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Gene Therapy Protocols


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Non-Viral Gene Delivery with Cationic Liposome–DNA Complexes

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Summary

A large amount of research activity worldwide is currently directed towards developing lipid- or polymer-based, non-viral gene vectors for therapeutic applications. This strong interest is motivated by their low toxicity, ease of production, ability to transfer large pieces of DNA into cells, and lack of immunogenic protein components. Cationic liposomes (CLs) are one of the most powerful non-viral vectors. In fact, CL-based vectors are among the prevalent synthetic carriers of nucleic acids currently used in human clinical gene therapy trials as well as in cell transfection applications for biological research. Our understanding of the mechanisms of action of CL–DNA complexes is still in its infancy. However, the relevance of a few crucial parameters, such as the lipid/DNA charge ratio (ρ_{chg}) and the membrane charge density of lamellar complexes (σ_{M}), is well established. To arrive at true comparisons of lipid performance, one must optimize both these parameters using a reproducible, reliable transfection assay. In this chapter, we aim to provide the reader with detailed procedures for liposome formation and transfection. It is our hope that the use of such optimized protocols will improve the comparability of transfection data obtained with novel lipids.

Key Words: Cationic liposome; cationic lipid; transfection; lipofection; non-viral; gene therapy; gene delivery.

1. Introduction

There currently is a large amount of interest in the development and improvement of new methods to deliver genes, i.e., DNA, to cells, motivated

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mainly by the promises of gene therapy. Most current methods of gene therapy start with a vector, containing the therapeutic gene, which has the ability to transfer the gene into cells. This vector is then applied in either *ex vivo* or *in vivo* transfer methods (1). In the *ex vivo* method, cells derived from patient tissue are transfected *in vitro*, possibly selected for successful gene transfer, and then returned to the patient. The *in vivo* method may involve either systemic delivery, e.g., by *i.v.* injection, or local application of the vector, e.g., by injection into tumors (2,3).

Gene delivery vectors may be divided into two major classes. Viral vectors are based on replication-deficient engineered viruses, which include retroviruses and adenoviruses among several others (4). Their main advantage is high gene transfer efficiency, both *in vitro* and *in vivo*. There are, however, safety concerns associated with the use of viral vectors, which have been highlighted by a few recent setbacks (5,6). In a prominent example, 3 of 11 patients developed a leukemia-like disease caused by insertional mutagenesis. This marred the first clinical success of gene therapy, which had managed to correct X-linked severe combined immunodeficiency (SCID-X1) with an engineered retrovirus vector (7-9).

Synthetic vectors, which are based on lipids, polymers, peptides, or combinations of these (1,10-14), are gaining importance as a safe alternative to viral vectors (15). They have other advantages as well: their preparation is facile and straightforward and several parameters can be tuned to optimize transfection results; they need not contain immuno-stimulating peptide or protein components; last but not least, they do not impose a size limit on their genetic cargo. In fact, while viral vectors have a maximum carrying capacity of about 40,000 base pairs, cationic liposome (CL) vectors have successfully been employed to deliver human artificial chromosomes, with a size between 6 and 10 million base pairs, to mammalian cells (16). Currently, more than 20% of open clinical trials of gene therapy worldwide use synthetic vectors, with CL carriers employed in about one-third of those trials (17).

1.1. Liposomes

Liposomes or vesicles are closed shells of lipid bilayers, which may form spontaneously when a lipid film is exposed to water (18,19). The liposomes used for gene delivery typically contain at least two types of lipid, one cationic and one neutral. Small liposomes of a fairly uniform size distribution are the preferred starting material for the preparation of CL-DNA complexes. These small liposomes are typically prepared by hydrating a lipid film and treating the resulting aqueous solution (which will contain a variety of structures, including large and small liposomes as well as multilamellar vesicles) with ultrasound or extruding it several times through filters with a small (200 nm) pore size. For

effective liposome formation, the temperature at which hydration of the lipid film is performed needs to exceed the lipid's gel-to-liquid-crystal transition temperature, T_C . Above its T_C , the hydrophobic chains of a lipid are in a liquid rather than in an ordered state, resulting in fluid, flexible membranes.

1.2. Structures of CL-DNA Complexes

The first application of CLs as gene vectors resulted from a landmark study by Felgner and collaborators (20). It was originally thought that DNA would simply wrap around CLs to form the complexes. In reality, the equilibrium structures of CL-DNA complexes result after major rearrangements, as determined by synchrotron X-ray diffraction. To date, three equilibrium structures of CL-DNA complexes have been discovered. These are shown in Fig. 1.

The lamellar (L_α^C) phase of CL-DNA complexes is the most widely found. It consists of stacked lipid bilayers with DNA intercalated in between (21-24). As an example, lipid mixtures of DOTAP (2,3-dioleoyl-1-trimethylammonium propane, a monovalent cationic lipid) and neutral DOPC (1,2-dioleoyl-*sn*-glycero-phosphatidylcholine) form lamellar DNA complexes throughout the entire composition range (DOTAP mole fraction).

The commonly used neutral co-lipid DOPE (1,2-dioleoyl-*sn*-glycero-phosphatidylethanolamine) can lead to the formation of a different structure, which is shown in Fig. 1 (middle) (25). In this inverted hexagonal (H_{II}^C) phase, the DNA molecules are enveloped in inverse lipid micelles and assembled on a

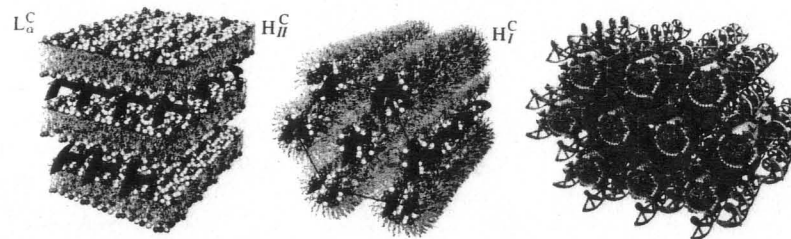


Fig. 1. The three known equilibrium structures of cationic liposome (CL)-DNA complexes. (Left) The lamellar, L_α^C , phase of CL-DNA complexes with alternating lipid bilayers and DNA monolayers. (Middle) The inverted hexagonal, H_{II}^C , phase of CL-DNA complexes with DNA chains coated by inverse micelles and arranged on a hexagonal lattice. Adapted from ref. (32). (Right) In the hexagonal, H_I^C , phase of CL-DNA complexes, cylindrical lipid micelles are assembled into a hexagonal lattice, with DNA arranged in the interstices and forming a honeycomb lattice. Schematics of the L_α^C and H_{II}^C phases reprinted with permission from ref. (25). Schematic of the H_I^C phase reprinted with permission from ref. (26). Copyright 2006 American Chemical Society.

hexagonal lattice. The main reason for the formation of this different structure is that DOPE is a cone-shaped (rather than cylinder-shaped) lipid, which confers a negative spontaneous curvature to membranes.

Very recently, we have shown that a novel cationic lipid with a large, highly charged headgroup (16+), termed MVLBG2, gives rise to a third structure of CL-DNA complexes when combined with DOPC. In this structure, named H_1^c , cylindrical lipid micelles are assembled into a hexagonal lattice, with DNA arranged in the interstices to form a honeycomb lattice (26).

1.3. Important Parameters of CL-DNA Complexes

CL-DNA complexes spontaneously form when solutions of DNA and small CLs (of 50–100 nm diameter) are mixed. This self-assembly process is driven by entropy gained from the release of counterions, which were tightly bound to the highly charged DNA and liposomes (27,28), into solution, as the charges on DNA and liposomes compensate each other (29,30).

A few compositional parameters of CL-DNA complexes of established importance affect their transfection efficiency (TE; a measure of the vector's ability to deliver the gene into cells) strongly. One of these is the lipid/DNA charge ratio (ρ_{chg}) (31). For all lipids investigated in our laboratory to date, TE increases with ρ_{chg} up to a saturation value; this behavior is independent from the ratio of cationic to neutral lipid in the membrane. The investigated lipids cover headgroup charges from +1 to +16 and varied headgroup structures. The onset of the saturation depends on the cationic lipid. For example, as shown in Fig. 2, $\rho_{\text{chg}} = 3$ lies in the saturated regime for DOTAP, whereas a group of recently synthesized dendritic lipids required at least $\rho_{\text{chg}} = 4.5$ (32). Fig. 2 displays the transfection efficiencies of complexes with 60 mol% cationic lipid for DOTAP (+1), MVLG2 (+4), MVLBG1 (+8), and MVLBG2 (+16) at various values of ρ_{chg} .

A second key parameter affecting TE of CL-DNA complexes is the membrane charge density (σ_M), which varies with the ratio of cationic and neutral lipid in the membrane. The membrane charge density provides a lipid-independent measure of how cationic a membrane is, because it is defined simply as the cationic charge per unit area. For example, two membranes, each containing the same molar fraction of a cationic lipid, may exhibit very different values of σ_M if the two cationic lipids carry a different charge (assuming their headgroup areas are the same). At the same time, σ_M of two membranes containing very different molar fractions of cationic lipid may be similar, if the lipids bear very different charges. To calculate the membrane charge density, one needs to know the effective charge of the cationic lipid in DNA complexation [which can be obtained using an ethidium bromide based assay (33)] and the lipid's headgroup area, which we use as a fitting parameter.

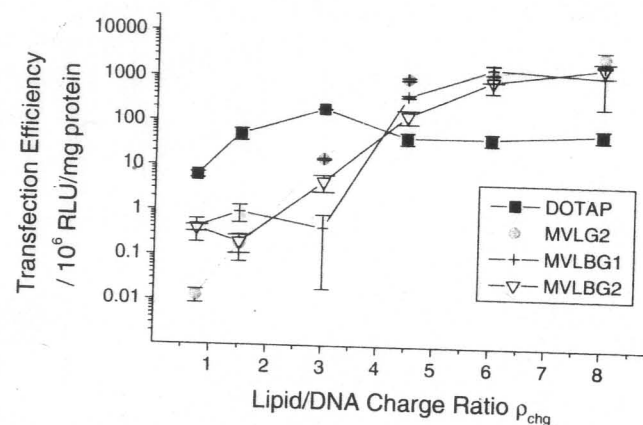


Fig. 2. Transfection efficiency (TE), determined according to the protocol described below, of DNA complexes containing 60 mol% cationic lipid at various lipid/DNA charge ratios for DOTAP (+1), MVLG2 (+4), MVLBG1 (+8), and MVLBG2 (+16). The amount of DNA is constant for all data points. Reprinted with permission from ref. (32). Copyright 2006 American Chemical Society.

The remarkable usefulness of the parameter σ_M is illustrated in Fig. 3. The plot on the left shows TE for DNA complexes of several lipids, with headgroup charges ranging from +1 to +5, as a function of the cationic lipid/DOPC molar ratio (33,34). The amount of DNA was kept constant for all data points. All cationic lipids exhibit a maximum in TE as a function of lipid composition: at 65 mol% for MVL2 (+2), 70 mol% for MVL3 (+3), 50 mol% for MVL5 (+5), 55 mol% for TMVL5 (+5), and 90 mol% for DOTAP (+1). This result is of note with a view to literature results which often only compare one or two ratios of cationic and neutral lipid: although the optimized TE is similar for all lipids, this TE appears at different molar ratios. Thus, testing only a few ratios is inadequate to fully assess the potential of a new lipid. The optimal molar ratios result in a TE up to three orders of magnitude larger than that of complexes that transfect poorly.

The plot on the right of Fig. 3 shows the same TE data, now plotted versus σ_M . Remarkably, a notable simplification takes place and all the data points merge onto a single curve. This demonstrates that the membrane charge density is a universal parameter and a predictor of TE for lamellar (L_α^c) CL-DNA complexes. The resulting universal curve reveals an optimal charge density of $\sigma_M^* = 17.0 \pm 0.1 \times 10^{-3}$ ($e/\text{\AA}^2$) (33).

X-ray diffraction shows that DOTAP as well as the MVLs form lamellar (L_α^c) cationic lipid/DOPC-DNA complexes. Notably, the TE of DOTAP/DOPE-

containing complexes, which exhibit the H_{II}^C phase in the low- σ_M region labeled Regime I in Fig. 3, is independent of σ_M . This deviation from the universal curve is indicative of a distinctly different transfection mechanism for the inverted hexagonal phase, which has also been confirmed by other methods (35).

Considering the data for DOTAP/DOPE–DNA complexes, it may be tempting to conclude that DOPE is a generally better choice of co-lipid which eliminates the need for optimizing σ_M . However, independence of TE from σ_M is a property of complexes in the H_{II}^C phase, not of DOPE-containing complexes. New lipids, and in particular multivalent lipids with their larger headgroups (which can even be large enough to impose the H_I^C structure in mixtures with DOPC), may result in lamellar rather than inverted hexagonal DNA complexes when mixed with DOPE. Even with DOTAP, complexes containing larger fractions of cationic lipid exhibit the lamellar phase. Furthermore, DOPE has turned out to be unsuitable for in vivo applications where cholesterol, which promotes lamellar complexes, has gained importance. It is thus important to dispel the widespread belief that complexes containing DOPE rather than DOPC as the neutral lipid are always higher transfecting (36–38). We have repeatedly shown that this assessment is not true if lipid composition is optimized, at which point DOPC-containing (lamellar, L_α^C) complexes transfect as well as the best DOPE-containing (H_{II}^C) complexes (33–35).

The mechanistic implications of the data shown in Fig. 3 and detailed mechanisms for transfection with both lamellar and inverted hexagonal CL–DNA

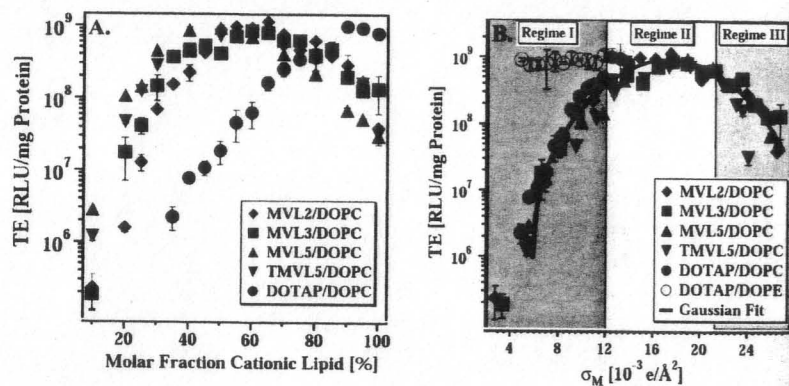


Fig. 3. (A) Transfection efficiency (TE) as a function of molar fraction of cationic lipid for cationic liposome (CL)/DOPC–DNA complexes prepared with MVL2 (+2, diamonds), MVL3 (+3, squares), MVL5 (+5, triangles), TMVL5 (+5, inverted triangles), and DOTAP (+1, circles). (B) The same TE data plotted against the membrane charge density, σ_M . The solid line is a Gaussian fit to the data. Data for DOTAP/DOPE complexes (open circles, H_{II}^C phase) are also shown. Reprinted with permission by John Wiley & Sons, Inc. from ref. (33).

complexes have been discussed elsewhere (33,35,39–41). In practice, to arrive at true comparisons of lipid performance, one must optimize crucial parameters such as ρ_{chg} and σ_M for each lipid using a reproducible, reliable transfection assay. In the protocols below, we aim to provide the reader with detailed procedures for liposome formation and cell transfection. We expect from the reader a general knowledge and competence in the culture of adherent cell lines as described, e.g., in ref. (42). We hope that these optimized protocols will help yield transfection data for novel lipids that allow for true comparisons of different lipids and formulations.

In the following, we detail the liposome preparation and cell transfection protocol that has evolved in our group. Lipids are mixed in chloroform solution for homogenous mixing, dried to prepare a lipid film, and subsequently hydrated and sonicated to form small liposomes. These are combined with plasmid DNA encoding luciferase and the resulting complexes transferred onto cells. After incubation, cells are harvested and the amount of expressed luciferase protein is measured. Luciferase, the protein generating the firefly's bioluminescence, allows measurement of the expressed protein levels by means of a light-emitting assay, providing for a very large dynamic range.

2. Materials

2.1. Liposome Preparation

1. Small glass vials (2 ml or 4 ml; Fisher Scientific, Pittsburgh, PA, USA) (see Notes 1 and 2).
2. Larger glass vials or measuring flasks for solvent stock solutions (Fisher) (see Note 2).
3. Plastic syringes and Teflon membrane filters (0.2 μm , Whatman, Florham Park, NJ, USA).
4. Low-conductivity water (18 M Ω /cm; from Millipore, Billerica, MA, USA).
5. Neutral (e.g., DOPC or DOPE) and cationic (e.g., DOTAP) lipid(s) (Avanti Polar Lipids, Alabaster, AL, USA). Store at -20°C . (see Note 3).
6. Chloroform and methanol (ACS grade, from EM Science, Gibbstown, NJ, USA). Inhalation, ingestion and skin contact should be avoided. They should be handled in a fume hood.
7. Ultrasound generator (e.g., Vibracell from Sonics & Materials, Newtown, CT, USA).
8. Incubator (at 37°C).

2.2. Cell Transfection

1. 150-mm Cell culture dish.
2. 1.5-ml Centrifuge tubes ("Eppendorf tubes").
3. 50-ml Centrifuge tubes.

4. Hemocytometer (Sigma, St. Louis, MO, USA).
5. 24-well culture plates (2 cm²/well).
6. pGL3 Control Vector (Firefly Luciferase) Plasmid DNA (Promega, Madison, WI, USA) (*see Note 4*).
7. L-Cells (mouse fibroblast cells; from ATCC, Manassas, VA, USA; ATCC number: CCL-1) (*see Note 5*).
8. Serum-free medium: DMEM (Dulbecco's Modified Eagle's Medium; Invitrogen, Carlsbad, CA, USA).
9. Supplemented medium: 5% Fetal Bovine Serum (Invitrogen) in DMEM.
10. 1× Phosphate-buffered saline (PBS; Invitrogen).
11. Trypsin buffer (or TrypLE; both Invitrogen).
12. Liposome solutions (prepared as described in **Subheading 3.1.**) (*see Note 6*).
13. Passive Lysis Buffer (5×; Promega).
14. Luciferase Assay System (Promega).
15. Protein concentration assay reagent (BioRad, Hercules, CA, USA).

3. Methods

3.1. Liposome Preparation

1. For each neutral and cationic lipid, weigh out an appropriate amount of lipid in a larger glass vial (*see Note 7*).
2. Dissolve each lipid in chloroform at a concentration of 0.6 mM (*see Notes 8–11*).
3. In small glass vials, combine the appropriate volumes of organic solutions of neutral and cationic lipid(s) to make the desired lipid mixtures (*see Notes 12 and 13*).
4. Remove the solvent, using a stream of dry nitrogen gas in a fume hood (*see Note 14*).
5. Once this has yielded a film on the surface of the vial and no visible liquid remains, place the vial in a desiccator attached to an oil pump and incubate in a vacuum overnight (*see Note 15*).
6. To the dried lipid films, add the amounts of water required to prepare a solution of the desired concentration. The typical concentration of liposomes for transfection purposes is 0.6 mM.
7. Close the vials tightly and incubate them at 37°C (or above T_C of the used lipid) for 4–8 h. Longer times (8 h) are usually advantageous for facile liposome formation, but ester bonds in the lipids may be hydrolyzed upon more prolonged incubation in water, especially at elevated temperature (*see Note 3*).
8. Place the vials in a water bath (*see Note 16*) and tip-sonicate the lipid solution for 10 min or until clear (*see Notes 17–20*).
9. Aspirate the solution with a syringe and transfer it to a new container (glass vial or 1.5 ml centrifuge tube) by passing the solution through a filter (0.2 μm pores) (*see Note 21*).
10. Store the resulting liposome solutions at 4°C (*see Note 6*).

3.2. Cell Transfection

3.2.1. Day 1: Preparing Cells in 24-Well Plates

Starting with cells that are almost confluent on a 150-mm dish, release the adherent cells and reseed them in a 24-well plate:

1. Aspirate the medium from a 150-mm dish containing an almost confluent layer of cells (*see Note 22*).
2. Wash with 1× PBS and aspirate PBS (*see Note 23*).
3. Briefly coat the cells with 2 ml of trypsin buffer (*see Note 24*) and incubate the dish at 37°C for no more than 5 min.
4. Transfer the cells to a 50-ml centrifuge tube using 15 ml of supplemented DMEM (*see Note 25*).
5. Determine the cell density using a hemocytometer (*see Note 26*).
6. Add 500 μl of supplemented DMEM to each well of a 24-well plate that is to be used.
7. Calculate the required volume of cell suspension (*see Note 27*) and seed the 24-well plate(s) at 85,000 cells per well, ensuring an even distribution of cells in the well (*see Note 28*).
8. Several plates may be seeded at a given time. The plates are then incubated under the conditions used for maintenance of the cells for about 20 h (*see Note 29*).

3.2.2. Day 2: Transfection

1. Check the cells in the 24-well plates (*see Note 30*).
2. Calculate the volumes of liposome stock solutions required for all values of ρ_{chg} and σ_M to be investigated, starting from a constant amount of 1 μg pGL3 DNA (*see Note 31*). Add the lipid solution(s) to 1.5-ml centrifuge tubes (*see Note 32*).
3. Prepare the required amount of a solution of pGL3 plasmid DNA at 1 μg/250 μl in serum-free DMEM (*see Note 33*).
4. Dilute the liposome solutions to 250 μl with serum-free DMEM. After the lipid samples have been incubated for 5 min (never longer than 30 min!) (*see Note 34*), add 250 μl of the prepared DNA solution. Invert the closed tube 10 times to mix and centrifuge at 3000 rpm (604 g) for 30 s to collect all the solution in the bottom of the tube.
5. Incubate the samples at room temperature for 30 min to allow the CL-DNA complexes to form.
6. Remove a 24-well plate from the incubator. Processing 12 wells at a time, aspirate the medium and wash the cells once with 1× PBS (*see Notes 22, 23, and 35*).
7. After gently mixing the CL-DNA complex solution by repeatedly filling and emptying the pipette, add 200 μl to each well (*see Note 36*).
8. Repeat **steps 6 and 7** for more wells as desired.

9. Incubate the cells with the complex solution for 6 h under the conditions used for their maintenance (see Note 37). The plates should be left undisturbed during this time (see Note 38).
10. Processing 12 or 24 wells at a time, aspirate the complex solution, wash the cells once with 1× PBS, and add 500 µl of supplemented medium (see Note 35).
11. Incubate the cells for 20 h, i.e., the time required for one full cell cycle to occur, under the conditions used for their maintenance (see Note 39).

3.2.3. Day 3: Harvesting of Cells; Determining Luciferase and Total Protein Content

1. Freshly prepare a sufficient amount of 1× Passive lysis buffer (PLB): 150 µl of PLB for each well (see Note 33).
2. After aspirating the medium, wash the cells once with 1× PBS. Then add 150 µl of PLB to each well (see Note 40). After processing all wells, place the plates in a freezer overnight.
3. Thaw the plates for 30–45 min (see Note 41).
4. Completely transfer the content of each well into a 1.5-ml tube and centrifuge for 5 min at 5000 rpm (1677 g) (see Note 42).
5. Add 20 µl of the supernatant to a 12×75 mm disposable glass culture tube (see Note 43). Place the tubes in the luminometer and collect the light output readings using the luciferase assay.
6. Using a ultraviolet visible (UV/VIS) spectrophotometer, determine the protein concentration of the supernatant with the protein assay reagent as described by the reagent manufacturer (see Note 44). The light output readings from the luciferase assay are normalized with this number. The total cellular protein content is also a crude indicator of cell viability.

4. Notes

1. The desired final volume of liposome solution determines the size of the vial. In order for sonication of the liposome solution to be possible, a 4-ml vial needs to contain at least 200 µl of solution, whereas only about 50 µl are required in a 2-ml vial. These numbers should be confirmed by testing with plain water. Large volumes of liposome solutions can be prepared using round-bottom flasks and a rotary evaporator.
2. The vials should have Teflon-lined screw cap closures to minimize solvent evaporation.
3. Common lipids such as DOPC, DOPE, or DOTAP are available as powders or convenient chloroform solutions. They need to be warmed to room temperature before weighing; otherwise, water will condense on the lipid and in the container.
4. We propagate this plasmid in *Escherichia coli* as prescribed by Promega and purify it using a Qiagen (Valencia, CA, USA) Giga kit (following the directions for a "high yield" plasmid). The resulting aqueous solution may be stored for

- months in a refrigerator and for years at -20°C . The DNA concentration is measured spectrophotometrically at 260 nm (an absorbance of 1 at a path length of 1 cm corresponds to 50 µg/ml DNA).
5. For reliable assessment of the usefulness of a (new) lipid, it is important to test it in several different cell lines, e.g., originating from different species. Examples of commonly used cell lines are COS-1, HeLa, NIH-3T3.
 6. Lipids with ester bonds are subject to hydrolysis upon prolonged storage in aqueous suspension. Therefore, liposome solutions should be used within 1–2 months. They should be re-sonicated before use if stored for over a week or if opaque.
 7. Lipid powders can be hygroscopic and/or glue-like and thus tricky to weigh out. A method that has worked well in our laboratory is to use two spatulas: the first (with a wide blade) to remove some lipid from its container, and the second (thin blade, narrow tip) to transfer the lipid from the first spatula into the vial. Dissolving the lipid is facilitated by lightly depositing the lipid rather than smearing it onto the wall of the vial. Hygroscopic lipids need to be handled speedily or under a nitrogen atmosphere. In this case, it is imperative to adjust the concentration by the amount of solvent that is added rather than by weighing out a specific amount of lipid.
 8. The exact concentration of lipid is not crucial at this step, but should be at or above that of the desired concentration of the liposome solution (0.6 mM), so that the entire lipid film (see steps 4 and 5) will be wetted. Having identical molar concentrations for all lipids greatly facilitates the calculations for making lipid mixtures.
 9. Chloroform/methanol mixtures may be required to dissolve lipids with higher headgroup charge or Poly(ethylene glycol) (PEG) chains (2:1, 4:1, 9:1 are typical ratios). In this case, it is best to stepwise add the solvents, starting with part of the calculated amount of methanol, then adding part of the calculated amount of chloroform, etc. This allows an empirical determination of the optimal solvent composition while the solution is being prepared. Obviously, it is crucial to keep track of the total amount of solvent added.
 10. If feasible, organic solvents (especially chloroform) should be dispensed only using glass pipettes. If pipettes with plastic tips (only polypropylene should be considered) are used, the time that the solvent is in contact with the plastic must be minimized. In addition, it is crucial to fill and empty the tip once or twice before aspirating the amount to be measured. Otherwise, the vapor pressure of the solvent pushes part of it out of the tip.
 11. The chloroform or chloroform/methanol solutions may be stored at -20°C under argon in glass (never plastic) containers with Teflon-lined closure. Marking the level of liquid on the container is advisable, because solvent evaporation is the main reason for the limited shelf life (1–2 months) of the solutions.
 12. It may not be necessary to prepare liposome solutions for all desired ratios of neutral/cationic lipid. Mixing of two liposome solutions to achieve an intermediate concentration just prior to addition to DNA may be successful. However,

- this has first to be tested (i.e., must give same results, in structural characterization as well as in transfection) for a given lipid combination by preparing the desired concentrations both ways. For example, we have successfully prepared samples in the DOTAP/DOPC system covering the whole composition range in 5% or 10% steps using only liposome stock solutions of 100, 70, 40, and 10 or 100, 80, 60, 40, and 10 mol% DOPC.
13. These calculations are most conveniently performed and recorded using a spreadsheet program.
 14. A stream of dry nitrogen is directed into the vial(s) via Pasteur pipettes or plastic pipette tips of appropriate size. If preparing a series of lipid compositions, constructing a setup with multiple outlets is helpful. Care must be taken to adjust the speed of the nitrogen stream so it does not spill the solution. It also must not be too gentle a stream, which would lead to a thick film with little surface area at the bottom of the vial. To achieve the goal of forming a thin film, one may have to adjust the position of the vial with respect to the pipette over time.
 15. The vials may remain open at this stage or the caps placed very loosely on the vials.
 16. The water bath prevents heat buildup in the vial. The vial has to be secured very tightly to prevent it from falling into the water bath because of vibrations generated by the sonication.
 17. The sonicator tip has to be submerged in the lipid suspension. It should be as close to the meniscus of the suspension as possible for optimum performance but must not touch the wall of the glass vial. The amplitude of the ultrasound should be increased slowly and maintained at the highest level possible without causing the suspension to splash and spill. The sonicator tip should be cleaned with ethanol before processing the next sample.
 18. If the sonicator tip touches the wall of the vial, the glass may break. Because movement may occur during sonication, it is best to monitor the process. The tip touching the glass wall is usually apparent from a change in pitch of the sound generated by sonication.
 19. The noise pollution due to sonication can be minimized by encasing the whole setup in a cupboard or box.
 20. Alternate methods for the preparation of small liposomes are using a high-power ultrasound bath and extrusion (which is easily scaled up). For more information, see, for example, the websites of Avanti Polar Lipids (<http://www.avantilipids.com>) and Northern Lipids Inc. (<http://www.northernlipids.com>).
 21. This step sterilizes the solution and removes debris stemming from the sonicator tip. Very small amounts of lipid solution may be transferred to 1.5-ml tubes and briefly centrifuged to collect the debris at the bottom of the tube.
 22. We use a setup with a large washing flask connected to a water aspirator. The waste needs to be autoclaved before it can be disposed of. (b) Cells should never be allowed to reach confluency for the sake of reproducibility of the data.

23. All media and solutions that are applied to the cells need to first be warmed to room temperature or 37 °C in a water bath.
24. After adding the trypsin buffer to the cells and rocking the dish back and forth to cover the cells, immediately aspirate the trypsin buffer to minimize the amount that will remain on the cells.
25. Tilting the cell culture dish after removing it from the incubator will indicate whether the cells have successfully been dislodged from the surface (a slightly opaque film should slowly slide to the bottom of the flask). Using a pipette, the added DMEM should be aspirated and re-added to the flask a few times to completely remove the cells from the surface and ensure a homogeneous mixture.
26. Directions for using the hemocytometer: Pipet 20 μ l from a 50- μ l aliquot of the cell suspension onto the hemocytometer (between coverslip and grid). Count the number of cells in each of the nine large squares of the cytometer and take the average of that number. The result, multiplied by 10,000, gives the number of cells per ml of the suspension.
27. A typical numerical example: to yield 85,000 cells (n) per well, 56.7 μ l (V) of a cell suspension with 1.5×10^6 cells/ml (c) needs to be added to each well: $V = n/c$.
28. It is good practice to gently agitate the cell stock about every 12 wells to prevent the cells from settling in the centrifuge tube. To achieve even distribution of cells, one should circle the pipette over the well while the cell suspension is added. More importantly, once cells have been added to half or all of the plate, the plate is rocked first back and forth and then side to side. This movement should be quick and vigorous, but not so abrupt as to spill the content of the wells. It is vital to *never* swirl the plate, even avoiding rotational movement as the plates are transferred back to the incubator.
29. Again, the cells should not be allowed to reach full confluency. For reproducibility, it is important to perform experiments at similar levels of confluency, because this parameter affects the measured TE.
30. Cells should be homogeneously distributed in each well with around 80–90% confluency. If there appears to be any problem or irregularity with the cells, it is best to discard the plates and restart the experiment by seeding new 24-well plates.
31. This description assumes that every experimental data point will be performed in duplicate. To perform triplicates, scale each lipid sample up to a final volume of 700 μ l, calculating the amount of lipid required for 1.4 μ g DNA. For each well, 200 μ l of the lipid solution will be used eventually, but preparing a slight excess is necessary to account for pipetting inaccuracies and losses. All these calculations and pipetting schemes are most conveniently performed and recorded using a spreadsheet program.
32. It is prudent practice to agitate the liposome stock solution just prior to using it to ensure a homogenous solution.

33. To account for inaccuracies and losses in pipetting, it is sensible to prepare 20% more of this solution than the amount calculated from the number of samples.
34. Because of the salt content of DMEM, aggregation/fusion of liposomes due to decreased electrostatic repulsion will set in. The exact timescale will depend on the lipid mixture, which determines the zeta potential of the liposomes. In our experience, incubating for 5 min or less has avoided aggregation-related effects (a certain incubation time is unavoidable when processing a larger number of wells). Aggregation/fusion of small liposomes in the DOTAP/DOPC system starts to be significant at times of 30 min or more.
35. The reason for only processing 12 wells at a time is to prevent the cells from drying out. An alternative method (likely more suitable when getting familiar with the procedure) is to process two (or three if performing experiments in triplicate) wells at a time, aspirating the medium, washing with PBS, aspirating the PBS, and then adding the 200 μ l of complex solution.
36. Accomplish this step as quickly and as gently as possible. When pipetting the complex solution into the well, the tip of the pipette should be in contact with the side of the well rather than the bottom of the well (the cells) to prevent dislodging cells when adding the complexes.
37. This time (the time required for an optimum amount of complexes to be taken up by the cells) was determined by a series of experiments measuring TE as a function of incubation time, where TE first increases and then saturates or decreases (due to serum starvation of the cells). A time of 6 h universally works well for common cell lines as investigated in our and other laboratories.
38. Even very small hydrodynamic forces, such as those generated by taking out a plate, looking at it, and rocking it back and forth a bit, can have a notable (favorable but irreproducible) effect on complex uptake and, thus, TE.
39. Incubating for a full cell cycle allows for the machinery of the cell to transport the transferred DNA into the nucleus. Waiting for longer than one cell cycle is not advised because it may lead to cell crowding or daughter cells that do not retain the transferred DNA.
40. It is now no longer necessary to prevent the cells from drying out by processing only a part of the wells at a time.
41. Also thaw the luciferase assay kit at this time.
42. Complete removal of the contents of the well is ensured by repeatedly (about 10 times) aspirating and releasing the content with a pipette (set to about 200 μ l volume). Centrifugation pellets insoluble cell components.
43. This applies if using a Berthold Autolumat Plus luminometer. If using a different luminometer, follow the directions supplied with the instrument and the luciferase assay.
44. This can be done, for example, by mixing 20 μ l of supernatant and 800 μ l of protein assay reagent in 1-ml disposable cuvettes. These readings can also be done more efficiently in 96-well plates on a UV/VIS plate reader using 2 μ l of the supernatant with 198 μ l of reagent.

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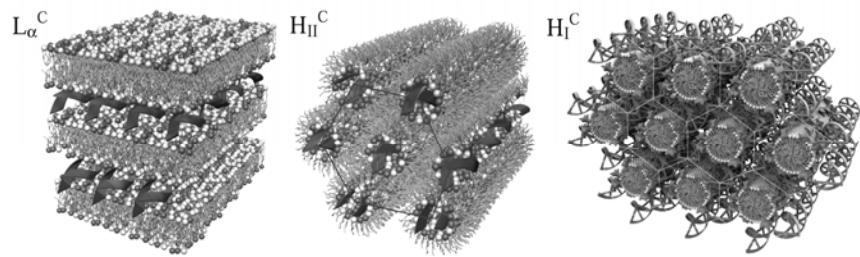


Fig. 1

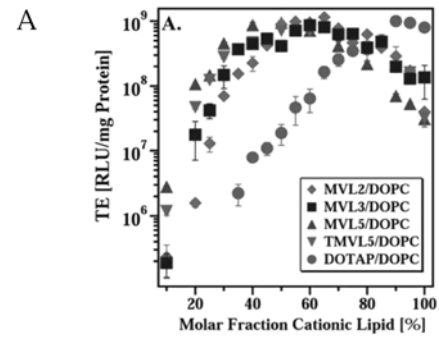


Fig. 3

