Chapter 2

Cationic Lipid-Based Nucleic Acid Vectors

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Abstract

The delivery of nucleic acids into cells remains an important laboratory cell culture technique and potential clinical therapy, based upon the initial cellular uptake, then translation into protein (in the case of DNA), or gene deletion by RNA interference (RNAi). Although viral delivery vectors are more efficient, the high production costs, limited cargo capacity, and the potential for clinical adverse events make nonviral strategies attractive. Cationic lipids are the most widely applied and studied nonviral vectors; however, much remains to be solved to overcome limitations of these systems. Advances in the field of cationic lipid-based nucleic acid (lipoplex) delivery rely upon the development of robust and reproducible lipoplex formulations, together with the use of cell culture assays. This chapter provides detailed protocols towards the formulation, delivery, and assessment of in vitro cationic lipid-based delivery of DNA.

Key words Nucleic acid delivery, Lipoplexes, Cationic lipids, Co-lipids, Particle size, Charge ratio, Transfection efficiency, Cytotoxicity

1 Introduction

The seminal work of Felgner, beginning in the late 1980s, heralded a new era in our understanding of nonviral gene delivery by employing cationic lipid-DNA complex (lipoplex) assemblies. This began with the synthesis and application of the cationic lipid *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) [1], and has continued to this day with a variety of cationic structures based on lipids [2, 3], polymers [4, 5], and dendrimers [6, 7], to name a few.

Although the use of viral vectors to deliver therapeutic genes remains the most effective approach for gene therapy, the high cost, complexity, limitations in cargo capacity, and potential for immunological complications make nonviral carriers an attractive alternative. In order to improve upon nonviral gene delivery through nanotechnology, an increasing effort has been witnessed in recent decades [8]. Comprehensive reviews detailing the breadth and scope of existing nonviral gene delivery systems are

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reported elsewhere [9, 10]. Herein, we confine our discussion to the use of cationic lipids as carriers of plasmid DNA (pDNA), considered the most promising and extensively studied vehicles for nucleic acids [11].

Despite the commercial availability of numerous cationic lipid vectors, investigations continue to explore new and novel lipid architectures [12]. The general cationic lipid design includes four common domains, a positively charged head group, a hydrophobic domain, a backbone region, and a chemical linker moiety to connect the various domains. The head group typically bears one or more cationic units, commonly amino-based moieties that facilitate binding to the genetic material. The backbone structure, often based on glycerol, influences the overall shape of the cationic lipid and thus the structure of the lipoplex. The hydrophobic domain plays a critical role in the assembly and organization of the lipoplex, and finally the chemical linker used to join these groups together is commonly an ether, ester, or amide bond.

Structural modifications within these four domains are employed to increase the cell tolerance of the cationic lipid, while at the same time enhancing the transfection efficiency. The cell tolerance, or biocompatibility of the lipids is typically addressed through the judicious choice of the chemical linker moiety such that the lipid is rendered biocompatible after successful delivery of the DNA cargo. Enhancing the transfection efficiency of a lipid-DNA formulation depends very much on the chosen route of administration—each of which presents unique barriers to be overcome [13]. As an example, for in vivo delivery using intravenous methods, the barriers to be considered when choosing an appropriate lipid design include poor circulation time due to opsonization followed by rapid clearance, a lack of cell selectivity, poor cellular uptake, endosomal escape, and nuclear entry. Generally, cationic lipid-based gene delivery is a highly inefficient and wasteful approach, and requires an improved understanding of structure efficiency relationships to achieve lower cytotoxicity together with higher transfection efficiency.

Attempts to overcome many of the barriers that have thus far impeded progress towards safe and efficient in vivo delivery of therapeutic nucleic acids, and moving beyond the discrete cationic lipid structure, include the emergence of purpose-designed lipoplex formulations of a modular nature [14–16]. Within these nanoparticle formulations, the modular components are chosen such that the nucleic acid cargo is condensed within functional concentric layers of chemical components designed for delivery into cells and intracellular trafficking, biological stability, and biological cell targeting.

Developments in lipoplex formulations and components that have moved the field towards more effective delivery include the use of neutral co-lipids such as cholesterol or 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) to facilitate membrane fusion and endosomal escape [17]. The application of polyethylene glycol (PEG) to modify the liposomal surface results in longer circulation times [18]. Additives, such as protamine or chloroquine, enhance transfection [19]. Finally, targeting ligands, such as carbohydrates [20] or folic acid [21], promote cell selectivity. Nanoparticles that include such modular components have enabled the functional in vivo delivery of nucleic acids to lung, liver, and tumors [22, 23].

Cell-based assays are employed to evaluate the relative in vitro transfection efficiency and cytotoxicity of lipoplexes based on cationic lipids. In support of this key data, additional assays and measurements determine particle size, DNA binding, and protection from degradative enzymes. Therefore, the methods employed in the lipoplex formulation, preparation, and characterization play a key role in the development of safe, efficient, and reproducible cationic lipid-based gene delivery systems.

Herein, we present materials and methods for the preparation of lipoplex formulations from pDNA and liposomes generated from thin films, together with key assay protocols employed in the evaluation of in vitro cytotoxicity and transfection efficiency. Specifically, the chapter describes lipid stock solution preparation, liposome formulation and lipoplex preparation, and the characterization of these nanoparticles based on particle size and zeta potential (ζ-potential). Furthermore, gel assay protocols are described which evaluate lipid-DNA binding and the capacity to protect the genetic material from enzymatic degradation over a range of cationic lipid:DNA molar charge ratios (CRs). Finally, protocols used to perform in vitro assays that evaluate cytotoxicity and transfection efficiency, over a range of CRs, are described. In all, this chapter presents a detailed set of protocols for the successful, reproducible preparation, characterization, and evaluation of lipoplexes, based on cationic lipids, for in vitro gene delivery.

2 Materials

The preparation of all solutions employed deionized water (dH_2O) and analytical grade reagents. Unless indicated otherwise, all solvents were obtained from Sigma–Aldrich (St. Louis, MO, USA) and all reagents were prepared and stored at room temperature (RT). Commercial assay solutions and kits are described below. When disposing waste materials, diligently follow all waste disposal regulations.

2.1 Cationic Liposome and Lipoplex Preparation and Characterization

1. Novel or commercial cationic lipid. Store as appropriate.
2. DOPE (Avanti Polar Lipids, Alabaster, USA). Store at −20 °C.
3. Cholesterol (Avanti Polar Lipids, Alabaster, USA). Store at −20 °C.
4. Dichloromethane (CH_2Cl_2).
5. Rotary evaporator.
6. Round-bottom flasks.
7. Polypropylene microcentrifuge tubes.
8. pDNA containing β-galactosidase (β-gal) gene, pCMVBeta Mammalian lacZnls12co (Marker Gene Technologies, Inc, Oregon, USA).
9. Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) for particle size determination by dynamic light scattering (DLS) and ζ-potential measurement, or Zetasizer APS (Malvern Instruments, Worcestershire, UK) for particle size determination by DLS at 25 °C.
10. Capillary cells for ζ-potential measurement.
11. Agarose.
12. Tris-borate-ethylenediaminetetraacetic acid (EDTA) buffer (TBE).
13. Ethidium bromide (EtBr).
14. 6× gel loading solution (0.25 % (w/v) bromophenol blue, 0.25 % (w/v) xylene cyanol FF, 40 % (w/v) sucrose in dH_2O).
15. 5 % (w/v) sodium dodecyl sulfate (SDS) in dH_2O.
16. Geliance 200 Gel Imaging System (PerkinElmer Life and Analytical Sciences, Shelton, CT, USA).

2.2 Cell Culture

1. Chinese hamster ovarian (CHO-K1) cell line (Health Protection Agency Culture Collections, Salisbury, UK).
2. Roswell Park Memorial Institute (RPMI) 1640 media (Gibco™ supplied by Life Technologies).
3. Phenol red-free Dulbecco's Modified Eagle Medium (DMEM) (Gibco™ supplied by Life Technologies) (*see* **Note 1**).
4. Phenol red-free Opti-MEM® reduced Serum Media (Gibco™ supplied by Life Technologies) (*see* **Note 1**).
5. Fetal calf serum (FCS).
6. 100× penicillin-streptomycin solution (10,000 U penicillin and 10 mg/mL streptomycin) (Gibco™ supplied by Life Technologies).
7. Amphotericin B.
8. Dulbecco's Phosphate Buffered Saline (PBS) (Gibco™ supplied by Life Technologies).

2.3 Transfection Experiments and Post-transfection Assays

1. BCA Protein Assay (Pierce Biotechnology, Rockford, IL, USA).
2. Beta-Glo® Assay System (Promega, Madison, WI, USA).
3. CellTiter96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA).
4. Absorbance and luminescence microplate reader.

3 Methods

The delivery of pDNA, whether it occurs via in vivo gene transfer for potential therapeutic treatments, or in vitro for cell culture applications, aims to achieve the expression of a protein that is lacking in the cells. In cell culture applications, an assessment of gene delivery efficiency depends upon the expression of the reporter gene (*see* **Note 2**) employed for this purpose and is matched to the specific application (*see* **Note 3**). This protocol describes the lipid-based delivery of a pDNA that contains a gene encoding the enzyme, β-gal. Upon successful cellular uptake, the relative efficiency of the delivery vehicle (or transfection efficiency) is assayed using 6-O-β-galactopyranosyl-luciferin, which the pDNA-derived β-gal cleaves to yield luciferin. The subsequent conversion of luciferin, mediated by luciferase in the presence of cofactors, results in the emission of light detected for quantitative analysis. The following protocols will be restricted to cationic lipid formulations that contain a single cationic lipid (*see* **Note 4**) combined in a 3:2 molar ratio with a neutral co-lipid. Finally, it is important to optimize each lipoplex formulation for each specific cell type (*see* **Note 5**). Here we outline protocols used in our laboratory that employ CHO cells (specifically, CHO-K1 cells) (*see* **Note 6**), an easy-to-transfect cell line that is the preferred choice for gene and genome-based research [24].

3.1 Lipid Ethanolic Stock Solutions

1. Dissolve a known amount of each lipid (cationic lipids and co-lipids) separately in CH_2Cl_2 in round-bottom flasks (*see* **Note 7**).
2. Remove the CH_2Cl_2, in each flask, on a rotary evaporator (*see* **Note 8**) at 35 °C bath temperature until a dry thin lipid film appears.
3. Dissolve the film in sufficient anhydrous ethanol (EtOH) to achieve a final 1 mM lipid EtOH stock solution (*see* **Note 9**). Subsequently store the solution at −80 °C until use.

3.2 Liposome Formulation from Thin Film

1. Combine 900 μL of 1 mM stock solution of cationic lipid (*see* Subheading 3.1) with 600 μL stock solution of the co-lipid in EtOH into a round-bottom flask to achieve a 3:2 molar ratio of cationic lipid to co-lipid, respectively (*see* **Note 10**).

2. Remove the organic solvent on a rotary evaporator (*see* **Note 8**) to obtain a thin film (*see* **Note 11**).
3. Dry the thin film under high vacuum for at least 2 h, or overnight, to remove all traces of organic solvent.
4. Hydrate the thin lipid film with a known amount (e.g. 450 μL) of sterile dH_2O (or Opti-MEM®) to achieve final hydrated multilamellar vesicle (liposome) stock solution with 2 mM cationic lipid concentration.
5. Upon addition of dH_2O or medium, warm the mixture above the lipid transition temperature for 15 min prior to vortexing for 30 s. Store the liposome stock solution overnight at 4 °C (*see* **Note 12**).

3.3 Liposome Particle Size Reduction

Liposome sonication, freeze-thaw, and extrusion are techniques employed, either separately or in combination, to reduce or homogenize liposome particle size. Each technique involves warming the sample above the phase transition temperature of the lipid.

3.3.1 Sonication

1. Sonicate the hydrated liposome stock solution (*see* Subheading 3.2) stored in the round-bottom flask for 30 min in a sonic bath in order to transform the multilamellar vesicles into liposomes of homogenous particle size (*see* **Note 13**).
2. Store the samples at 4 °C before use.

3.3.2 Freeze-Thaw

1. Transfer 450 μL of the multilamellar liposomes (*see* Subheading 3.2) into a 5 mL glass tube and lower it into liquid nitrogen (N_2) for rapid cooling.
2. After 2–3 min, transfer the frozen formulation to a water bath set at 60 °C and allow it to thaw for 3 min.
3. Freeze the preparation again in liquid N_2.
4. Repeat the freeze-thaw operation (above **steps 2** and **3**) ten times in total.
5. Store the samples in a refrigerator at 4 °C before use.

3.3.3 Extrusion

It is possible to extrude the multilamellar liposomes (*see* Subheading 3.2) at RT or above the phase transition temperature of the lipids (*see* **Note 14**). In the latter case, the extrusion of liposome formulations at elevated temperatures relies upon the use of the heating block provided with the extruder or a formulation preincubated in a water bath of adequate temperature.

1. Load 450 μL of the multilamellar vesicle solution into one of the two syringes and carefully place it into one end of the extruder (*see* **Notes 15** and **16**).
2. Place the second, empty syringe into the other end of the extruder.

3. Gently push the plunger of the filled syringe until the lipid solution has completely transferred into the second syringe.
4. Gently push the plunger of the second syringe until the lipid solution has completely transferred back into the original (first) syringe.
5. Repeat the above **steps 3** and **4** until the lipid solution has passed through the membrane a total of ten times or more (*see* **Notes 17** and **18**).
6. Inject the extruded lipid solution into a clean sample vial and store in a refrigerator at 4 °C before use.

3.4 Liposome Characterization

3.4.1 Particle Size

1. Transfer 450 μL of the final liposome solution into disposable square polystyrene cuvettes (dilute it to 1 mL with dH_2O) or in disposable 96-well plates (50 μL of liposome solution diluted to 100 μL with dH_2O).
2. Measure the liposome particle size (hydrodynamic diameter, dH) and polydispersity (*see* **Note 19**) by quasi-elastic DLS apparatus at 25 °C (*see* **Notes 20** and **21**).

3.4.2 ζ-Potential

1. Liposome suspensions should be prepared in 1 mM NaCl (i.e., in a low ionic strength medium).
2. Combine 350 μL of the liposome sample with 350 μL of 2 mM NaCl.
3. Transfer the sample to a 1 mL disposable syringe, dislodge any air bubbles, and insert the syringe to one of the ports on the ζ-cell.
4. Transfer the mixture into capillary ζ-cell and measure the ζ-potential at 25 °C (*see* **Notes 20** and **22**).

3.5 Preparation of Lipid/pDNA Complexes (Lipoplexes)

The formation of lipoplexes involves combining liposomes with pDNA (*see* **Note 23**).

1. Dilute 14.4 μL of pDNA (250 ng/μL in elution buffer) with 57.6 μL of Opti-MEM giving a final pDNA volume of 72 μL.
2. Dilute a set of five liposome suspensions in dH_2O such that the final concentration of net positive charge across the set is 0.5, 1.5, 3, 5, and 10 times higher than the phosphate concentration in the pDNA solution (*see* **Note 24**).
3. Combine equal volumes of the DNA solution with each of the liposome suspensions of varying concentration (*see* **Notes 25** and **26**), and then incubate at RT for 30 min to obtain lipoplex suspensions at molar CR (+/−) of 0.5, 1.5, 3, 5, and 10.
4. Take 48 μL of each lipoplex formulation for the gel assays.
5. Add 204 μL of Opti-MEM to each lipoplex formulation to reach a final volume of 300 μL/well (*see* **Note 27**).

3.6 Characterization of Lipoplexes

Measure particle size and ζ-potential of lipoplex suspensions as described above (*see* Subheading 3.4).

3.6.1 Particle Size Analysis and ζ-Potential

3.6.2 Gel Retardation Assay

Gel assays performed using lipoplexes prepared in different CRs (*see* **Notes 28** and **29**).

1. Transfer 20 μL of each lipoplex formulation into a polypropylene microcentrifuge tube.
2. Add 2 μL of 6× gel loading solution and mix.
3. Load 18 μL of each lipoplex sample onto a 1% agarose gel impregnated with EtBr and run at 105 V for 1 h in 1× TBE buffer (*see* **Note 30**).
4. Observe the pDNA bands using a gel imaging system.

3.6.3 DNase I Degradation Assay

A degradation assay characterizes the capacity of the cationic lipids to protect the genetic material from degradation by enzymes that the lipoplex could encounter outside the cells (in vivo) (*see* **Note 31**).

1. Transfer 20 μL of each lipoplex formulation into a polypropylene microcentrifuge tube, add 1 μL of DNase I solution, then incubate at 37 °C for 1 h.
2. After incubation, add 4 μL of 5% SDS solution and incubate for a further 30 min.
3. After incubation, continue as described in Subheading 3.6.2, **steps 2–4**.

3.7 Transfection Experiments and Post-transfection Assays

3.7.1 Cell Culture and Transfection Protocol

1. Grow the CHO cells in RPMI 1640 media supplemented with 10% FCS, 100 U/mL of penicillin/streptomycin, and 0.25 μg/mL amphotericin B.
2. 24 h before transfection, seed the cells onto an opaque and transparent 96-well plate at a density of (3×10^4 cells/cm^2) 1×10^4 cells per well and incubate at 37 °C in the presence of a 5% CO_2 atmosphere.
3. Once 80% confluence is reached (after approximately 24 h), remove old medium and wash cells with 100 μL of PBS.
4. Add 45 μL/well of each lipid-pDNA complex preparation at different CR (in triplicate) and incubate the plate at 37 °C in the presence of a 5% CO_2 atmosphere for 4 h.
5. After 4 h of incubation, remove the lipoplex-containing medium and wash the cells with PBS.
6. Add 100 μL/well of RPMI, then incubate at 37 °C in the presence of a 5% CO_2 atmosphere for an additional 44 h.

3.7.2 BCA Assay

1. 48 h after transfection, remove old medium and wash cells with 100 μL of PBS.
2. Add 10 μL/well of passive lysis buffer and incubate at RT for 30 min.
3. Dilute the contents of a Bovine Albumin Standard (BSA) ampule of Promega BCA kit using dH_2O to obtain serial dilutions with a range of 20–2000 μg/mL.
4. Use the calibration curve obtained from these dilutions to determine the cellular protein content per well.
5. Add 200 μL/well of BCA working solution, gently mix by pipetting, and incubate at RT for 1 h.
6. Read the absorbance at $\lambda = 562$ nm using a microplate reader.
7. Determine the cellular protein content per well by extrapolation from the standard curve.

3.7.3 β-Galactosidase Assay

1. 48 h after transfection, remove old medium and wash cells with 100 μL of PBS.
2. Add 50 μL/well of phenol red-free DMEM media, and mix thoroughly.
3. Add 50 μL/well of Beta-Glo™ working solution and mix thoroughly.
4. Incubate at RT for 1 h.
5. Read the luminescence at $\lambda = 562$ nm on a plate reader. Express β-Galactosidase activity as relative light units (RLU).
6. Normalize luminescence values with protein concentration (determined by the BCA assay described in Subheading 3.7.2) to afford RLUs/mg of proteins.

3.7.4 Cytotoxicity Assay

The cytotoxicity associated with the lipoplex formulations at CRs (+/−) ranging from 0.5 to 10 can be evaluated through the use of a standard assay. The assay described here is based on the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay [25]. Other methods for the evaluation of cell viability include the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay [26] (*see* **Note 32**) and the Alamar Blue assay [27], which employs a sensitive oxidation–reduction indicator that fluoresces and changes color upon reduction by living cells.

1. 48 h after transfection, remove old medium and wash cells with 100 μL of PBS.
2. Add 50 μL/well of phenol red-free DMEM media, and mix thoroughly.
3. Add 10 μL/well of CellTiter96® Aqueous One Solution Cell Proliferation Assay and mix by gentle rocking.

4. Incubate at 37 °C in the presence of a 5 % CO_2 atmosphere for 1 h.
5. Read the absorbance at $\lambda = 490$ nm using a microplate reader (*see* **Note 33**).
6. The percentage of viable cells is calculated from the absorbance ratio of treated to untreated cells (Cell viability (%) = [Cell viability of treated cells/Cell viability of untreated cells] × 100).

4 Notes

1. The presence of phenol red will affect the luminescent signal. Therefore, it is strongly recommended that phenol-red-free culture media be employed.
2. Commonly employed gene reporter systems include the following pDNA systems: β-gal; luciferase; or green fluorescent protein (GFP) using a pCMVTnT-GFP pDNA [28].
3. Fluorescent microscopy or fluorescence-activated cell-sorting (FACS) analysis of living cells are methods used to monitor GFP expression. The visualization of GFP by fluorescence microscopy facilitates the assessment of relative transfection efficiency, and thus enables a comparison between different formulations. It is possible to visualize a sample using either an inverted light microscope with epifluorescence optics or a confocal microscope. The possibility of making optical slices with a confocal microscope provide evidence that lipoplexes (or DNA released from them) are indeed inside the cells and not simply associated with the cell surface.
4. Mixed or binary cationic lipid formulations can lead to enhanced gene delivery, however, one must take care when working with binary cationic lipid formulations, as noted by MacDonald and coworkers [29].
5. The optimized molar ratio between the cationic lipid and the neutral co-lipid depends upon the cell line of choice.
6. A number of immortalized or primary cell lines are commercially available. The literature is replete with reports that describe nonviral in vitro gene transfer studies with different cell types.
7. For example, in the case of lipid L1 (molecular weight, MW of 437 g/mol) weigh out 4.37 mg and solubilize in 5 mL of CH_2Cl_2.
8. If the lipids are light sensitive, the use of a foil covered round-bottom flask will reduce light exposure during procedures such as rotary evaporation.
9. For the example lipid L1 mentioned above, solubilize the 4.37 mg thin film in 10 mL of EtOH.

10. The addition of components other than cationic and helper lipids occurs at the step of combining appropriate volumes of the separate EtOH lipid stock solutions. Examples of additional components include 3–5 % (molar ratio) of PEGylated lipid for "stealth" liposomal systems; and/or a lipid with an attached targeting ligand, if desired, for systems that are more complex.
11. For EtOH lipid stock solutions, rotary evaporate for approximately 1 h at 35 °C.
12. Before use, warm the hydrated stock solution to 37 °C and sonicate for 30 min.
13. Depending on the liposome concentration, the solution will be a transparent or milky dispersion.
14. For example, an Avanti Mini-Extruder (Avanti Polar Lipids, Alabaster, USA).
15. To reduce the dead volume, prewet the extruder parts by passing a syringe full of dH_2O or buffer through the extruder, and discard the liquid.
16. Extrusion of multilamellar liposomal suspensions will produce unilamellar liposomes with a pore size of 0.2 μm.
17. Polycarbonate membranes are intended for a single liposome preparation and should not be reused.
18. The final extrusion should fill the second syringe. This reduces the possibility of contamination with larger particles or foreign material.
19. Polydispersity index is a measure of the liposome or lipoplex size distribution. Methods that aim to reduce the size distribution (extrusion for example) are associated with a decrease in the polydispersity index value. Small size lipoplexes (<200 nm) are required for intravenous administration, whereas the administration of larger lipoplexes must occur via other routes (intraperitoneal for example).
20. Report the data as a mean ± standard deviation (SD) derived from three independent measurements.
21. Within the range of concentrations used in this protocol, the samples do not require predilution with water.
22. Cationic liposomes should have a positive ζ-potential in order to interact with negatively charged pDNA.
23. Unlike liposomal delivery of small organic molecules encapsulated within the aqueous core of the liposome, the combination of a cationic liposome and a large pDNA molecule results in a lipid-based complex characterized commonly by a lamellar, inverted hexagonal or cubic structure, or some combination of these morphologies. The determination of lipoplex

morphology commonly employs transmission electron microscopy (TEM) or small-angle X-ray diffraction (SAXD) studies. It is possible to observe the structure and morphology of lipoplexes using negatively stained TEM. A drop of liposome or lipoplex suspension is deposited over a carbon coated standard TEM copper grid, and then a droplet of the stain solution (generally uranyl acetate or phosphotungstic acid) is applied to the copper grid. The stained liposome or lipoplex suspension is observed on the grid, using a transmission electron microscope. SAXD protocols are dependent on beam source [30].

24. Lipoplexes of concentrations 0.081 mM, 0.243 mM, 0.486 mM, 0.81 mM, and 1.62 mM, corresponding to CRs (+/−) of 0.5, 1.5, 3, 5, and 10 respectively, are prepared from the 2 mM liposome stocks.
25. Greater transfection efficiency has been reported when the nonviral DNA complex is formed by addition of the cationic vector to the DNA solution, as opposed to the reverse [31].
26. The average weight of a single DNA base pair (bp) is 650 Da (Daltons or g/mol), and therefore the MW of a double-stranded DNA (dsDNA) molecule equals the number of base pairs multiplied by 650 Da. In terms of the negative charge, for every mole of base pair there are 2 mol of negative phosphorus groups. Therefore, the number of moles of negative phosphorus groups within a dsDNA molecule equals the number of moles of DNA multiplied by the number of bases within its structure. If the cationic lipid carries one positive charge, the number of moles of positive charge is equal to the number of moles of the lipid itself.
27. Transfection experiments are performed using each of the diluted lipoplex formulations and conducted in triplicates.
28. Based on constant DNA and an increasing amount of cationic lipid, employ a 1% TBE-agarose gel, which separates any remaining nucleic acid not incorporated into the particles. The absence of DNA bands indicates full association of the DNA with the cationic lipid.
29. In addition to the gel retardation assay, it is possible to perform a competitive binding assay to determine how tightly the DNA and cationic lipid are bound to one another. The negatively charged surfactant, SDS, is commonly employed and competes for lipid binding. In practice, the weaker the lipoplex, the greater the number of cationic lipid molecules that bind to SDS, thus leaving a greater amount of unbound DNA that is visualized on the gel.
30. The electric field impedes the migration of pDNA complexed with the cationic lipid.

31. The exposure of lipoplexes to DNase I leads to cleavage of the unbound and/or unprotected DNA into linear fragments. Separation of the DNA fragments from the cationic lipids, using a detergent, leads to the subsequent detection of the components by agarose gel electrophoresis. The presence of a DNA band is indicative of the proportion of the DNA protected via association with a cationic lipid.
32. In cytotoxicity assays, MTT and MTS function in a similar manner, which involves the reduction of a tetrazolium into a formazan product. The amount of formazan is directly proportional to the number of living cells. In the MTT assay, the formazan product is insoluble, and extra steps are required to dissolve the crystals resulting in the full destruction of cells. The formazan product formed using the MTS assay is soluble in tissue culture medium, hence, the medium can be used for the cell viability evaluation while the cells themselves can be used for other quantitative analyses, such as total protein BCA assay.
33. The absorbance of the converted dye correlates with the number of viable cells.

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