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Large scale production of DC-Chol cationic liposomes by microfluidization

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Abstract

In this report, we describe the large scale production and testing of DC-Chol cationic liposomes by microfluidization. These liposomes are produced in a GMP acceptable manner to a 500 ml batch size and are shown to be sterile. Further, when stored at 4°C, DC-Chol liposomes will retain their original size, remain suspended in solution, and retain activity for a period exceeding 1.5 years. In-process QA and QC procedures have identified problems in processing and methods to produce a final product of pharmaceutical quality have been developed to overcome these obstacles. Assays for product content (DC-Chol and DOPE assays), size, sterility, endotoxin determination, storage conditions and shelf life have been developed. Successful lots have been used in a human gene therapy clinical trial for cystic fibrosis at Oxford University as well as many pre-clinical experiments throughout the world. Implications for application to further gene therapy clinical trials as well as the development of liposome vector programs are discussed in detail. Copyright © 1996 Elsevier Science B.V.

Keywords: Cationic liposome; DC-Chol; Gene therapy; Microfluidizer; Nonviral vector

Abbreviations: DC-Chol, 3β[*N*-(*N'*,*N'*-dimethylaminoethane)-carbamoyl] cholesterol; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine; EU, Endotoxin Units; GMPs, good manufacturing practices; IU, international units; MLVs, multi-lamellar vesicles; QA, quality assurance; QC, quality control; QELS, quasi-elastic light scattering; SUVs, small unilamellar vesicles.

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1. Introduction

As the liposome community celebrates the 30 year anniversary of the initial publication describing the physical structure and behavior of liposomes (Bangham et al., 1965), finally two liposomal drug products have recently been approved by the US FDA, i.e. Doxil™ (liposomal

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doxorubicin, Sequus Pharmaceuticals) and Abelcet™ (liposomal amphotericin B, The Liposome Co.). It has often been suggested that the effective scale-up and quality control measures required by the FDA have been major obstacles in preventing the marketability of a liposomal dosage form. Drug delivery via conventional liposomal dosage forms involves the encapsulation of a drug within the liposome. Quality control and process validation measures set tight guidelines as to the extent of encapsulation, the removal of free unencapsulated drug, and the extent of leakiness (the drug leaking out of the liposome) of the finished product. Unfortunately, much of the knowledge from the scale-up to the quality control of these preparations are proprietary information and have not been reported in the literature in any detail.

Cationic liposomes, in contrast to conventional liposomes (drug encapsulated within the liposome), are likely to have an easier time in adhering to quality control and validation requirements. The cationic liposome is a delivery vehicle for a negatively charged moiety, usually a protein (Debs et al., 1990; Nair et al., 1992; Walker, 1992), peptide (Bennett et al., 1992; Chiang et al., 1991), or oligonucleotide (Brigham et al., 1989; Burger et al., 1992; Felgner and Ringold, 1989; Malone et al., 1989; Muller et al., 1990; Weiss et al., 1989). In the case of gene therapy, cationic liposomes are produced at a known concentration and purity, having achieved acceptable limits for sterility, endotoxin, and other quality control standards. The cationic liposomes are mixed with DNA and a complexation occurs due to electrostatic interactions. At present, this complexation event is accomplished immediately prior to administration as the dose, ratio of DNA to liposome, and type of DNA to be used for a defined disease is patient specific. It would be difficult to envision (or futuristic at best) such a demand that treatments for diseases would be standardized and commercially available as ready-to-use dosage delivery systems. Currently, the demand for a cationic liposomal formulation would simply entail the production of the empty liposome.

Various methods are available for the production of liposomes (Bangham et al., 1974; Szoka and Papahadjopoulos, 1980; Deamer and Uster, 1983; Gregoriadis, 1984; Hope et al., 1986; Lichtenberg and Barenholz, 1988) but many of these methods tend to be variable or unsuitable for large scale production. Sonication, for example, produces liposomes of varying sizes and does not lend itself to scaling (Perrett et al., 1991). Probe sonication is scalable but presents problems due to degradation of lipids, metal particle shedding, and aerosol generation (Riaz et al., 1989). High pressure extrusion by means of a homogenizer (Bachmann et al., 1993) or microfluidization (Mayhew et al., 1984; Washington and Davis, 1988; Gregoriadis et al., 1990) have been shown to produce homogenous, reproducible samples which are scalable to production size batches (Barenholz, 1992). The microfluidizer uses the principles of fluid dynamics to produce liposomes in a continuous process which is compatible with pharmaceutical good manufacturing practices (GMPs) (Mayhew et al., 1984).

The production of liposomes using homogenizers and microfluidizers has been attempted for 10 years prior to this report. The major disadvantage to these methods of preparation is that they produce small unilamellar vesicles (SUVs) after a single pass during processing. The primary drawback to SUVs is that they have a low encapsulation efficiency due to a small aqueous volume (Szoka and Papahadjopoulos, 1980) and tend to leak their content of encapsulated drug more readily than multi-lamellar vesicles (MLVs). Cationic liposomes, however, do not share this drawback as their mechanism of action is by complexation of the active compound to the surface of the liposome by charge interaction, instead of by encapsulation of the active compound inside the liposome. For the production of cationic liposomes, the microfluidizer is ideally suited for this purpose.

2. Materials and methods

2.1. Lipids

DC-Chol was synthesized by the one step reaction of dimethylethylene diamine with cholesterol

chloroformate according to the published method (Gao and Huang, 1991). DOPE was purchased from Avanti Polar Lipids, of Alabaster, Alabama. Deionized, distilled water which has been filtered and autoclaved was used throughout unless otherwise noted.

2.2. DNA

pRSV-Luc plasmid DNA (De Wet et al., 1987) was used for all transfection experiments. The plasmid contained a luciferase gene driven by the Rouse Sarcoma Virus promoter. The plasmid DNA was prepared by using a modified version of the Sambrook method (Sambrook et al., 1989) and isolation/purification by ethidium bromide and cesium chloride gradient ultra-centrifugation.

2.3. DC-Chol liposomes

DC-Chol and DOPE (in chloroform) were mixed in a 250 ml round-bottom flask at a 3:2 molar ratio, dried to a thin film in a stream of nitrogen gas, and allowed to vacuum desiccate for several hours to remove any residual chloroform. The lipid film was hydrated overnight with water and briefly sonicated to remove the lipid off the glass walls of the vessel. The liposomes were produced by microfluidization using an M-110S microfluidizer (Microfluidics Corp.) at a pressure of 90 psi to a size (average mean diameter) below 200 nm. The liposomes were passed through a 0.2 μm filter (nylon or cellulose nitrate) to provide sterilization and further diluted with water to a final concentration of 2 μmol/ml (1.2 mg total lipid/ml).

2.4. Microfluidizer

An M-110S small volume microfluidizer was purchased from Microfluidics Corp. (Newton, MA). The microfluidizer is powered by a 5 foot nitrogen gas cylinder. This supplies a pressure of 90 psi into the piston which provides pressure of approximately 17 000 psi in the interaction chamber and a flow rate of 100 ml/min. The microfluidizer is cleaned by recycling 95% ethanol, followed by passing of five samples of water to remove the residual ethanol. The microfluidizer is primed with water and the liposome batch is passed through and collected without recirculation. Briefly, the sample is placed in the reservoir and is pumped through the interaction chamber, through a cooling loop and returned to the reservoir for recirculation or allowed to exit the system. This process is repeated until a liposome of a desired size is achieved. The interaction chamber is packed in ice in order to remove the heat produced during microfluidization.

2.5. Quasi-elastic light scattering (QELS) analysis

The size of the liposomes were determined by using a Coulter (Hialeah, FL) N4SD sub-micron particle analyzer with a wavelength of 633 nm. The samples were prepared by diluting 10–15 μl of the liposome sample in 500 μl of deionized distilled water and counted/integrated for 200 seconds. The counts per second were within the range of 5×10^4 to 1×10^6 as per the manufacturers instructions. A unimodal analysis was used for all samples.

2.6. Phosphate assay

A phosphate assay (Kates and Morris, 1986) was performed on the liposomes to assay for DOPE content, as each molecule of DOPE contains one phosphate group. Briefly, 100 μl of the sample was placed in a test tube and digested with 400 μl of 70% perchloric acid. A marble was placed over each test tube (to allow for venting without loss of volume) and placed in a 180°C sand bath for 30 min. After cooling, 200 μl of a 5% ammonium molybdate solution, 200 μl of amidol reagent (1% amidol, 20% sodium bisulfite), and 4.2 ml of water were added to each tube, covered with a marble, and immersed in boiling water for 7 min. Once cool, the absorbance was read at 750 nm. A standard phosphate solution was prepared using monobasic potassium phosphate (32.25 mM). All samples were performed in triplicate and assayed prior to microfluidization and filtration, as well as post-filtration.

2.7. DC-Chol assay

The amount of DC-Chol was quantified by using a modified Liebermann-Burchard Test (Rendina, 1971). This assay is specific for a 3-hydroxysteroid with C-5 unsaturation, but cannot differentiate DC-Chol from cholesterol. Briefly, 100 μl samples of liposomes were lyophilized to remove the water, and were later reconstituted with chloroform. To each sample, 900 μl of acetic anhydride and 100 μl of sulfuric acid were added to the sample and mixed. This produced a green color and the absorbance was read at 625 nm. As in the phosphate assay, all samples were performed in triplicate and assayed prior to microfluidization and filtration, as well as post-filtration. The DC-Chol assay is linear within a range of 50–2000 μg with an r^2 value of 0.998 (data not shown).

2.8. Endotoxin test

Each batch of liposomes were tested for the presence of endotoxin by using a QCL-1000 chromogenic limulus amebocyte lysate (LAL) kit by Bio-Whittaker (Walkersville, MD). The kit was used according to the manufacturers instructions. Endotoxin levels are quantifiable by this colormetric assay and can be expressed as endotoxin units (EU) of international units (IU) (1 IU = 0.75 EU).

2.9. Transfection

Chinese hamster ovary (CHO) cells were maintained in F-12 (Ham) nutrient mixture (Gibco BRL) with 10% fetal bovine serum (Hyclone), 1% penicillin, streptomycin, and L-glutamine in a 5% CO_2 atmosphere at 37°C. The cells were seeded onto a 48 well plate (Falcon) and incubated overnight. DC-Chol liposomes were diluted in 1 ml of Hank's balanced salt solution (Gibco BRL) and DNA was diluted in 1 ml of CHO-S-SFM II (Gibco BRL). Equal volumes of the liposome and DNA solutions were mixed and 0.5 ml of the mixture was added to the cells which were at 50–60% confluency. The cells were incubated with the DNA/liposome complex for 4–8 h after which time the cells were aspirated and fresh F-12 media (supplemented with FBS and antibiotics) was added. The cells were further incubated for 28–44 h prior to being assayed for luciferase activity, using an AutoLumat LB 953 (Berthold) luminometer with a Luciferase Assay System (Promega). The media was aspirated from each well being careful not to disturb the cell monolayer. The cells were washed with 0.9% NaCl and lysed with 100 μl of lysis buffer (0.05% Triton X-100, 0.1 M Tris, 2 mM EDTA). The lysate was collected and spun at 14 000 RPM for 5 min and 4 μl of the supernatant was added to a luminometer tube and counted for 20 s. Each sample was assayed in triplicate.

The luciferase activity was normalized to the level of protein in the sample by using a Coomassie Plus Protein Assay Reagent (Pierce) as per the manufacturer's instructions.

3. Results

3.1. Endotoxin

The liposomes were tested for the presence of endotoxin. Low levels of endotoxin were detected in all batches of liposomes (Table 1). This level was comparable to that found in the water supply which consisted of distilled/deionized water. This problem was easily corrected by using Sterile Water For Injection, USP which is tested as being negative for the presence of endotoxin and was used in lots # 18 and # 19, resulting in a dramatic decrease in detectable endotoxin levels.

3.2. Sterility

Each batch of liposomes is examined for microbial growth at the time of manufacture, after long term stability at 4°C, and at storage at room temperature. Liposomes were spread on a nutrient agar plate and incubated at 37°C for 3 days. No bacterial growth has been found. This is to be expected as the lipids are initially in a chloroform solution. The only steps which could introduce bacterial contamination to the product is upon the introduction of water for hydration and dur-

ing processing in the microfluidizer. Since the water has been filtered and autoclaved (we now use Sterile Water For Injection, USP), and the microfluidizer has been thoroughly cleaned with ethanol, no bacterial growth is expected.

3.3. Filtration

Sterilization is achieved by filtration through a 0.2 μm filter. Three filter compositions (nylon, cellulose nitrate, and cellulose acetate) were tested. Liposomes were tested for DOPE content by a phosphate assay prior to filtration and after filtration through each filter. Membranes composed of nylon and cellulose nitrate showed minimal loss (2.7%) in DOPE content after filtration. However, the filter composed of cellulose acetate resulted in a 18.2% loss of DOPE content upon filtration (Table 2). It is presumed that this loss was the result of adsorption of the lipid on the cellulose acetate membrane. Filters composed of nylon or cellulose nitrate were used for all sterile filtration of all liposome batches. Loss of lipid upon filtration was tested and found to be uniform with respect to DC-Chol and DOPE content. The liposomes were sterile filtered and packaged in sterile vials under laminar flow conditions.

Table 1
Endotoxin levels of various liposome lots

Sample[a]	Endotoxin units/ml (mean ± S.D.)	International units/ml (mean ± S.D.)
Lot # 1	3.212 ± 0.172	2.409 ± 0.129
Lot # 2	7.531 ± 0.315	5.648 ± 0.236
Lot # 4	8.738 ± 0.245	6.554 ± 0.184
Lot # 5	5.988 ± 0.554	4.491 ± 0.416
Lot # 6	7.705 ± 0.140	5.778 ± 0.105
Lot # 7	11.416 ± 0.075	8.562 ± 0.056
Lot # 8	1.026 ± 0.164	0.770 ± 0.123
Lot # 10	3.156 ± 0.294	2.367 ± 0.220
Lot # 11	2.963 ± 0.189	2.222 ± 0.142
Lot # 12	3.635 ± 0.152	2.727 ± 0.114
Lot # 13	0.670 ± 0.325	0.502 ± 0.244
Lot # 14	11.166 ± 0.254	8.375 ± 0.190
Lot # 15	11.041 ± 0.175	8.281 ± 0.131
Lot # 16	1.788 ± 0.173	1.341 ± 0.130
Lot # 17	1.574 ± 0.377	1.181 ± 0.283
Lot # 18	0.212 ± 0.087	0.159 ± 0.065
Lot # 19	0.019 ± 0.051	0.014 ± 0.038
DD water	10.234 ± 0.162	7.675 ± 0.121
SWFI	0.020 ± 0.041	0.015 ± 0.031

[a] Deionized, distilled water was used in lots # 1–17. Sterile Water For Injection, USP (SWFI) was used for lots # 18 and # 19.

Table 2
Loss of DOPE content upon filtration

Filtration condition	μmol phosphate/ 100 μl	% loss on filtration
Pre-filtration	0.110 ± 0.003[a]	-
0.2 μm nylon filter	0.107 ± 0.006	2.7
0.2 μm cellulose nitrate filter	0.107 ± 0.002	2.7
0.2 μm cellulose acetate filter	0.090 ± 0.001	18.2

[a] Mean ± S.D. ($n = 3$).

3.4. Storage conditions

Aliquots of DC-Chol liposomes were packaged in 1–2 ml sterile vials and stored at 4°C and room temperature. The samples stored at 4°C exhibited activity in transfection and no aggregation/precipitation for all batches. As shown (Fig. 1), the mean diameter of all batches remained below 200 nm. The liposomes (lot # 11) which were stored

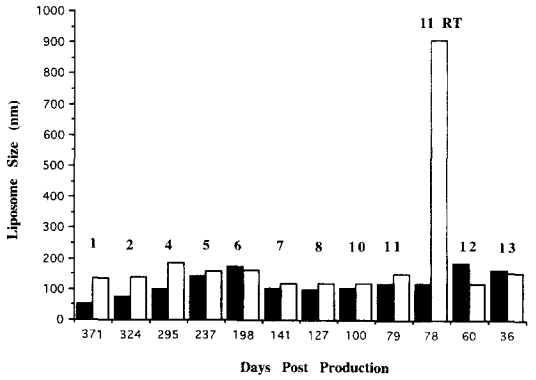

Fig. 1. Colloidal stability of liposomes over time. All lots were stored at 4°C, except for a sample of lot # 11 (11 RT) which was stored at room temperature. The numbers over the bars represent the lot numbers. ■, size of liposomes at the time of production; □, size of liposomes after testing (days post production listed under the bar).

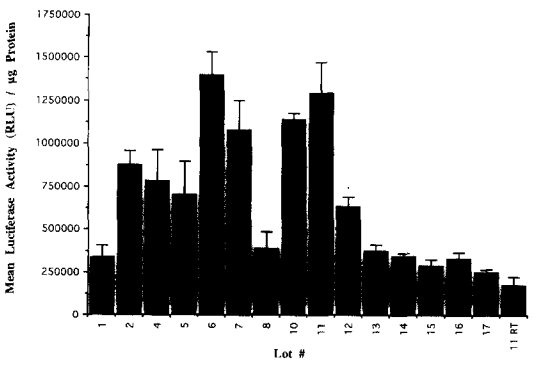

Fig. 2. Transfection activity of all lots. CHO cells were transfected with 1 μg of DNA (pRSV-Luc) and 10 nmol of DC-Chol liposomes (per well) from various lots in 0.5 ml of media. The transfection was allowed to proceed for 8 h with a 50.5 h total incubation in a 48 well plate. The assay for reporter gene was as stated in Section 2. This assay was performed over 525 days (about 1.5 years) after the date of production of the first lot. The data is presented as the mean of a triplicate sample with the standard deviation of that mean.

at room temperature, although still showed some transfection activity (Fig. 2), had precipitated within a period of 2 months. The precipitate could be redispersed but remained flocculated. This was confirmed by QELS as the mean diameter had grown to 950 nm in size.

3.5. Transfection activity

Transfection was performed as noted in the experimental section. All batches were transfected at the same time such as to eliminate all transfection variables (incubation time, transfection time, cell confluency, etc.). Due to the aforementioned transfection variables, it is difficult to compare the results of past transfections to those of current transfections. It is therefore impossible to assess an increase or decrease in transfection activity over time. The only comparisons which can be made is to transfect all lots at one time and compare different lots to each other. All lots have been shown to be active in transfection (Fig. 2). There appears to be a large variability in the extent of transfection activity which cannot be explained by any variable except for the age of the batch. No conclusion can be drawn by examining variables such as different lots of raw materials, liposome size, batch size, filtration or processing time.

4. Discussion

The interaction chamber is the functional component of the microfluidizer. Its design is based on a submerged jet design with fixed microchannels and ceramic passages. As the sample is pumped into the interaction chamber at high pressures, the flow of the sample is separated into two micro-channels (2×100 μm) and recombined at a later point. The shearing force produced upon recombination results in laminar flow, turbulence, and cavatational forces that are responsible for the reduction in particle size of the sample (Vuillemard, 1991). Liposomes processed through the microfluidizer have been shown to result in a single bilayer membrane with a small homogeneous vesicle size (Mayhew et al., 1984). It is processed under mild conditions, is cost effective, and can be easily scaled-up (Bachmann et al., 1993).

The processing time for the liposomes during microfluidization varied from batch to batch. The main objective of microfluidization is to produce liposomes with a small mean diameter such that they could be easily passed through a 0.2 μm filter to provide sterilization. Sterile filtration appeared to be the method of choice to provide sterilization (Freise, 1984) as it has been reported that degradation products can be formed during heat (Kikuchi et al., 1991) or ionizing irradiation (Konings, 1984). Also, it has been recently reported that liposomes with a mean diameter less than 300 nm can readily be sterilized by simple filtration through a 0.2 μm bacterial filter (Goldbach et al., 1995). Many of the earlier batches produced (lots 1–12) required multiple passes (15–25) through the microfluidizer to provide adequate size reduction for sterile filtration. The number of passes was reduced by increasing the processing pressure from 60 to 90 psi and through adequate hydration (from several hours to overnight) of the lipids. It is well known that hydration, more than any other step, influences

the type (number of layers, size, entrapped volume) of liposome formed (Martin, 1990). The liposomes are produced in sterile water instead of a buffered solution. This is primarily due to the fact that the liposomes are charged and their activity is based on electrostatic interactions. Dilution in a buffer would provide counterions and diminish the interaction between the cationic liposomes and the anionic component (DNA or protein) of interest. Also, smaller vesicles are produced when made in water instead of a buffer. This can be attributed to vesicle aggregation induced by salts or an increased lipophilicity of the lipids in the presence of electrolytes (Gregoriadis et al., 1990; Talsma et al., 1989). It is important to keep the number of passes through the microfluidizer to a minimum as there is the production of heat during processing and the danger of vesicle regrowth. It has been reported that cholesterol containing vesicles exhibit a maximal size reduction after 5–10 cycles and further recirculation resulted in regrowth of the vesicles which could not be resolved by further processing (Bachmann et al., 1993). Presently, adequate size reduction can be achieved by a single pass through the microfluidizer but processing for 3–5 cycles is typically performed to provide a smaller mean diameter and allow for less loss during filtration.

From a processing standpoint, it is desirable to keep the volume of liposomes to a minimum when working with the microfluidizer and filtering the final product. This is achieved by producing concentrated liposomes and later diluting them to their desired concentrations. However, due to the introduction of water into the sample from the washing steps, the calculated concentration of the DC-Chol liposome batch differs from the theoretical concentration. Also, large liposomes can be lost upon filtration, further decreasing the concentration. It was therefore necessary to develop a method to quantify the amount of DC-Chol and DOPE present in the liposomes. The DOPE assay was known from the literature while the DC-Chol assay was modified from existing cholesterol assays. The DC-Chol assay is a rapid colormetric assay which is very sensitive and easy to perform. It was assumed that any loss of content (DC-Chol or DOPE) was uniform (i.e., the loss of DC-Chol and DOPE was proportional to the ratio of their initial concentrations and that one component was not lost preferentially in respect to the other). This was confirmed by performing both assays, and there was uniform loss of each component, proportional to the original concentrations. This test for content is an essential step in the QA testing of our liposomes.

Of the 19 batches produced, two batches failed a QC step and were rejected. Lot # 3 was unable to produce liposomes by microfluidization which were smaller than 200 nm. As a result, filtration resulted in a 33% loss of active ingredients and the final concentration fell below that of the desired concentration. The difficulties in size reduction were attributed to poor hydration of the lipid film. Lot # 9 was also rejected due to an non-uniform loss of content. A large batch size was attempted (700 ml working volume to be diluted to 1000 ml) and this volume proved to be too difficult to process. Liposome size reduction was difficult but manageable. However, after processing, it was noted that a considerable loss of DC-Chol had occurred (68% loss) without a corresponding loss of DOPE (8% loss) content. The reason for this discrepancy is unclear. Preprocessing assays show that the correct amounts of each component were present prior to microfluidization. Reasons for this phenomena are purely speculative and this occurrence has not been seen in any subsequent lots.

Of the other 17 batches which successfully passed all of the QA and QC tests, all batches of liposomes remained active in transfection for a period of over 1.5 years when stored at 4°C. This stability is partially due to the nature of the liposomes. By introducing the cationic charge into the liposomes, close approximation of the liposomes is prevented due to charge repulsion and therefore the opportunity for aggregation is decreased (Martin, 1990). In regards to the functionality of the liposomes, the data in Fig. 2 appears to suggest that the liposomes become more active in transfection over time. This may be possible due to some chemical degradation of the DOPE either through oxidation or hydrolysis. The oxidation of phospholipids may be minimized by the handling of lipids under an inert atmosphere, such

as nitrogen or argon (Riaz et al., 1989; Szoka and Papahadjopoulos, 1980). Hydrolysis may occur at the ester bonds (C-2 position) linking the fatty acid to the glycerol moiety and is easily controlled by regulating the temperature as hydrolysis rates are negligible at low temperatures but become significant at higher (> 40°C) temperatures (Martin, 1990). The interaction chamber is packed in ice in an attempt to keep the temperature at a minimum but it is difficult to measure or control the actual temperature inside the interaction chamber itself. If the DOPE is hydrolyzed to a lyso-PE, this could result in a greatly enhanced permeability of the liposomes (Riaz et al., 1989). Historically this manifests itself as instability of liposomes and release of the encapsulated contents. However, in cationic liposomes, this increase in permeability may result in the passage of more DNA into the cells, allowing for increases in expression rates and transfection activity, as seen in Fig. 2. The differences in activity cannot be explained by differences in DOPE lots as the same lot of DOPE was used from the last 10 DC-Chol liposome batches.

In summary, we have been able to produce cationic liposomes (DC-Chol:DOPE) in a large scale, sterile, pharmaceutically acceptable manner. Further, an assay for the quantitation the DC-Chol has been developed and been shown to be a necessary QA procedure for the successful manufacture of these liposomes. These liposomes have been shown to remain sterile, retain their original size (no aggregation/precipitation), and remain active for a period exceeding 1.5 years when stored at 4°C. This scale-up will allow for the mass production of this formulation and will allow DC-Chol to become a therapeutic reagent of pharmaceutical quality, and support the gene therapy clinical trials throughout the world. Currently, this process is being used to support a Phase I (single and multiple dose) clinical trial for cystic fibrosis at Oxford University.

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