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Report

New Cationic Lipid Formulations for Gene Transfer

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Purpose. To develop appropriate dosage forms of DNA for gene delivery.

Methods. 3β[N-(N′, N′ dimethylaminoethane) carbamoyl] cholesterol (DC-Chol) was mixed either with Tween 80 alone, or with additional lipid components including castor oil and phosphatidylcholine (PC) or dioleoylphosphatidylethanolamine (DOPE) to make different lipid formulations. The particle size and the physical stability of the formulations upon mixing with plasmid DNA containing the luciferase cDNA were examined using laser light scattering measurement. The transfection activity of the DNA/lipid complexes was tested in presence or absence of serum using a cell culture system.

Results. We demonstrated that many favorable properties as a gene carrier could be achieved by formulating DNA into new dosage forms using Tween 80 as the major emulsifier. Compared to the cationic liposomes, these new formulations transfected different cell lines with an equivalent or higher efficiency. Not only are they resistant to serum, but also form stable DNA complexes which could be stored for longer periods of time without losing transfection activity.

Conclusions. Cationic lipids formulated into different lipid formulations using Tween 80 as a surfactant appeared to have more favorable physical and biological activities than traditional cationic liposomes as a carrier for gene delivery.

KEY WORDS: gene transfer; gene therapy; cationic lipid; transfection.

INTRODUCTION

Gene therapy is a new approach for the treatment of inherited and acquired diseases. It generally requires a delivery system to transfer a gene sequence into the target cells to achieve therapeutic benefits for the recipient. Two major strategies are currently used to accomplish gene delivery: one uses a virus as a vector and the other uses a non-viral vector as a carrier (1–5). The major advantage of the viral vector is its high efficiency in gene transfer. However, the disadvantage of this system relates to the concerns about its safety with respect to the possibility of recombination with endogenous virus to mutate into a deleterious infectious form. Furthermore, a virus-based delivery system induces an immune response against the intrinsic viral antigens, rendering repetitive treatments with the same delivery vehicles less likely (6). For these reasons, development of non-viral vectors has drawn increasing attention as an alternative carrier for gene delivery.

One of the most promising non-viral gene delivery systems developed so far is cationic liposomes (7 and 8 for review). Cationic liposomes employ specific types of cationic lipids as the functional component to bring DNA molecules into the cell. Many different cationic lipids have been synthesized and shown activity in delivering genes into the cells both in vitro and in vivo (7–12). Two liposome formulations containing either DC-Chol or N-[1-(2,3-dimystyloxy)propyl]-N,N-dimethyl-N-(2-hydroxyethyl)ammonium bromide (DMRIE) have been administered to patients in human clinical trials for the treatment of cancer and cystic fibrosis (11,12).

Despite the fact that many of the cationic lipids in the form of liposomes showed good gene transfer activity, one of the problems associated with such a system is the lack of physical stability of DNA/liposome complexes. Upon mixing the DNA with cationic liposomes at an optimal DNA to liposome ratios at which the maximal transfection activity occurs, large DNA/liposome complexes are formed. Although such problem can be solved by preparing the complexes at lower concentration or using only freshly prepared samples, it is a problem for in vivo studies or clinical trials where a high concentration of the complexes is usually required to accommodate the relatively large dose of DNA needed for treatment.

To overcome such a problem, we have explored the possibility of formulating DNA molecules into different lipid formulations using Tween 80 as one of the lipid components. Our approach is based on the hypothesis that by including Tween 80 into the cationic lipid formulations, the branched polyethylene glycol moieties of Tween 80 on the surface of the lipid particles will provide a steric barrier and minimize DNA-induced aggregation, therefore to provide physical stability of DNA/lipid complexes. We report here the characteristics of these new lipid formulations regarding to their transfection activity, physical stability and potential application as new lipid formulations for gene delivery.

MATERIALS AND METHODS

Materials

DC-Chol was synthesized according to Gao and Huang (13). Tween 80 and castor oil were purchased from Fisher Scientific Inc. DOPE and egg PC were obtained from Avanti Polar Lipids. pCMV-Luc, a luciferase expression plasmid vector driven by the immediate early promoter of the cytomegalovirus was constructed in Dr. Leaf Huang's laboratory. The preparation and purification of plasmid DNA were carried out according to the standard procedures (14).

Preparation of Lipid Formulations

The lipid suspensions were prepared according to previously described procedures (15). Briefly, Tween 80 diluted in chloroform was mixed with DC-Chol with or without castor oil and additional lipid components at desirable ratios. The organic solvents were evaporated under a stream of nitrogen gas. The lipid film was then vacuum desiccated at 4°C overnight to remove residual organic solvent. One ml of phosphate buf-

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ABBREVIATIONS: DC-Chol, 3β[N-(N′, N′-dimethylaminoethane)carbamoyl] cholesterol; DOPE, dioleoylphosphatidylethanolamine; FBS, fetal bovine serum; PBS, phosphate buffered saline; PC, phosphatidylcholine from egg yolk; RLU, relative light unit.

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fered saline (pH 7.4) was then added and the mixture was allowed to hydrate for 1 hour at room temperature. The lipid suspension was then mixed using a vortexer and subsequently homogenized for 3–4 min using a tissue tearer at a speed of about 20,000 rpm. The average size of the lipid particles was around 200 nm as measured by 90° laser light scattering using a Coulter N4SD submicron particle sizer (Coulter Electronic).

Preparation of DC-Chol/DOPE Liposomes

Small unilamellar liposomes of approximately 100 nm in diameter were prepared by sonication of the hydrated mixture of DC-Chol and DOPE (1:1, weight ratio) according to Gao and Huang (13).

Cell Culture and Transfection

Four cell lines that are derived from different origin were used for this study. Murine melanoma BL-6 cells were cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS). Human embryonic kidney 293 cells and murine melanoma Fo cells were cultured in DMEM medium supplemented with 10% FBS. Chinese hamster ovarian (CHO) cells were cultured in F12 medium supplemented with 10% FBS. For transfection, 5×10^4 cells per well were plated in a 48 well plate and allowed to grow for 24 hours before the transfection. The pCMV-Luc plasmid DNA was diluted in 125 μl of serum free CHO-S-SFM medium (Life Technologies, Inc.). The lipid mixtures or DC-Chol/DOPE liposomes were diluted in 125 μl of Hank's balanced salt solution (HBSS). The diluted DNA and formulations were combined and incubated at room temperature for 5–10 min. To test serum sensitivity, FBS was added to the DNA mixture with a final concentration of 20% before being added to the cells. Cells with transfection reagents were incubated for 5 hours. Transfection medium was then replaced with growth medium containing 10% FBS. Cells were cultured for an additional 2 days before the level of gene expression was determined.

Luciferase Assay

Cells were washed twice with PBS and incubated at room temperature for 10 min in the presence of 100 μl lysis buffer (0.1 M Tris-HCl/ 0.05% Triton X-100/2 mM EDTA, pH 7.8) and then centrifuged at $12,000 \times g$. Ten μl of supernatant was then taken for the luciferase assay using the luciferase assay kit (Promega) in a luminometer (AutoLumat LB953 from EG& G, Berthhold). Protein concentration in the supernatant was determined by a standard Coomassie blue assay (Pierce). The total extracted proteins from each well were used to indicate the toxic effect of the different formulations on the transfected cells.

RESULTS

Lipid Composition Dependent Transfection

Ten different formulations were tested for their transfection activity in BL-6 cells. The resulting data are presented in Table 1. It is clear from these results that all the formulations tested are active in transfecting BL-6 cells except the one without cationic lipid (#5). However, other lipid components included in the formulations significantly modulated the total transfection activity. For example, substituting DOPE by the same amount of PC in the formulation containing either castor oil/Tween 80/ DC-Chol (#1 vs #6) or castor oil/Tween 80 (#4 vs #8), the total luciferase activity obtained from the transfected cells was 2–3 fold lower. This suggested that DOPE is better than PC under the experimental conditions. Similar conclusions can also be inferred for the formulations without oil (#2 and #7). Formulations without phospholipids (#3) exhibited very high transfection activity. More importantly, substitution of DOPE by Tween 80 from the most commonly used DOPE/DC-Chol liposomes (#9) exhibited the second highest transfection activity, better than that of DOPE/DC-Chol liposomes. The total amount of proteins recovered from the cells were about the same for all formulations tested.

Transfection Activity Depends on the Ratio of DNA to Cationic Lipid

To further characterize these new formulations, four formulations including #1 (Castor Oil/PC/Tween 80/DC-Chol), #3 (Castor Oil/Tween 80/DC-Chol), #6 (Castor Oil/DOPE/Tween 80/DC-Chol) and #10 (Tween 80/DC-Chol) were selected and tested for their transfection activity at various conditions. Figure 1 shows that the maximal transfection activity for all four formulations was obtained at a DNA to DC-Chol ratio of 1:6 (w/w). The highest luciferase activity ($\sim 3.5 \times 10^8$ RLU/well) was obtained from cells transfected with formulation #6, followed by formulation #10 (2.2×10^8 RLU/well). Formulations #1 and #3 showed similar transfection activity with luciferase activity being 1.3×10^8 RLU/well at the optimal DNA to DC-Chol ratio. To confirm such an optimal ratio, a series of transfection experiments was performed in which the amount of plasmid DNA used was fixed (2 μg) while the total amount of formulation was varied from 4 (3 μg DC-Chol) to 28 μl (21 μg DC-Chol). As shown in Figure 2, except formulation #3, the optimal transfection activities were seen at DNA to DC-Chol ratio of 1:6 (w/w). The DNA to DC-Chol ratio that exhibited the optimal transfection activity for #3 was about 1:7.5 (w/w). The results shown in Figure 2 and those in Figure 1 suggest that the optimal DNA to DC-Chol ratio for transfection activity was about 1:6–1:8 by weight.

Transfection Activity in the Presence of Serum and in Different Cell Lines

All of the above described transfection experiments were carried out in a serum-free medium using BL-6 cells. To determine if these new formulations exhibited the same transfection activity in different cell lines and whether their transfection activity is sensitive to serum, we have selected three additional cell lines and tested the transfection activity of the four selected formulations. Fo cells, CHO cells and 293 cells were transfected at an optimal DNA to DC-Chol ratio, as obtained from BL-6 cells, and the luciferase activity was compared among different cell lines with or without serum. Results of these experiments are shown in Table 2. It is evident that all three cell lines showed a high level of gene expression, ranging from about 4×10^6 to 10^9 RLU per about 10^6 cells transfected. The 293 cells exhibited the highest level of gene expression in comparison to those of Fo and CHO cells, indicating the difference in transfection activity of each formulation for the different cell lines.

Table I. Effect of Lipid Composition on Transfection Activity[a]

Formulation Number	Composition (mg)					RLU/Well $\times 10^{-7}$	Total Proteins (μg/well)
	Castor Oil	PC	DOPE	Tween 80	DC-Chol		
1	0.25	0.25	—	0.125	0.75	15.9 ± 2.5	57 ± 1
2	—	0.25	—	0.125	0.75	3.2 ± 0.6	62 ± 1
3	0.25	—	—	0.125	0.75	17 ± 0.7	48 ± 2
4	0.25	0.25	—	—	0.75	2.4 ± 0.1	62 ± 3
5	0.25	0.25	—	0.125	—	0	61 ± 1
6	0.25	—	0.25	0.125	0.75	36.3 ± 3.2	55 ± 4
7	—	—	0.25	0.125	0.75	14.0 ± 0.5	61 ± 2
8	0.25	—	0.25	—	0.75	4.0 ± 0.5	59 ± 1
9[b]	—	—	0.6	—	0.6	10.7 ± 2.1	58 ± 3
10	—	—	—	0.25	0.75	25.9 ± 1.2	54 ± 4

[a] Lipid components were mixed with the indicated amounts and formulations were prepared in 1 ml of PBS (pH 7.4). Transfections in BL-6 cells were performed using 2 μg of pCVM-Luc and 16 μl of each formulation, according to the procedure described in the method section. Ten μl of cell extracts from transfected cells were used in the assay for the luciferase activity. The level of enzyme activity in each well is presented as RLU for the total amount of extracted proteins.

[b] This is the DC-Chol/DOPE liposome formulation.

Importantly, the level of gene expression obtained in the presence of serum was either at a similar level or higher. In the presence of serum, the highest increase (~19 fold) in luciferase activity was obtained when formulation #6 was used with CHO cells. Among the four formulations tested, formulation #10 appeared to be the most active in transfection.

Physical and Biological Activity of DNA/Lipid Complexes as a Function of Storage Time

To our knowledge, almost all cationic liposome-based transfection reagents are in a two vial formulation. DNA and cationic liposomes are usually mixed right before the transfection experiment, due to the formation of large aggregates upon mixing the negatively charged DNA and positively charged liposomes. As can be seen in Figure 3, these new formulations behave quite differently. First of all, formulations #1 and #10 formed relatively small complexes with DNA; the average particle size of the complexes, as measured by laser light scattering, ranged from 200–400 nm, and remained small even after 10 days at 4°C. Formulations #6 and #3, on the other hand, formed larger complexes with DNA, being 600 and 900 nm in diameter, respectively. In contrast, DC-Chol/DOPE liposomes formed large aggregates (1,800 nm) on day 1, and grew even larger

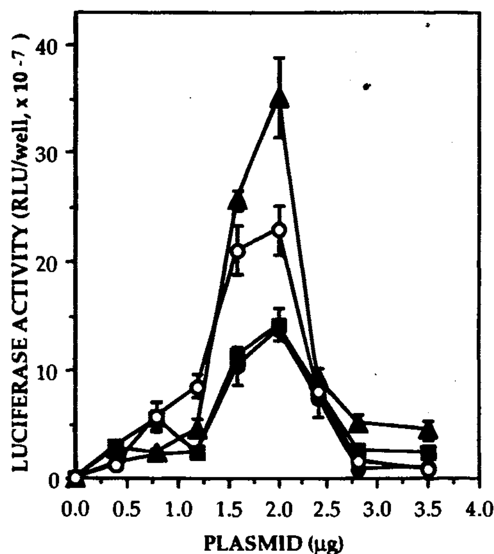

Fig. 1. Transfection activity of selected formulations as a function of DNA to DC-Chol ratio. Sixteen μl of each formulation containing 12 μg of DC-Chol were mixed with different amounts of plasmid DNA, and BL-6 cells were transfected in the absence of serum. The total luciferase activity in the extracted proteins from the transfected cells was expressed as RLU per well. Formulations are composed of Castor Oil/PC/Tween 80/DC-Chol (2:2:1:6, w/w) (#1, ●); Castor Oil/Tween 80/DC-Chol (2:1:6, w/w) (#3, ■); Castor Oil/DOPE/Tween 80/DC-Chol (2:2:1:6, w/w) (#6, ▲) and Tween 80/DC-Chol (2:6, w/w) (#10, ○). Data represent the mean (SD) (n = 3).

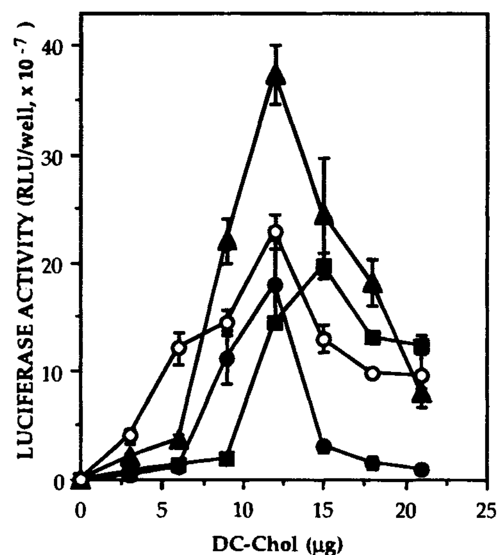

Fig. 2. Optimal DNA to DC-Chol ratios of selected formulations for transfection. The amount of DNA plasmid was fixed at 2 μg in each well. The level of gene expression in transfected BL-6 cells with different formulations was examined at various amounts of formulations. Castor Oil/PC/Tween 80/DC-Chol (2:2:1:6, w/w) (#1, ●); Castor Oil/Tween 80/DC-Chol (2:1:6, w/w) (#3, ■); Castor Oil/DOPE/Tween 80/DC-Chol (2:2:1:6, w/w) (#6, ▲) and Tween 80/DC-Chol (2:6, w/w) (#10, ○). Data represent the mean (SD) (n = 3).

Table II. Transfection Activity of Selected Formulations in Different Cell Lines[a]

Formulation[b]	Luciferase Activity (RLU/well, $\times 10^{-7}$)					
	F_0 Cells		CHO Cells		293 Cells	
	− serum	+ serum	− serum	+ serum	− serum	+ serum
#1	8.3 ± 3.7	13.2 ± 7.0	8.0 ± 0.7	5.9 ± 0.4	33.0 ± 7.8	37.0 ± 3.4
#3	2.7 ± 0.2	1.6 ± 0.9	1.6 ± 1.6	10.4 ± 1.3	33.3 ± 11.3	44.2 ± 1.2
#6	8.7 ± 2.1	12.0 ± 2.1	0.4 ± 0.0	7.5 ± 0.4	27.9 ± 7.0	34.7 ± 2.1
#10	22.0 ± 3.3	13.2 ± 1.1	0.4 ± 0.1	1.5 ± 0.3	68.0 ± 17.0	168.0 ± 17.0

[a] Conditions used were identical to those in Table 1. For serum effect, the serum concentration in the transfection reagent was 20%.
[b] For composition, see Table 1.

(>4,000 nm) on day 3 and precipitated out from the solution. Thus, these new formulations can form complexes with DNA in which the physical stability is much improved over that of the cationic liposomes.

The transfection activity of the DNA/lipids complexes, as a function of storage time, was tested in BL-6 cells. These experiments were performed in the presence of 20% serum. As seen in Figure 4, the DNA/lipid complexes for all four selected formulations showed relatively high transfection activity even 10 days after the complex formation. The level of gene expression on days 4, 7 and 10 when compared to that on day 1, formulation #6 (Castor Oil/DOPE/Tween 80/DC-Chol), appeared to have an increased transfection activity upon storage. For example, at day 1, the luciferase activity obtained was about 6×10^7 RLU/well while, after storing the complexes for 10 days at 4°C, it increased to 2×10^8 RLU/well when the same amount of DNA and formulation was used. In contrast, the transfection activity of formulation #1 appears to decrease with an increase in storage time. No obvious changes in transfection activities for formulation #3 and #10 were seen upon storage.

DISCUSSION

We have demonstrated in this paper that lipid formulations composed of DC-Chol and Tween 80 with or without additional lipid components can serve as efficient vehicles for DNA transfer. In addition to their high transfection activity in the absence of serum (Table 1), these formulations are found to be equally or more active in the presence of serum (Table 2). More importantly, these formulations form relatively small and physically stable DNA/lipids complexes and their biological transfec-

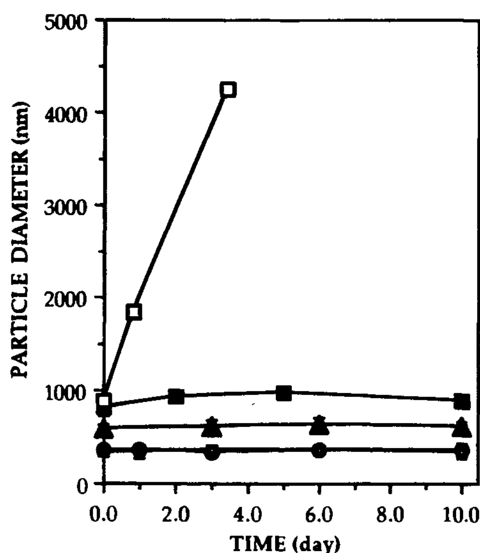

Fig. 3. Physical stability of DNA complexes with different formulations as a function of time. Sixteen μl of each formulation diluted in 125 μl of PBS and mixed with the same volume of PBS containing 2 μg of pCMV-Luc at room temperature. The average particle size of the DNA complexes with each formulation was measured using a laser light scattering particle size analyzer at different time after storage at 4°C. The temperature of the samples was equilibrated to room temperature before each measurement. Castor Oil/PC/Tween 80/DC-Chol (2:2:1:6, w/w) (#1, ●); Castor Oil/Tween 80/DC-Chol (2:1:6, w/w) (#3, ■); Castor Oil/DOPE/Tween 80/DC-Chol (2:2:1:6, w/w) (#6, ▲), Tween 80/DC-Chol (2:6, w/w) (#10, ○) and DC-Chol/DOPE liposomes (#9, ❑). Data represent the mean (SD) (n = 3).

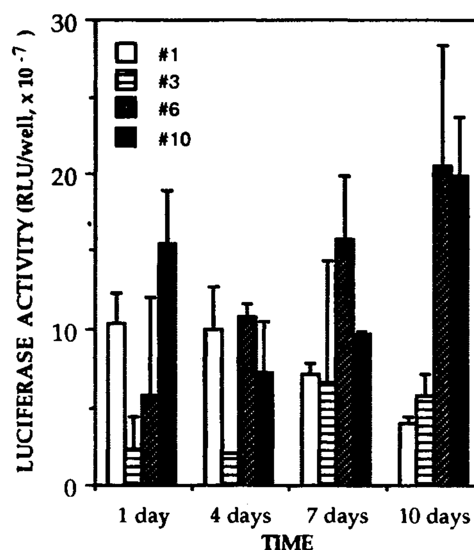

Fig. 4. Transfection activity of DNA complexes with selected formulations as a function of storage time. Transfection was performed with 2 μg of pCMV-Luc plasmid and 16 μl of the original formulations (12 μg DC-Chol) according to our standard transfection conditions (see methods for detail). The transfection was done with BL-6 cells at the same time for all of the samples and in the presence of 20% serum. Castor Oil/PC/Tween 80/DC-Chol (2:2:1:6, w/w) (#1, ❑); Castor Oil/Tween 80/DC-Chol (2:1:6, w/w) (#3, ⊟); Castor Oil/DOPE/Tween 80/DC-Chol (2:2:1:6, w/w) (#6, ▨) and Tween 80/DC-Chol (2:6, w/w) (#10, ■). Data represent the mean (SD) (n = 3).

tion activities do not change upon storage (Figure 3 and 4). These results would suggest that it is possible to prepare a "gene drug" in a single vial as a ready-to-use formulation for transfection.

One of the common ingredients used in these new formulations was Tween 80. Tween 80 is a synthetic, and one of the most commonly used, nonionic surfactants in the pharmaceutical industry. The most attractive feature of this molecule is its activity in preventing the formation of large DNA complexes with the lipid formulations. As shown in Figure 3, the average diameter of the DNA complexes with four selected formulations remained the same when stored at 4°C for 10 days. Such activity is most likely due to its branched polyethylene oxide head groups, which may form a steric barrier and prevent the DNA induced aggregation of the lipid particles.

While it is evident that these new formulations were effective in delivering genes inside the cells, the mechanisms by which the level of gene expression is regulated needs to be further investigated. It has been hypothesized that the reason that DOPE is a preferred helper lipid for cationic liposome-mediated DNA transfer, is due to its fusogenic property that facilitates the transfer of DNA from the endosome to the cytosol (7). The fact that a high transfection activity was also observed with the formulation composed of Tween 80/DC-Chol (formulation #10) indicates that Tween 80 may have a similar activity to that of DOPE. Otherwise, the mechanisms involved in the transfer of DNA molecules into cells with cationic liposomes and Tween 80 containing formulations would have to be different.

In summary, we have shown in this communication that, in addition to cationic liposomes, other types of lipid formulations can also be used to facilitate the gene transfer into the cells. These new delivery systems have many advantages over the currently used liposome formulations. First, these formulations with appropriate amounts of surfactant, such as Tween 80, showed high transfection activities which are not inhibited by serum. In fact, it was commonly observed that the transfection activity of these new formulations was higher in the presence of serum (for example, 20% FBS). Second, these formulations did not show obvious toxicity at the amounts commonly used for transfection. Third, the surfactants used in the formulations are approved by the FDA for use in humans. Being synthetic substances, they can be prepared in large quantities and in high purity. They can also be purchased from commercial sources at a relatively low price. These formulations can be easily prepared without involving the sophisticated procedures and instrumentation. Fourth, the formulations appear to be stable for storage. In the absence of DNA, no changes in physical properties (particle size) and biological activity (transfection activity) of the formulations have been observed after storage for 6 months (data not shown) at 4°C. Finally and most importantly, these formulations form a stable complex with DNA with no change of particle size and transfection activity after 10 days at 4° C (Figures 3 and 4). With these stable formulations of DNA that do not have to be prepared freshly, the gene drug can be prepared and shipped ready to use. Furthermore, because of their resistance to serum, these formulations may also serve as an effective vehicle for gene transfer in various conditions.

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