Aerosolization of Lipoplexes Using AERx[®] Pulmonary Delivery System

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ABSTRACT The lung represents an attractive target for delivering gene therapy to achieve local and potentially systemic delivery of gene products. The objective of this study was to evaluate the feasibility of the AERx Pulmonary Delivery System for delivering nonviral gene therapy formulations to the lung. We found that "naked" DNA undergoes degradation following aerosolization through the AERx nozzle system. However, DNA formulated with a molar excess of cationic lipids (lipoplexes) showed no loss of integrity. In addition, the lipoplexes showed no significant change in particle size, zeta (ζ) potential, or degree of complexation following extrusion. The data suggest that complexation with cationic lipids had a protective effect on the formulation following extrusion. In addition, there was no significant change in the potency of the formulation as determined by a transfection study in A-549 cells in culture. We also found that DNA formulations prepared in lactose were aerosolized poorly. Significant improvements in aerosolization efficiency were seen when electrolytes such as NaCl were added to the formulation. In conclusion, the data suggest that delivery of lipoplexes using the AERx Pulmonary Delivery System may be a viable approach for pulmonary gene therapy.

KEYWORDS: Aerosol, Gene therapy, Formulation, Plasmid, Lipoplex, Fluorescence assay.

INTRODUCTION

Recent clinical successes in the treatment of severe combined immunodeficiency (SCID),¹ hemophilia,² and heart disease³ suggest that gene therapy is starting to prove itself and that there will be rekindled clinical and pharmaceutical interest in it. Gene delivery to the lung is especially attractive because of its immediate accessibility by inhalation and the wide range of chronic and life-threatening conditions that could be treated. Lung diseases are prominent among the disorders for which hu-

man gene therapy protocols have been approved.⁴ The rate-limiting factor for gene therapy in general is the efficient delivery of the genetic material to the target cell populations. Effective means to deliver genes via the pulmonary route will likely evolve through a combination of an efficient delivery device and a delivery vector.

Aqueous formulations of gene therapies are intuitively attractive, as gene vectors have been mainly formulated as such. There have been previous reports of jet and ultrasonic nebulizers that have been evaluated for gene therapy.⁵⁻⁸ One of the potential drawbacks of these systems is that during the aerosolization process the liquid undergoes multiple passes that could compromise stability and increase the odds of contamination. Another disadvantage of conventional jet nebulizers is generally their low efficiency and poor reproducibility of delivery, which could also significantly increase cost of treatment.⁹

The AERx Pulmonary Delivery System has unique features that guide the patient to breathe in an optimal manner each time a dose is taken, which enhances reproducibility and efficiency of delivery. The AERx System delivers aerosolized medication from a dosage form comprising a blister containing 50 μ L of liquid drug formulation and a micromachined nozzle array. The aerosol is generated by extruding the formulation under pressure through the array of holes. The AERx System can also incorporate a temperature controller to minimize the effects of ambient air conditions and enhance the generation of aerosol droplets optimal for pulmonary targeting.¹⁰⁻¹⁹

In this study we evaluated the feasibility of aerosolizing a prototype gene therapy formulation comprising a cationic lipid complexed with plasmid DNA (lipoplex). The study assessed stability of plasmid DNA and lipoplexes following extrusion through the nozzle array and aerosolization, in vitro transfection efficiency of the lipoplexes following extrusion, and emitted dose of the lipoplex and other prototype nonviral gene therapy formulations following aerosolization through the AERx System. A new fluorescence-based assay was developed to quantify the emitted dose of the lipoplex formulation from the AERx System.

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MATERIALS AND METHODS

Chemicals

The expression vector p-CMV-SEAP, containing the cDNA encoding secretory alkaline phosphatase (SEAP) under the control of the cytomegalovirus (CMV) promoter/enhancer, was provided by Aldevron, Inc (Fargo, ND). The analysis of gene product was performed using Phospha-Light secreted alkaline phosphatase reporter gene assay system (Applied Biosystems, Foster City, CA). Lyophilized preparations of 3 β -[N-(N',N'-dimethylaminoethane-carbomol)]cholesterol:L- α -dioleoyl phosphatidyl ethanolamine (DC-Chol:DOPE) at a mole ratio of 6:4 were custom-synthesized by Avanti Polar Lipids (Alabaster, AL). Rhodamine-DHPE (cat L-1392), fluorescein isothiocyanate (FITC, cat F-143), and Bodipy-FL-DHPE (cat D-3800) were obtained from Molecular Probes (Eugene, Ore). Some 47-mm Gelman glass fiber filters were obtained from Pall Corporation (Ann Arbor, MI). The polyethyleneimine-DNA (PEI-DNA) formulation was a gift from Dr. Charles Densmore at Baylor College of Medicine.

Preparation of and formulations with plasmid DNA

The lyophilized DC-Chol:DOPE was first hydrated in 10% lactose solution and extruded through 100-nm polycarbonate filters to achieve desired size characteristics. The liposomes were formulated with p-CMV-SEAP by adding the lipid to a diluted solution of plasmid DNA in 10% lactose using mild vortexing in a final volume of 300 μ L. In formulations containing NaCl, the NaCl was added about 10 minutes after mixing the lipid with the DNA. The majority of studies employed lipoplexes formulated at DNA:lipid ratios of 1:6 (wt/wt) and 1:1.6 (wt/wt). Preparation of the PEI-DNA²⁰ and DNA-Artificial viral envelope (DNA-AVE)²¹ formulations has been previously described. Formulations prepared for emitted-dose quantification were spiked with a fluorescent probe prior to loading in AERx dosage forms.

AERx dosage form

The dosage form is a 3-layer laminate consisting of a 50- μ L blister container, a lid layer, and a nozzle layer that contains an array of laser micromachined holes (Figure 1). The blister layer is loaded with 50 μ L of formulation prior to heat sealing (50-psi pressure using 340°F top die, 70°F bottom die temperatures and a 0.8-second dwell time) with the lid and nozzle layer.

Figure 1. MPEG file representation of AERx operation available via online article version.

AERx Pulmonary Delivery System

A schematic showing operation of the AERx Pulmonary Delivery System is shown in Figure 1. The physical and mechanical details of the AERx System have been described previously.¹⁰ Briefly, the aerosol generation is done in about 1 second by mechanical extrusion by the piston of the aqueous formulation through an array of micron-sized laser micromachined holes. The extrusion causes the formation of aqueous jets that break up into droplets of sizes that relates to the sizes of the holes in the nozzle. The temperature controller warms up the dilution air to enhance stabilization of small, respirable droplets. The prototype system used for this study is a scaled-down version designed for inhalation delivery to dogs. For the majority of the studies described, the system was run at a flow rate of 15 L/min.

Collection of formulation for evaluation of stability

Stability following dosage form preparation. The AERx dosage forms were filled with 50 μ L of lipoplexes and stored at 2-8°C for up to 2 weeks. At specific time intervals, the formulation in dosage forms was analyzed for particle size, ζ potential, and plasmid integrity using agarose gel electrophoresis.

Stability during the process of aerosolization. Stability of the lipoplex formulations was evaluated at 2 points during the aerosolization process: (1) following extrusion through the nozzle array, and (2) following aerosolization.

Stability following extrusion through nozzle array. The stability of p-CMV-SEAP, p-CMV-SEAP/DC-Chol:DOPE

lipoplexes formulated at DNA:lipid 1:1.6 (wt/wt) and DNA:lipid 1:6 (wt/wt) immediately following extrusion through the nozzle array was assessed (in this equipment, the liquid jets were collected in a container before they broke up into aerosol droplets). The collected materials were characterized for particle size using dynamic light scattering (NICOMP 380, Particle Sizing Systems) using an intensity-weighted distribution analysis, for ζ potential using electrophoretic light scattering (NICOMP 380, Particle Sizing Systems), and for complex stability and DNA integrity using agarose gel electrophoresis. Integrity of the plasmid DNA in the lipoplexes was assessed by first solubilizing the lipid using sodium dodecyl sulphate (SDS) followed by agarose gel electrophoresis.

Stability following aerosolization. Lipoplex formulations were aerosolized and collected on a shortened version of a cascade impactor (Series 20-800 Mark II, Thermo Andersen). To evaluate the effect of temperature on complex stability, aerosolization was carried out at room temperature (temperature controller off) and at temperature controller settings of 80°F and 150°F. The formulations were reconstituted in water and assayed for particle size, ζ potential, and by agarose gel electrophoresis as described above.

Cell culture and in vitro transfection

A-549 cells were plated in 24-well plates at 10^5 cells/well in DME 21-H (4.5 g/L glucose) supplemented with 10% fetal calf serum and penicillin and streptomycin. The culture medium was aspirated about 24 hours after seeding cells and rinsed once with 1 mL of 1X phosphate buffered saline. Then 500 μ L of serum/antibiotics free medium was added, followed by 3 μ g of formulated DNA. The cells were allowed to incubate for 6 hours followed by aspiration of the medium. Fresh medium with serum and antibiotics was then added, and cells were allowed to incubate for 48 hours. At 48 hours, the cells were lysed using 200 μ L of lysis buffer (Tris buffer [pH 8.0], 0.5% Triton X, 1mM dithiothreitol) and analyzed for gene product.

Emitted-dose measurements

A fluorescence-based assay was developed to quantify emitted doses of the lipoplex formulations from the AERx System. A series of fluorophores including rhodamine-DHPE, fluorescein isothiocyanate, and Bodipy-FL-DHPE were evaluated for stability with the lipoplex formulations. Particle size and ζ potential were measured in each of these formulations following addition of the fluorophores. FITC was selected as the probe of choice based on the stability analysis. For emitted-dose quantification, the lipoplexes were mixed with the fluorescent probe FITC, loaded into AERx dosage forms, and aerosolized onto standardized Gelman filters. The filters were

rinsed with 10 mL of rinsing solution. The rinsate was analyzed for fluorescence using TECAN SpectroFluor Plus (excitation = 490 nm, emission = 525 nm). Emitted dose was calculated as the percentage of total dose loaded in the AERx dosage form leaving the device using standard curves generated with known amounts of FITC spiked onto filters. Parameters including rinsing media for filters and FITC loading dose were optimized to maximize assay efficiency.

Optimization of recovery efficiency off filters. To evaluate recovery efficiency, filters spiked with known amounts of FITC/lipoplex were rinsed with 10 mL water or ethanol:water mixture; the rinsate was analyzed for fluorescence as described above. Recovery efficiency was calculated as a percentage of FITC dose spiked onto the filter.

Data correlation with existing validated assays. To evaluate the correlation with existing validated emitted-dose assays, FITC was spiked into standardized nonlipoplex protein (DNase) or small molecule (cromolyn) formulations and aerosolized onto filters. The filter rinsates were then assayed simultaneously for fluorescence and either DNase content by high-performance liquid chromatography (HPLC) or cromolyn content by absorption spectroscopy (UV).

HPLC assay for DNase. The samples were analyzed using an Agilent 1100 HPLC system following final preparation of the sample solutions in auto sampler vials. The HPLC system consisted of a binary pump, a variable wavelength detector set at 214 nm, a column oven set to 35°C, and an auto sampler tray chilled to 5°C. The isocratic mobile phase was an aqueous solution of 0.01M sodium phosphate at neutral pH set at a flow rate of 0.6 mL/min. Standard and sample solutions were injected into a BioRad BioSil 250 SEC column guard (7.8 x 80 mm) in 50- μ L aliquots, with an inline prefilter installed prior to the column head. The standards established a calibration curve at the beginning of each run. The total run time for each injection was 7.5 minutes; the DNase peak elutes at 4.0 minutes followed immediately by the excipient peak at 5.0 minutes. Using the calibration curve, the peak area is converted to amount of DNase.

Absorption spectroscopy assay for cromolyn. The samples along with standards were plated in replicates on an acrylic UV transparent 96-well flat-bottom microtiter plate. A Molecular Devices THERMOMax microplate reader was used to analyze the samples at 340 nm using the SOFTmaxPRO program version 2.1.1. The SOFTmaxPRO program automatically converts the absorbance of the samples into emitted-dose values based on the calibration curve from the plated standards.

RESULTS

Stability of lipoplex on storage in AERx dosage forms

As shown in Figures 2A and 2B, there was no significant change ($P > .2$ for lipoplex 1:1.6 [wt/wt], $P > .1$ for lipoplex 1:6 [wt/wt]) in surface charge or particle size of the prototype formulation on storage in the dosage forms. In addition, complex stability and DNA integrity as determined using agarose gel electrophoresis were maintained (data not shown).

Figure 2. Stability of lipoplex formulations stored in AERx dosage forms. 50 μ L of lipoplex formulations at DNA: lipid 1:1.6 (wt/wt) and DNA:lipid 1:6 (wt/wt), DNA concentration 100 μ g/mL, were loaded into AERx dosage forms. The formulations were analyzed for particle size (A) and ζ potential (B) prior to dosage form preparation (time 0 days) and following 3, 7, 10, and 14 days in storage. Each data point is the average of 5 dosage forms. Data were analyzed using analysis of variance. * $P > .2$, no statistical significance between time points; † $P > .1$, no statistical significance between time points.

Effect of complexation with cationic lipids on the stability of DNA during aerosolization process. As discussed in the Materials/Methods section, stability of the formulations was evaluated immediately following extrusion through the nozzle array and following aerosolization.

Formulations were analyzed for particle size and ζ potential. Complex stability and integrity of the plasmid DNA were assessed using agarose gel electrophoresis.

Particle size/ ζ potential. As shown in Table 1, there was no significant change in the particle diameter or ζ potential of lipoplexes formulated at DNA:lipid 1:1.6 (wt/wt) or DNA:lipid 1:6 (wt/wt) following extrusion through the nozzle array. As shown in Table 2, there were slight increases in particle size with no detectable aggregation following aerosolization. The ζ potential of the aerosolized particles was maintained.

Complex stability and DNA integrity. We found that naked DNA was markedly degraded upon extrusion through the nozzle array (Figure 3A, lanes 1, 4). The heat-sealing procedure used for dosage form preparation did not contribute to this degradation (data not shown). However, integrity of DNA in lipoplexes formulated at DNA:lipid 1:6 (wt/wt)—ie, with a molar excess of cationic lipid—was intact (Figure 3B, lanes 3, 6). In addition, there was no change in the efficiency of complexation of the lipoplexes following extrusion through the nozzle array (Figure 3A, lanes 3, 6). As seen in the SDS-treated samples, DNA in lipoplexes formulated at DNA:lipid 1:1.6 (wt/wt)—ie, with a molar excess of DNA—showed some loss in supercoiled content upon extrusion (Figure 3B, lanes 2, 5).

Upon aerosolization, naked DNA was found to undergo appreciable degradation at all temperature controller settings evaluated (Figure 4A). In contrast, complexation with cationic lipids at DNA:lipid 1:6 (wt/wt) resulted in complete protection of the naked DNA during the aerosolization process (Figure 4B).

In vitro transfection. P-CMV-SEAP and p-CMV-SEAP/DC-Chol:DOPE lipoplexes formulated at DNA:lipid 1:6 (wt/wt) were extruded through the nozzle array. In vitro transfection efficiency of the extruded samples was compared to unextruded controls in A-549 cells. As shown in Figure 5, transfection levels with lipoplex formulations were significantly higher ($P < .0066$) than with naked DNA. There was no significant change in transfection efficiency of lipoplexes following extrusion through the nozzle array.

Development of fluorescence-based assay to quantify emitted dose

FITC was selected as the fluorescent probe based on favorable stability profile when mixed with the lipoplex formulations, as described in the Materials/Methods section.

Table 1. Characterization of Lipoplexes on Aerosolization Through AERx Following Exit Through the Nozzle Array*

Formulation	Particle size (nm)		ζ potential (mV)	
	Preextrusion	Postextrusion	Preextrusion	Postextrusion
Lipoplex 1:1.6 (w/w)	362.1 $\pm$ 186.5	379.7 $\pm$ 193.3	-28.0	-26.6
Lipoplex 1.6 (w/w)	513.1 $\pm$ 276.6	425 $\pm$ 201.0	27.2	22.1

* A total of 20 AERx dosage forms containing lipoplex, DNA concentration 100 μ g/mL, were aerosolized using AERx.

Table 2. Characterization of Lipoplexes on Aerosolization Through AERx Following Aerosolization*

Formulation	Particle size (nm)	ζ potential (mV)
Lipoplex (1:6, wt/wt) preaerosolization	195 $\pm$ 82	22.7
Lipoplex (1:6, wt/wt) postaerosolization, TC off	418 $\pm$ 291	23.2
Lipoplex (1:6, wt/wt) postaerosolization, T = 80°F	255 $\pm$ 157	24.7
Lipoplex (1:6, wt/wt) postaerosolization, T = 150°F	393 $\pm$ 264	21.8

* A total of 20 AERx dosage forms containing lipoplex, DNA concentration 100 μ g/mL, were aerosolized using AERx. TC indicates temperature controller.

Fluorescence intensity of samples on storage. Fluorescence intensity of lipoplex samples spiked with FITC was evaluated to ensure that there would be no loss of fluorescence intensity for the time period required to carry-out the assay. As shown in Figure 6, the samples tested showed no significant ($P > .05$ at 0.2 μ m/mL, $P > .8$ at 0.1 μ m/mL, $P > .3$ at 0.05 μ m/mL FITC concentration) loss in intensity over the 5-hour time period of storage at room temperature.

Optimization of recovery efficiency off filters. Recovery efficiency off FITC off Gelman filters was low and variable using initial conditions (Table 3). A significant improvement ($P < .014$) in recovery efficiency was obtained with a 30:70 ethanol:water mixture. Further increases in the ethanol:water ratio did not result in significant changes in the recovery efficiency (data not shown). There was also a concentration-dependent improvement in recovery efficiency with increasing amounts of FITC spiked into the formulation (Table 4).

Data correlation with existing validated assays. As shown in Table 5 and 6, there were good correlations between emitted-dose values generated using the fluorescence assay and the validated HPLC assay for DNase ($r = 0.987$, $n = 8$) and spectroscopy-based assay for cromolyn ($r = 0.962$, $n = 7$).

Figure 3. Effect of extrusion through the AERx nozzle array on DNA integrity. Different formulations were collected following extrusion through the nozzle array and analyzed by agarose gel electrophoresis without (A) and with (B) SDS. Lane 1: p-CMV-SEAP preextrusion; lane 2: lipoplex DNA:lipid 1:1.6 (wt/wt) preextrusion; lane 3: lipoplex DNA:lipid 1:6 (wt/wt) preextrusion; lane 4: p-CMV-SEAP postextrusion; lane 5: lipoplex DNA:lipid 1:1.6 (wt/wt) postextrusion; lane 6: lipoplex DNA:lipid 1:6 (wt/wt) postextrusion.

Effect of formulation composition on aerosol performance

Figure 4. Effect of aerosolization through the AERx nozzle array on DNA integrity. P-CMV-SEAP and Lipoplex DNA:lipid 1:6 (wt/wt) were collected following aerosolization at different temperature controller (TC) settings: off, 80°F, and 150°F. The pre- and post-aerosolized DNA (A) and lipoplex (B) samples were analyzed by agarose gel electrophoresis. Lane 1: p-CMV-SEAP pre-aerosolization; lane 2: p-CMV-SEAP aerosolized, TC off; lane 3: p-CMV-SEAP aerosolized, TC at 80°F; lane 4: p-CMV-SEAP aerosolized, TC at 150°F; lane 5: lipoplex pre-aerosolization; lane 6: lipoplex aerosolized, TC off; lane 7: lipoplex aerosolized, TC at 80°F; lane 8: lipoplex aerosolized, TC at 150°F; lanes 9-12: lanes 5-8 + SDS.

Figure 5. Effect of extrusion through AERx nozzle array on transfection efficiency in cultured A-549 cells. A-549 cells were plated in 24-well plates at 10^5 cells/well. The cells were incubated with p-CMV-SEAP and lipoplex DNA:lipid 1:6 (wt/wt), 3 μ g/well. At 48 hours, the cell supernatant was analyzed for SEAP gene product. The values are mean $\pm$ standard deviation for 3 sets of wells. Statistical significance is indicated by Student *t* test.

* $P \leq .0066$ statistical significance compared to naked DNA control;

† $P > .1$, no statistical significance between pre- and postextruded lipoplex.

We found that both water and lipoplex formulations gave low emitted-dose values. However, when 8.5mM was added to the formulation, there was a significant increase ($P < .0025$) in the emitted-dose values over lipoplex controls (Figure 7). A further increase in NaCl concentration to 15mM resulted in even higher emitted doses ($P < .03$), but an even higher concentration in the formulation (34 mM) did not result in additional improvement in emitted-dose values in this formulation. Emitted-dose values $> 50\%$ were achieved with the lipoplex formulation.

To test the feasibility of using AERx as a delivery system for a variety of nonviral gene therapy systems, we evaluated the efficiency of aerosolization of two additional prototype formulations using AERx—ie, DNA-AVE and PEI-DNA formulations. In studies carried out separately, PEI-DNA formulations gave emitted-dose values of $45\% \pm 5.8\%$, and DNA-AVE formulations gave emitted-dose values of $59\% \pm 3.7\%$ with an MMAD (mass median aerodynamic diameter) of $1.61 \mu\text{m}$ and a GSD (geometric standard deviation) of 1.40.

Figure 6. FITC fluorescence intensity in presence of lipoplex over time. FITC at varying concentrations was spiked into lipoplex DNA:lipid 1:6 (wt/wt), 50 μ g/mL. Fluorescence intensity was measured at specific time intervals for up to 5 hours. Data were analyzed by analysis of variance.

* $P > .3$, no statistical significance between time points at 0.05 μ g/mL FITC concentration;

† $P > .8$, no statistical significance between time points at 0.1 μ g/mL FITC concentration;

‡ $P > .05$, no statistical significance between time points at 0.2 μ g/mL FITC concentration

Table 3. Effect of Rinsing Medium on Recovery Efficiency off Filters*

FITC Concentration in Lipoplex	% FITC Recovery Using Water Rinse	% FITC Recovery Using Ethanol:Water Rinse†
0.1 µg/mL	74.7 ± 8	92.4 ± 0.2
0.05 µg/mL	77.8 ± 3.9	82.8 ± 2.8
0.025 µg/mL	80.2 ± 10.3	85.5 ± 0.7

*Filters spiked with known amounts of FITC/lipoplex mixture were rinsed with either water or ethanol:water (30:70) mixture. Rinsates were characterized for fluorescence, and emitted dose was calculated. The values are mean ± standard deviation (n = 5) for each condition. The data were analyzed using analysis of variance.

†P < .014, statistical difference to water rinse control group.

Table 4. Effect of FITC Dose on Recovery Efficiency off Filters*

FITC Concentration in Lipoplex	FITC Recovery† (% Spiked Dose)
0.4 µg/mL	101.5 ± 3.0
0.1 µg/mL	92.6 ± 0.2
0.05 µg/mL	84.8 ± 2.8

*Filters spiked with known amounts of FITC/lipoplex mixture were rinsed with either water or ethanol:water (30:70) mixture. Rinsates were characterized for fluorescence, and emitted dose was calculated. The values are mean ± standard deviation (n = 5) for each condition. The data were analyzed using analysis of variance.

†P < .0019, statistical significance between groups.

Table 5. Data Correlation of Fluorescence Assay With HPLC Assay*

Sample #	Fluorescence Assay (%)	HPLC Assay (%)
1	50.56	49.2
2	6.51	3.7
3	31.23	22.6
4	13.48	9.5
5	47.7	51.1
6	4.49	2.3
7	21.3	19.6
8	15.50	11.7

*FITC was spiked into standardized non-lipoplex protein (DNase) or small molecule (cromolyn) formulations and aerosolized onto filters, which were then assayed simultaneously for fluorescence and DNase content by HPLC.

Table 6. Data Correlation of Fluorescence Assay With UV Assay*

Sample #	Fluorescence Assay (%)	UV Assay (%)
1	41.3	46.0
2	66.2	73.1
3	60.5	69.7
4	59.3	58.3
5	64.9	79.0
6	54.8	56.4
7	49.8	60.2

*FITC was spiked into standardized nonlipoplex protein (DNase) or small molecule (cromolyn) formulations and aerosolized onto filters, which were then assayed simultaneously for fluorescence and cromolyn content by UV.

Figure 7. Effect of NaCl addition on emitted dose. The formulations were loaded into AERx dosage forms and aerosolized using AERx. The values are mean $\pm$ standard deviation ($n = 5$). Emitted dose was evaluated using fluorescence assay. The lipoplex formulation used for this study was DNA:lipid 1:1.6 (wt/wt).

* $P \leq .0025$, statistical difference to lipoplex control group (Student t test);

† $P \leq .03$, statistical difference to lipoplex + 8.5mM NaCl (Student t test).

DISCUSSION

There are numerous previous reports on the use of ultrasonic and jet nebulizers for delivering gene therapies to the lung.^{5-7, 20, 22} Nebulizers are relatively easy to use and no coordination is required, as the patient is instructed to breathe tidally. However, a disadvantage of nebulizers is their low efficiency and poor reproducibility of delivery. The amount of aerosol reaching the lung from a jet or ultrasonic nebulizer has been estimated by several researchers at no more than about 10%, even when the nebulizer is operated to dryness.²³ A significant contributor to this inefficiency is that nebulizers operate on the basis of continuous nebulization; thus, a large proportion of the dose will never reach the patient. To improve delivery efficiency, it is important to deliver the aerosol only during the inhalation cycle. Aerosol delivered at the beginning of an inspiration can fill distal parts of the lung, while aerosol inhaled at the very end of the inspiration is likely to deposit predominantly in the oropharynx and central airways. The AERx System is designed to sense the flow rate and volume and determine if it is optimum to deliver the targeted emitted dose. The patient's inspiratory flow rate is measured and integrated. The actuation of the aerosol generation can occur only at the preprogrammed inspiratory flow rate and inspired volume to prevent delivery at suboptimal conditions. Another critical issue for inhalation drug delivery is the potential for contamination with pathogenic microorganisms. Published reports on nebulizers show that contamination can be traced to multidose liquid packaging.^{24,25} Sterile unit-dose disposable systems such as the AERx dosage form overcome this problem. The AERx

System thus has unique features that are highly desirable for delivering gene therapies.

The AERx System has been previously used for the pulmonary delivery of both small molecules such as morphine²⁶ and fentanyl²⁷ and proteins such as insulin (molecular weight [MW] = 6 kDa),²⁸ rhDNase (MW = 37 kDa),¹¹ and IL-4R (MW = 54 kDa).²⁹ It has generally been yielding emitted doses in the range of 60% to 75%, the vast majority of which has been shown to deposit in the lung.¹⁹ Studies in normal fasting volunteers inhaling an aqueous solution of insulin from AERx show that reduction in glucose levels is at least as reproducible as that achieved by subcutaneous injection, in terms of both magnitude and time to maximum reduction in glucose levels.²⁸ In a clinical study to evaluate pharmacokinetics of inhaled fentanyl delivered via the AERx System, plasma concentration time courses for the AERx System and intravenous route were comparable.²⁷ In human clinical trials with IL-4R, AERx was approximately 3 times as efficient as the PARI LC STAR nebulizer in depositing drug in the lung. Median area under the curve (AUC) for AERx was found to be 7.66 times higher than that for the PARI nebulizer. The higher AUC for AERx was attributed to a combination of device efficiency and deposition pattern (central to peripheral deposition) in the lung.²⁹ We propose that aerosolization using the AERx System would enable rapid and efficient noninvasive delivery of the gene therapies to the lung.

The scope of this work was to evaluate the feasibility of delivery of particulate gene therapy formulations using the AERx System. A prototype lipoplex formulation was selected as a model formulation. Lipoplex formulations have been previously reported to be stable in sterile water for as long as a year.³⁰ We performed short-term stability studies (2 weeks) to ensure that the heat-sealing procedure and storage in dosage form components did not destabilize the formulation. There was no significant change in particle size or ζ potential ($P > .2$ for lipoplex 1:1.6 [wt/wt], $P > .1$ for lipoplex 1:6 [wt/wt]) of the lipoplexes over this time period. In addition, complex stability and DNA integrity were maintained. We found that "naked" DNA undergoes degradation following extrusion and aerosolization by the AERx System. In contrast, DNA in lipoplexes formulated with a molar excess of cationic lipid remained intact following extrusion and aerosolization. In addition, the lipoplexes showed no change in ζ potential or degree of complexation following extrusion. There was also no significant change ($P > .1$) in the potency of the lipoplex formulation following extrusion as determined by a transfection study in A-549 cells in culture. The data suggest that complexation with cationic lipids has a protective effect on the formulation following extrusion through the nozzle array and subsequent aerosolization. Such a protective effect using cationic lipids has been reported previously in the literature in nebulized formulations.^{5,7}

A key determinant of delivering gene therapy formulations using the AERx System is aerosolization efficiency. Previous attempts to develop assays to quantify emitted dose as a measure of aerosol performance using HPLC and absorption spectroscopy were unsuccessful. These results were in part due to low sensitivity and interference from the lipid-based components in the formulation. Also, any conformational changes in the formulation following aerosolization could have interfered with the analysis. A fluorescence-based assay was developed because of its potential for high sensitivity and circumvention of analytical errors resulting from changes in the formulation following aerosolization. A fluorescent probe (FITC) was spiked into the formulation prior to aerosolization and used as a tracer to track the formulation. In our initial work with this assay, we found that water and lipoplex formulations prepared in lactose gave low emitted-dose values. This effect may have been due to electrostatic charging, which is known to increase aerosol deposition on the walls of inhalation devices. Rosell et al.³¹ have shown that aerosols of pure water, which were generated by the AERx System, yielded a high electrostatic charge and small amounts of electrolytes suppressed this effect by increasing the electrical conductivity of the liquid jet emitted from the dosage form nozzle. The increased conductivity of the liquid jet presumably allows for charges to flow back toward the dosage form, before the liquid jet breaks up into droplets, thereby reducing the net charge on the droplets. We proposed that raising the electrolyte content in the lipoplex formulation would suppress aerosol charging by this same mechanism and improve emitted dose. To test this hypothesis, small amounts of an electrolyte (NaCl) were spiked into the formulation prior to aerosolization. The added electrolytes significantly improved the emitted dose ($P < .0025$) and reduced its variability. Electrolytes have been added to a number of other formulations with similar results.³² To test the feasibility of using AERx to aerosolize a variety of nonviral gene therapy formulations, two additional prototype formulations were selected, ie PEI-DNA (PEI, a polycationic polymer) and DNA-AVE formulations (DNA encapsulated in modified lipid-based system). Emitted-dose values with the PEI-DNA formulation were $45\% \pm 5.8\%$, DNA-AVE formulations showed emitted-dose values of $59\% \pm 3.7\%$, with an MMAD of $1.61 \mu\text{m}$ and GSD of 1.40.

CONCLUSION

The rate-limiting factor for gene therapy is the delivery of genetic material to target cell populations. Effective means to deliver genes via the pulmonary route will likely evolve through a combination of an efficient delivery device and an efficient delivery vector. The performance characteristics of the prototype lipoplex and other nonviral formulations with the AERx System suggest that this system can potentially be used for administering DNA-based drug products to the airways for the treatment of respiratory disorders. Clearly, the applicability

and ease of administration using AERx can be enhanced by the development of more potent formulations, which could potentially enable delivery of the target dose in single inhalation.

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