 0.05$) from 183±2.7 nm at time zero to 113±3.9 nm at the second sampling point. After 3 months, an increase in particle size was observed (129 nm) in comparison to the value at the 2-month time. Nevertheless, the particle size remained within the range 100-200 nm at all sampling points.

The influence of storage on the zeta potential of lyophilized formulations was monitored through the three-month stability study [Figure 6-B]. In all lyophilized formulations, a positive zeta potential was maintained during storage. Formulations $[P/G/L\text{-}S]_{lyp}$ showed significant ($p < 0.05$) increase in zeta potential values upon storage with a maximum increase observed at the three month sampling point (46±5.1 mV). The zeta potential for formulations $[P/G/L\text{-}T]_{lyp}$ remained steady during storage (ranging from 20 to 24 mV), whereas, the $P\text{-}[G/L\text{-}S]_{lyp}$ formulation fluctuated slightly during storage. Finally, the zeta potential of lyophilized lipoplexes of $P\text{-}[G/L\text{-}T]_{lyp}$ showed a significant increase (20%, $p < 0.05$) after one month of storage followed by a significant, continuous decrease with time to the 3-month value of 20.8+6.6 mV.

Ethidium bromide binding

The pDNA band was not observed in all lyophilized formulations after three months of storage at 25 ˚C/75% RH. This observation provided evidence that the complete interaction of the pDNA with the cationic gemini surfactant was maintained throughout the study [Figure 7-B].

Moisture content

The moisture content of lyophilized formulations was determined to assess the efficiency of the freeze-drying process and to evaluate the effect of storage on the moisture content. Following the freeze-drying cycle, the moisture content in all formulations was less than 2% (w/w). After three months of storage at 25 ˚C, the moisture content in the lyophilized cake increased by 50-170% compared to the values reported just after the lyophilization [Table 3].

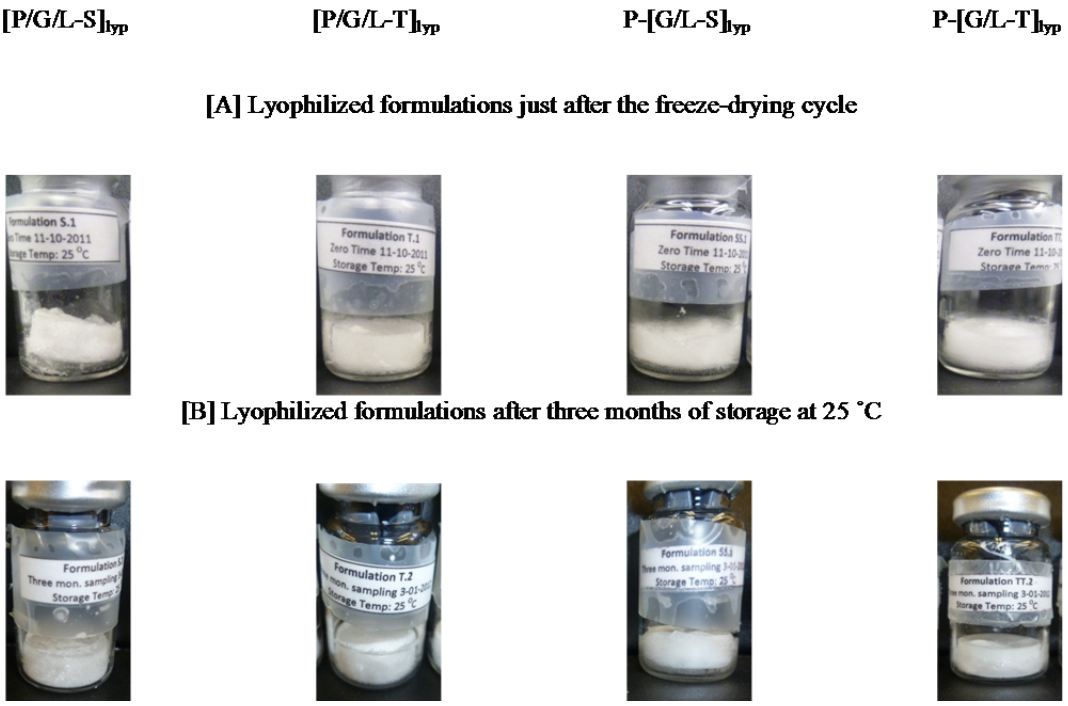

Figure 5. The appearance of lyophilized cake of four formulations [A] just after the freeze drying and [B] after three months of storage at 25 ˚C.

Table 3. Moisture content of lyophilized formulations (%w/w)

Formulation	Before storage	After storage
$[P/G/L\text{-}S]_{lyp}$	1.8±0.2	2.8±0.2
$[P/G/L\text{-}T]_{lyp}$	1.5±0.3	3.1±0.5
$P\text{-}[G/L\text{-}S]_{lyp}$	1.3±0.2	3.5±0.2
$P\text{-}[G/L\text{-}T]_{lyp}$	1.5±0.3	3.0±0.1

Values are shown as the average of three measurements of each formulation after three months of storage at 25˚C ± standard deviation.

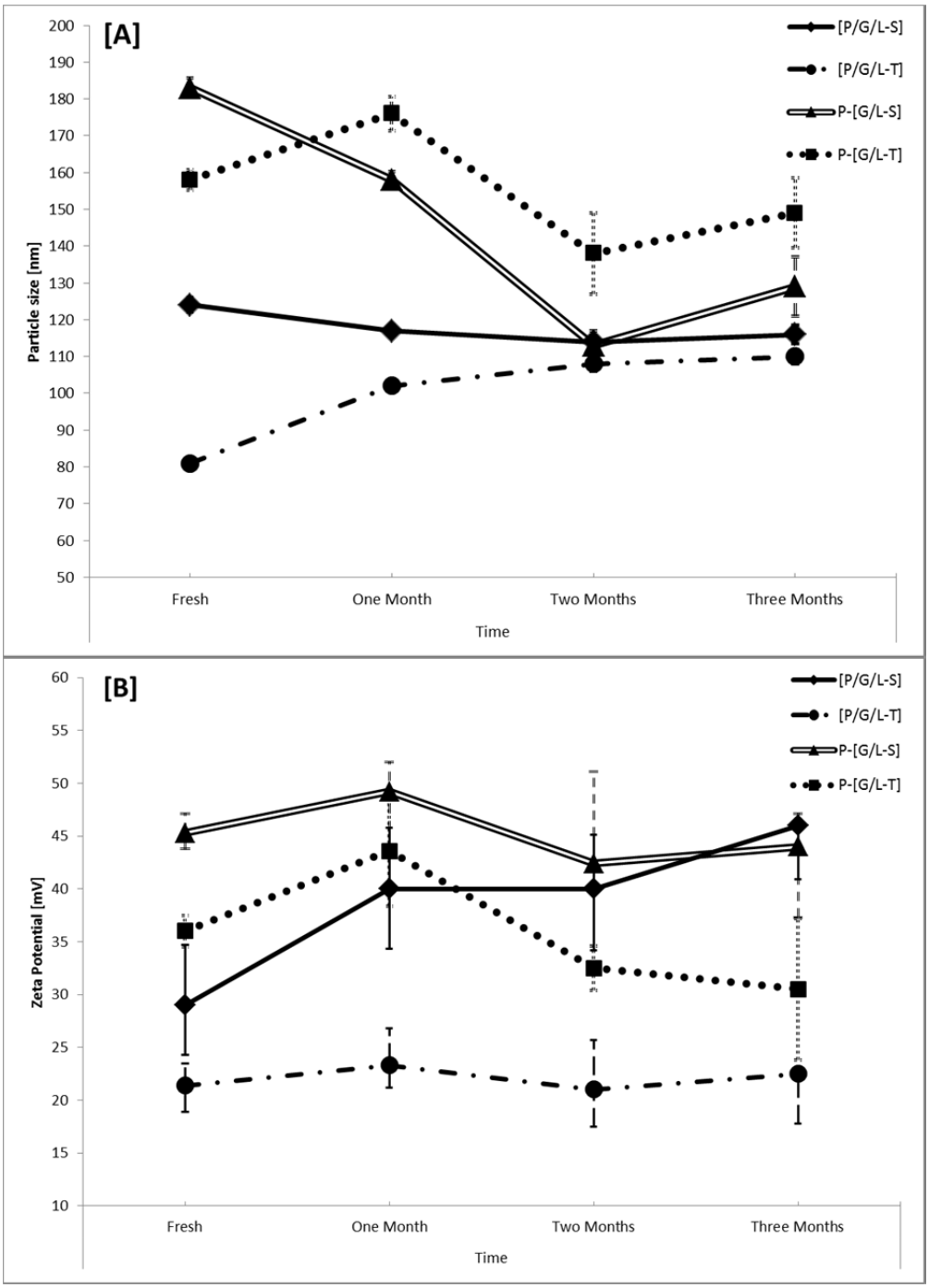

Figure 6. The influence of time on [A] the particle size and [B] zeta potential stored at 25 ˚C. Results are average of three samples of each formulation (n=3), error bar ± SD.

In vitro transfection activity

Upon storage at 25 ˚C, formulations P-[G/L-S]$_{lyp}$ and P-[G/L-T]$_{lyp}$ were able to preserve the transfection levels of their corresponding fresh non-lyophilized formulations for up to two months of storage [Figure 8]. After three months, formulations [P/G/L-S]$_{lyp}$, P-[G/L-S]$_{lyp}$ and P-[G/L-T]$_{lyp}$ showed a significant decrease in transfection activity with maximum reduction reported for formulations P-[G/L-S]$_{lyp}$ and P-[G/L-T]$_{lyp}$ (> 70%

and 80% loss in activity, respectively, Figure 8). The transfection activity of $[P/G/L\text{-}S]_{lyp}$ was reduced by approximately 40% compared to the original activity of non-lyophilized [P/G/L-S]. Formulation $[P/G/L\text{-}T]_{lyp}$ was the most stable formulation in terms of retaining its starting transfection activity, with no significant change observed throughout the three month period of study.

DISCUSSION

The purpose of this work was to evaluate freeze-drying as a technique to improve the stability of pDNA/gemini surfactant lipoplexes and to investigate the influence of the lyophilization and storage conditions on the essential physiochemical properties and *in vitro* transfection activity of the lipoplexes. We have developed in recent years a series of cationic gemini surfactants as a chemical carrier for DNA delivery (4, 9, 43, 44). Although a significant improvement in gene expression activity and improvement in the cellular safety profile have been achieved, the instability of the pDNA/gemini surfactant lipoplexes in aqueous formulations remained a concern. Due to several physical and chemical factors, freshly prepared formulations showed loss of transfection activity after one week of storage at room temperature (results not shown).

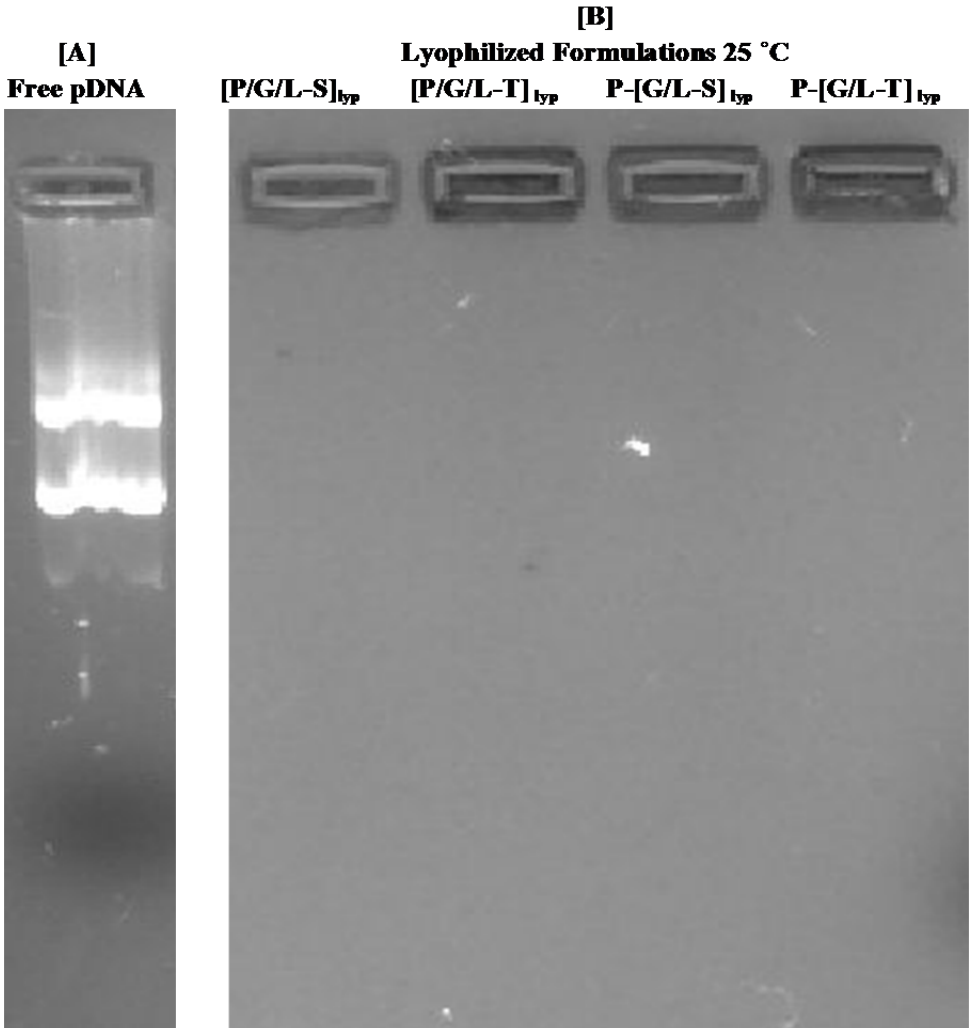

Figure 7. Ethidium bromide binding assay using agarose gel electrophoresis [A] free pDNA 0.5 µg, [B] lyophilized formulations stored at 25 ˚C for three months.

The physical instability of the lipoplexes is a result of changes in physiochemical properties such as particle size and positive zeta potential. When stored at room temperature, these positively charged particles tend to form micro-sized aggregates as a function of random collisions, Brownian motion, and gravity forces (23, 45). These changes can cause the loss of the supra-molecular structure of the lipoplexes leading to the leakage of the pDNA from the lipoplexes and loss of its biological activity (23, 45). The chemical stability of the different components of the lipoplexes (i.e., pDNA, gemini surfactant, DOPE) depends on the storage environment, namely pH of the formulation, temperature and the presence of metal contaminants (20-22, 32, 46). All these factors can lead to loss of the integrity of the lipoplexes and reduction of the gene delivery efficiency. Thus we investigated different formulation strategies to optimize the physiochemical stability of the lipoplexes and evaluate whether lyophilization could preserve their structural integrity and transfection efficiency.

As mentioned previously, the lyophilization process includes three stress steps that can destabilize the lipoplexes: freezing, drying (dehydration) and rehydration. Aggregation of the lipoplexes and a shift to negative zeta potential upon lyophilization/rehydration were observed when formulations were prepared without cryoprotectant agents (Appendix 1). For that reason, we evaluated several cryo-/lyo-protectants to determine whether they could stabilize and protect the lipoplexes during the freezing and lyophilization processes.

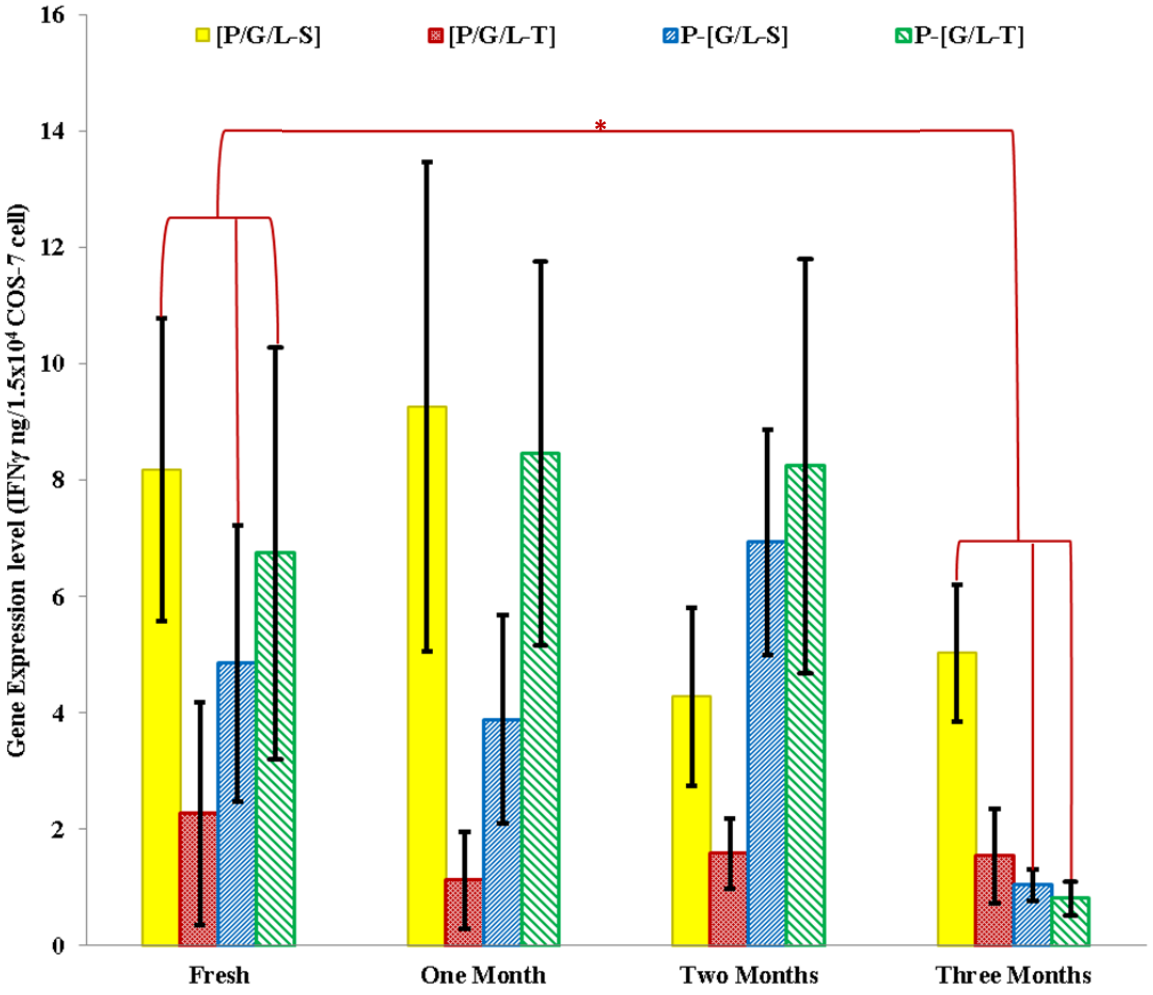

Figure 8. *In vitro* transfection activity of the lyophilized formulations stored at 25 ˚C for three months (ELISA-IFNγ). Results are the average of three samples of each formulation (n=3), error bars ± SD. * Indicates significant at $p < 0.05$ level.

The physiochemical properties that were examined included the particle size and zeta potential of the lipoplexes as several previous studies had reported a strong relation between the physiochemical properties of lipoplexes and cellular uptake and consequently, transfection activity (17, 47, 48). In the pilot evaluation of the cryoprotectant activity of different agents (Appendix 1), we assessed a number of stabilizing agents as a function of concentration and different combinations. These agents included monosaccharaides (glucose), disaccharides (sucrose, trehalose, lactose), polymers (polyethylene glycol) and simple polyol (glycerin). Disaccharide sugars, sucrose and trehalose, effectively maintained the particle size and the positive surface charge of the lipoplexes after lyophilization, similar to previous assessments that evaluated these agents during the lyophilization of chemically based gene delivery vectors (34, 39, 49, 50). Based on these findings, sucrose and trehalose were selected as lyo-/cryo-protectant agents to investigate the factors affecting the long-term stability of lyophilized gemini surfactant based lipoplexes.

The physiochemical and biological properties of four different lyophilized formulations were evaluated for stability at room temperature (25 ˚C). Fresh formulation [P/G/L-S] prepared in 9.25%, our standard formulation, showed the highest gene expression activity among all fresh formulations evaluated in this work. The replacement of sucrose by 10% trehalose in the formulation [P/G/L-T] caused significant changes in physiochemical properties, as the particle size and zeta potential both decreased. Additionally, CD results showed that the [P/G/L-T] altered the native structure of pDNA in a different manner compared to standard formulation [P/G/L-S]. As a result, the transfection activity was severely hampered resulting in a low level of IFNγ expression (60% reduction compared with [P/G/L-S]). The reduction in the particle size can be attributed to the pH-active imino group of the 12-7NH-12 gemini surfactant. Previous work, evaluating the structural and transfection activity of 12-7NH-12 gemini surfactant, found that a pH-dependent transition in the size of lipoplexes occurred at pH 5.5 and the size significantly decreased at higher pH values (43).In the present work, the pH value of [P/G/L-T] formulation was 7.4 as compared to fresh [P/G/L-S] formulation (pH 5.8) [Table 2-A]. Given these results, we hypothesize that trehalose produces strongly compacted lipoplexes that hinders the release of the pDNA after the cellular uptake, thus causing a lower level of gene expression. This hypothesis will be tested in future studies by evaluating the cellular trafficking of the lipoplexes using fluorescently tagged gemini surfactant or helper lipid and tracking the lipoplexes by confocal laser scanning microscopy or fluorescence microscopy (44, 51).

Formulations P-[G/L-S] and P-[G/L-T] were prepared initially to investigate the effect of the lyophilization process and storage conditions on the gemini surfactant/DOPE [G/L] lipid structure [Method B, Table 1]. They have the same chemical composition as formulations [P/G/L-S] and [P/G/L-T], respectively.

However, elimination of the pDNA from the lyophilized complex could permit the determination of whether any alteration in transfection activity, upon lyophilization and storage, is a result of DNA degradation or due to changes in the [G/L] polymorphic structure. The modification of the preparation method led to changes in the physiochemical properties and transfection activity. Fresh formulations P-[G/L-S] and P-[G/L-T] formed lipoplexes with larger particle size and greater positive zeta potential values compared to formulations [P/G/L-S] and [P/G/L-T], prepared by method A. In the standard formulation method [Method A, Table 1], the primary lipoplexes are formed by the electrostatic interaction between the gemini surfactant and pDNA producing the [P/G] lipoplexes. After the formation of the P/G lipoplexes, addition of DOPE vesicles can induce polymorphic changes in the lipoplex structure by producing a lipid bilayer packed in lamellar, cubic or inverse hexagonal morphologies (8, 43). The excess of the gemini surfactant (10 to 1 positive to negative charge ratio) provides the positive surface charge for the P/G/L system. On the other hand, the first step in the preparation of P-[G/L] systems [Method B, Table 1] involves the formation of [G/L] vesicles by the incorporation of the gemini surfactant into the DOPE film. Therefore, it is assumed that different polymorphic structures were induced in which the gemini surfactant molecules with DOPE form a bilayer lipid membrane and the positively charged gemini surfactant molecules are distributed in both layers. The addition of pDNA to the [G/L] bilayer system caused the formation of

lipoplexes with larger particles that are more positively charged than the [P/G/L] systems. The [G/L] lipid systems were able to completely interact with the pDNA as no pDNA bands were observed in ethidium bromide gel-electrophoresis [Figure-4-B] and this interaction caused changes in the pDNA structure as the CD spectra of pDNA was also shown to be altered. It is important to note that, CD spectra obtained from fresh P-[G/L-S] and P-[G/L-T] formulations were nearly identical but differed significantly from the spectra of the fresh [P/G/L-S] and [P/G/L-T] formulations [Figure 2-B,C].

These modifications in physiochemical properties caused significant changes in the biological activity as observed by the levels of gene expression. In the case of P-[G/L-S] formulation, the transfection activity was considerably reduced (40%) in comparison to [P/G/L-S]. Conversely, formulation P-[G/L-T] showed a substantial improvement in gene expression level compared to [P/G/L-T]. Since not all the gemini surfactant molecules in [G/L] vesicles were available to completely interact with the pDNA, the lipoplexes formed in the P-[G/L] formulations resulted in less compacted pDNA. Therefore, the lower gene expression activity of the P-[G/L-S] systems could be related to the loose DNA-compaction that could cause premature release of the DNA before nuclear internalization. On the other hand, the subordinate pDNA compaction caused by [G/L-T] improved the transfection activity of P-[G/L-T] lipoplexes in comparison to highly compacted [P/G/L-T] lipoplexes.

Influence of lyophilization on the lipoplexes properties

A major focus of in this work was to examine the effect of the lyophilization process on the transfection efficiency of the lipoplexes. A significant improvement in gene expression activity was observed for three formulations upon freeze-drying; $[P/G/L-T]_{lyp}$, $P-[G/L-S]_{lyp}$ and $P-[G/L-T]_{lyp}$ [Figure 3]. The $P-[G/L-S]_{lyp}$ and $P-[G/L-T]_{lyp}$ formulations showed more than a 40% increase in gene expression levels compared to the activity of standard fresh formulation [P/G/L-S] .

The results indicated that both particle size and zeta potential increased after lyophilization in all formulations [Table 2-B]. Additionally, the lyophilization altered the CD spectrum of pDNA in a different manner compared to the CD spectra obtained from fresh formulations [Figure 2, D and E]. We believe that these modifications were caused by changes in the lipid phase arrangement as a result of the freezing and dehydration cycles of the lyophilization protocol. In fact, previous studies reported relatively similar increases in transfection activity of cationic lipid/DNA complexes after freezing or lyophilization stresses. For instance, cationic hydroxyethylated cholesterol (DMHAPC-Chol) co-formulated with helper lipid DOPE as a system for gene delivery showed a four to five-fold increase in gene expression activity after lyophilization compared to the fresh non-lyophilized liposomes (52). The improvement in gene activity profile was correlated to the degree of hydration of the phosphate head groups of DOPE leading to polymorphic phase change as observed by NMR spectra of hydrated and dehydrated lipid systems (52). Similarly, a three-fold increase in transfection activity of the DOTAP:DOPE system was observed after a freeze-thaw cycle compared to freshly prepared lipoplexes in the absence of the cryoprotectant (27). However, lyophilization did not increase the transfection efficiency of the lipoplexes prepared with sucrose as cryoprotectant, the activity remained at the level of the fresh formulation (27). The improvement in gene expression activity was only reported in lipoplexes co-formulated with DOPE and the increase of transfection activity of frozen formulation was justified by possible structural alteration of DNA/lipid complexes induced by the freezing stress (27). It should be noted that in both studies, no cryo- or lyo-protectant agents were employed during the freezing/drying cycles.

In the present study, improvements of transfection activity were only observed for three lyophilized formulations and only when disaccharide sugars (sucrose or trehalose) were used. The improvement can be attributed to the alteration in the supramolecular structure of the lipoplexes induced by the disaccharide sugars during the freeze-drying cycle. A number of studies have proposed different hypotheses that explain the protective mechanisms of lyo-/cryo-protectant agents during the freeze-drying process of biopharmaceutical products. These hypotheses include: preferential exclusion (53), vitrification (54), particle isolation (55) and water replacement hypotheses (56, 57). Most of the studies that employed the lyophilization technique to stabilize

chemically mediated gene delivery vectors, explained the physiochemical stabilization and any observed changes resulted from lyophilization cycles using these hypotheses (24, 29, 42). The water replacement hypothesis is a well-established hypothesis proposing that lyoprotectant sugars are able to form hydrogen bonds with the lipid phase of liposomes and replace the surrounding water molecules leading to stabilization of the structure of lipid membrane during the dehydration phase (56, 57).

Based on the water replacement hypothesis, we believe that the disaccharide sugars (sucrose and trehalose) formed a hydrogen bond with the C=O and P=O moieties of the DOPE head group, more favorably than with the nitrogen of the quaternary ammonium head group or the nitrogen atom of the spacer region of the gemini surfactant (58, 59). In addition, DOPE is more abundant on the outer surface of the lipoplexes compared to 12-7NH-12 molecules (1 to 10 molar ratio). This interaction facilitated the preservation of the original supramolecular structure of the fully hydrated system during the drying step. Additionally, the effect of replacing water molecules by sugar can explain the increase of the particle size upon lyophilization. Consequently , after rehydration of lyophilized formulations, we assume an alteration of the surface properties of $[G/L]_{lyp}$ systems occurred due to partial rehydration of the DOPE molecules imposing the formation of an inverted hexagonal phase rather than the cubic or lamellar phases (60, 61). Previous studies indicated that the [P/G/L] systems exist in a mix of polymorphic phases (i.e., lamellar, cubic and hexagonal) (8). It was established that the inverted hexagonal phase is responsible for high transfection in *in vitro* studies (62, 63). Thus, upon rehydration, the [P/G/L] system might assume more hexagonal structure rather than a polymorphic assembly. We plan to investigate the assembly of the rehydrated formulations in the future by small and wide angle X-ray scattering.

Another potential explanation for the increase in the transfection activity might be due to the stress resulting from the freezing step (i.e., cryoconcentration effect) and the interaction of sugar molecules with [G/L] lipid phase. It was established that this effect could cause fusion of the lipid bilayer membrane (64, 65). Based on this, it is possible that during the freezing cycle, free 12-7NH-12 vesicles (or free molecules) present in the fresh formulations, particularly in the case of P-$[G/L-S]_{lyp}$ and P-$[G/L-T]_{lyp}$, were incorporated in the G/L vesicles. Upon rehydration and the addition of pDNA, the lipoplexes might have formed at a higher apparent +/– charge ratio compared to fresh P-[G/L-S] and P-[G/L-T] (27). In fact, this mechanism can explain the increase in zeta potential values that are observed after lyophilization/rehydration cycle [Table 2-B].

Stability study

We evaluated the stability of four lyophilized formulations stored at 25 ˚C for three months to investigate the ability of lyophilization to improve the shelf stability of gemini surfactant lipoplexes. It was found that the ability of the formulations to preserve the transfection activity was dependent on the formulation method and the nature of the protectant sugar.

During the stability study, the lyophilized cake in all formulations maintained the original solid aspect and no shrinking or collapse of the lyophilized cakes was observed [Figure 5-B]. Additionally, the lyophilized products were reconstituted to form clear dispersions with no aggregation or large particles. All lyophilized formulations retained particle size within the original size range (100-200 nm) and positive zeta potential values throughout the three months [Figure 6]. The lyophilized formulations containing the pDNA (i.e., $[P/G/L-S]_{lyp}$ and $[P/G/L-T]_{lyp}$) were able to preserve adequate levels of gene expression up to three months of storage. Formulation $[P/G/L-S]_{lyp}$ maintained the same transfection activity of fresh formulation (time zero) for one month of storage and approximately 60% of original activity after three months of storage [Figure 8]. Formulation $[P/G/L-T]_{lyp}$,with trehalose as freeze-drying protectant agent, was able to preserve almost 70% of the gene expression activity of the fresh [P/G/L-T] formulation at the end of the study [Figure 8]. We believe that the partial loss of the transfection activity of $[P/G/L-S]_{lyp}$ and $[P/G/L-T]_{lyp}$ formulations is due to conformational changes in the lipoplex structure, particularly in the presence of sucrose cryoprotectant.

While formulations P-$[G/L-S]_{lyp}$ and P-$[G/L-T]_{lyp}$ stored at 25 ˚C were able to maintain their transfection activity for two months, during the third month both formulations lost more than 60%

of their transfection activity. As there was no difference between these formulations and the $[P/G/L\text{-}S]_{lyp}$ and $[P/G/L\text{-}T]_{lyp}$ formulations lyophilized and stored with the pDNA, we believe that the loss of the activity after three months of storage is a result of the loss of the [G/L] bilayer arrangement resulting from the freeze-drying cycle rather than DNA degradation.

The moisture content of lyophilized formulations showed an increase with time for most of the formulations upon storage [Table 3]. However, no correlations were observed between the changes in the moisture content and the physiochemical properties and biological activity of the lyophilized formulations during the stability study. In fact, Yu and co-workers, found that there is no correlation between the biological activity of lyophilized DC-Cholesterol:DOPE-based lipoplexes and the level of moisture content when samples were stored for three months at room temperature (66).

CONCLUSION

We evaluated the practicality of lyophilization to formulate cationic gemini surfactant-based lipoplexes with long-term stability at room temperature. Both trehalose and sucrose were useful as lyoprotectant agents to stabilize the physiochemical properties of lipoplexes during the freeze-drying. Substantial enhancements in transfection efficiencies of gemini surfactant/DNA lipoplexes after lyophilization of G/L systems were observed. These observations were attributed to more balanced compaction of the DNA and the possible formation of inverted hexagonal phase lipoplexes. Lyophilization appears to be acceptable as a formulation technique to prepare highly efficient gemini surfactant-based lipoplexes. The stability study at 25 ˚C showed that the lyophilized $[G/L]_{lyp}$ systems formulated with sucrose and trehalose can be stored at room temperature for up to two months without significant changes in physiochemical properties or gene expression activity. The loss of transfection activity upon storage is most probably due to the conformational changes in the supramolecular structure of the lipid phase that result during the lyophilization process.

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Appendix 1. The components of selected formulations and the influence of lyophilization on the physiochemical properties (size distribution and zeta potential).

ID#	Stabilizing agents solution used to prepare DOPE film	Fresh Formulation		Lyophilized Formulation	
		Particle Size (PDI) Nm	Zeta potential mV	Particle Size (PDI) nm	Zeta potential mV
0	No stabilizing agent	145±1 (0.11)	- 1±3	642±247 (0.71)	-11±3
1	9.25 % Sucrose	76.1±0.6 (0.19)	30±2	125±2 (0.18)	29±1
2	9.25 % Sucrose + 0.5%Glycerin	81±2 (0.19)	32±2	99±2 (0.20)	38±1
3	9.25 %Sucrose + 1%Glycerin	79 ±2 (0.19)	30±2	96±3 (0.21)	37±3
4	10 % Trehalose	97±3 (0.19)	24±1	162±9 (0.18)	24.3±0.6
5	10 %Trehalose + 0.5%Glycerin	107±2 (0.22)	28±2	109±3 (0.17)	35±3
6	10 % Trehalose + 1%Glycerin	109±3 (0.17)	17±2	126±3 (0.19)	33±1
7	9.25 % Sucrose + 0.5% Tween 80	34.6±0.7 (0.49)	3±3	100±0.5 (0.52)	2±4
8	1% Tween 80	54±1 (0.25)	- 6±4	192±47 (0.56)	- 1.9±0.2
9	5% PEG 1450	88.1±0.5 (0.29)	- 16±7	301±5 (0.43)	-23±7
10*	9.25 % Sucrose	80±0.1 (0.23)	31±1	131.6 ± 5.47 (0.23)	24.0 ± 1.0

Values are shown as the average of 4 measurements; (PDI) is indicated for size distribution measurements. Formulations (0-9) prepared with pDNA:12-7NH-12:DOPE [P/G/L] lipoplexes in the indicated stabilizing solution. * In formulation (10); pDNA:12-7NH-12 lipoplexes [P/G] lyophilized and rehydrated with DOPE suspension.