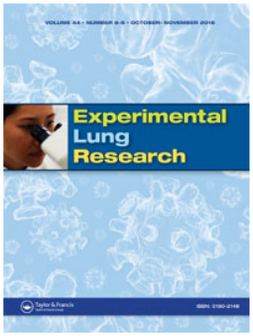

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RESEARCH-ARTICLE

Delivery of nucleic acids to *ex vivo* porcine airways using electrospray

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ABSTRACT

Aim of the Study: Nucleic acid-based therapies have the potential to provide clinically meaningful benefit across a wide spectrum of lung disease. However, *in vivo* delivery remains a challenge. Here we examined the feasibility of using electrospray to deliver nucleic acids to both porcine tracheal tissue sections and whole lung *ex vivo*. **Materials and Methods:** The effect of electrospray solution, emitter gauge, flow rate and voltage on plasmid DNA integrity was examined by analyzing supercoiled:open circle structure ratio by gel electrophoresis. Optimal parameters were used to deliver luciferase DNA and mRNA and siRNA-FITC to tracheal tissue sections. Luciferase mRNA was delivered to whole porcine lungs *ex vivo* using a catheter and bronchoscope system. Luciferase activity and fluorescence were analyzed by luminometry and microscopy respectively. **Results:** The incidence of DNA plasmid nicking was greatest in a low salt solution without ethanol compared with 1% and 20% ethanol with salt. From a range of emitters tested, a 32 gauge emitter produced the best supercoiled:open circle structure ratio, likely because less voltage was required to produce a stable electrospray with this emitter. Lower flow rates also showed a trend towards reduced DNA nicking. GFP DNA electrosprayed at 5 kV and 6 kV resulted in lower levels of GFP expression in A549 lung cells following lipofection compared with 3 kV and 4 kV. Optimised parameters of 20% ethanol solution, 32 gauge emitter, low flow rates and voltages of 3–5 kV, nucleic acid molecules were successful for delivery of luciferase DNA and mRNA as well as siRNA-FITC to porcine tracheal tissue sections and for delivery of luciferase mRNA to whole porcine lungs via bronchoscope. **Conclusions:** We report *ex vivo* delivery of nucleic acids to porcine lung tissue via electrospray and bronchoscopic electrospray delivery of nucleic acid to an *ex vivo* porcine lung model.

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Gene delivery; transfection; gene therapy; electrospray; cystic fibrosis

Introduction

Nucleic acid-based therapies have the potential to provide clinically meaningful benefit across a wide spectrum of lung disease. However, despite decades of investigation of viral and non-viral methods of nucleic acid delivery to the lungs, no treatments have yet been approved for clinical use.[1] Issues with adverse effects and efficiency of delivery have hampered progress and the airway mucus gel layer remains a significant barrier to both viral and non-viral vectors.[2] In addition to long-standing gene therapy strategies where a gene is delivered to replace a defective gene or provide a therapeutic effect, emerging techniques such as gene editing hold promise as new treatment approaches. For these techniques it may be necessary to deliver mRNA, short DNA sequences and/or proteins.[1] Therefore, new delivery strategies are needed to address challenges seen with traditional gene delivery approaches and to ensure that progress with new techniques can be translated to the lungs.

Non-viral methods of gene delivery, including chemical and physical methods, have benefits as they are less immunogenic than viral technologies and have fewer regulatory hurdles for *in vivo* use.[3] However, the absence of a successful benchmark therapy involving non-viral vectors indicates that further challenges remain for these applications. Aerosolization has long been viewed as a desirable route for gene delivery to the lungs and can be achieved by several methods.[4] The most common method employs a spray nozzle through which fluid passes and is acted upon by mechanical forces that atomize the liquid. For delivery to the lung, sonication is widely used

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whereby high frequent vibration through a nebulizer nozzle plate produce small droplets of a liquid. However, because drugs are inspired, there is a high amount of drug lost and poor targeting of delivery.[4] Furthermore, current aerosol delivery methods such as jet or ultrasonic nebulizers can cause shearing of the DNA resulting in a poor efficiency of gene delivery.[5] The ability to deliver nucleic acid-based drugs in a non-viral, targetted manner to the airways in a way that preserves activity of the molecules is therefore desirable.

Electrospray, also known as electrostatic spray, is an alternative method of atomizing a liquid that is well-established in many fields including soft ionization mass spectrometry.[6] An electrospray occurs when tiny quantities of electrical charge are applied to a fluid as it passes through a conducting emitter such as a needle. The potential difference generated between the charged solution and a ground electrode generates an electric field drawing the liquid towards the ground electrode. The meniscus generated forms a characteristic cone, known as a 'Taylor' cone. When the voltage threshold overcomes the surface tension of the solution, the tip of the Taylor cone dissociates, or atomizes, into droplets forming either a jet or a plume of charged microdroplets. While other methods of spray generation utilize gravity and droplets decelerate once formed, within a certain distance, the electrospray droplets accelerate towards a surface.[7] In addition, because the droplets are charged, an electrospray is more controllable than other forms of spray and therefore lends itself to targeted delivery. These features have led some investigators to examine electrospray as a method for gene delivery although reports are few and inconsistent, possibly due to significant differences between electrospray configurations, solutions and parameters. While successful delivery of plasmid DNA has been reported in cultured A549 lung cells and mouse skin using electrospray, Zeles-Hahn et al. did not observe transfection of luciferase plasmid DNA in a study of tracheal/bronchial epithelial cells cultured at air-liquid interface.[8,9] Another electrospray-based technique is 'bio-electrospray' whereby cells are electrosprayed onto a surface such as an extracellular matrix-like scaffold in a targeted and controlled manner. First reported in 2006, bio-electrospray is being investigated for applications such as tissue engineering and regenerative medicine and we have previously reported successful bio-electrospray of human mesenchymal stem cells.[10]

It is well understood that solutions must have optimal conductivity, surface tension and viscosity levels in order to form a stable Taylor cone and plume during electrospraying.[8] Therefore, solutions typically contain low concentrations of salts as well as solvents such as ethanol. However, the effect of these components on DNA integrity during electrospraying has not been examined. Here we report optimization of electrospray parameters for DNA solutions and successful delivery of plasmid DNA, mRNA and siRNA to porcine tracheal explant tissue cultured at an air-liquid interface using low voltage electrosprays with a bench-top instrument. We subsequently used a catheter-based electrospray device to assess the feasibility of electrospray atomization for the delivery of nucleic acids directly to the lung in a targeted manner, via bronchoscopy, in a porcine *ex vivo* lung model.

Materials and methods

Cell culture

A549 human lung cell line (Cat. No. 86012804, Sigma-Aldrich, Wicklow, Ireland) was routinely cultured in DMEM (Gibco, Thermo Fisher Scientific, Dublin, Ireland) supplemented with 5% fetal bovine serum (Gibco) and 2 mM L-glutamine (Gibco).

Electrospray studies

The electrospray device comprised a silent air compressor, syringe pump, laser and camera for visualization of the Taylor cone and plume and a control unit (Avectas, Dublin, Ireland). For DNA integrity studies, pEGFP plasmid (Clontech Laboratories, Saint-Germain-en-Laye France) solutions were sprayed into a 24-well cell culture plate (Costar, Sigma-Aldrich) that contained the ground electrode which was a copper wire shaped into a ring. DNA was diluted in a low salt solution (137 nM NaCl, 1.47 nM KH_2PO_4, 8.10 nM

Na_2HPO_4, 2.68 nM KCl), 1% ethanol/H_2O or 20% ethanol/H_2O, all chemicals from Sigma-Aldrich. DNA quantification was carried out using the Nanodrop (Thermo Fisher Scientific). DNA samples were run on a 1% electrophoresis gel, stained with ethidium bromide and scanned using a Gel Doc™ XRS System and Quantity One® software (Bio-Rad Laboratories, Watford, UK).

Lipofection

pGFP solution was electrosprayed into an empty culture dish, retrieved, quantified using the Nanodrop analyzer and 400 µg DNA was lipofected into A549 cells seeded into 24 well tissue culture plates (Sarstedt, Nümbrecht, Germany) at 5 x 10^4 cells per well. Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific) was used to transfect pGFP plasmid into A549 cells according to manufacturer's instructions. GFP expression was determined using an Accuri flow cytometer (BD Biosciences, Wokingham, UK) at 24 hr post-transfection.

Electrospray delivery to cultured porcine tracheal explants

Pig lungs were obtained from a local abattoir. Cold ischemic time was limited to 90 min. The trachea was dissected into sections approximately 20 × 50 mm, rinsed with phosphate-buffered saline (PBS) and 3–4 wash cycles in 1:1 RPMI 1640:DMEM, 200 units/ml penicillin, 200 µg/ml streptomycin, 2.5 µg/ml amphotericin, 50 µg/ml gentamicin (all Sigma-Aldrich) were performed. Each wash cycle included changing wash media, 10 minutes of agitation on a cell shaker and 1 hr incubation at 37˚C, 5% CO_2. Tracheas were cut into 10 mm^2 segments, including epithelium, mucosa and cartilage. Tissue segments, epithelial layer facing upwards, were placed onto 5 mm high sterile agarose (1% w/v) plugs in 12-well plates (Costar, Sigma-Aldrich). Culture media (wash media plus 10 mM L-glutamine and 10% FBS) was added so that the basal section of the tissue section was submerged. Explants were incubated overnight at 37˚C, 5% CO_2, in a humidified atmosphere. For delivery, the electrospray emitter was positioned above a grounded base plate, 15 mm from the epithelial surface of the tissue. Voltage was adjusted between 3–5 kV to achieve a stable Taylor cone and diffuse plume depending on environmental conditions (humidity/temperature). Nucleic acids were resuspended in delivery solution 20% ethanol/H_2O. Porcine tissues were placed epithelial surface up, in the center of a base plate, below the electrospray emitter and nucleic acid solutions were delivered over 2–3 minutes. For pGLuc (New England Biolabs, Ipswich, MA, USA), three doses 5 µg were delivered over three consecutive days. For luciferase mRNA (TriLink Biotechnologies, San Diego, CA, USA) and siRNA-FITC (Thermo Fischer Scientific) the total treatment was administered with a single spray. Following nucleic acid delivery, the tissue segments were placed on agarose plugs in fresh culture medium. For pGLuc and luciferase mRNA activity, supernatant was analyzed using the Biolux™ Gaussia Luciferase Assay (New England Biolabs) according to manufacturer's instructions. Fluorescence was analyzed using the Olympus CKX41 microscope (Olympus, Stansfield, UK).

Electrospray delivery to whole porcine lung ex vivo

The porcine heart-lung block was prepared by cannulating the superior vena cava and perfusing culture medium supplemented with 7% albumin and 0.5% dextran through the pulmonary vasculature with a peristaltic pump (Masterflex, Gelsenkirchen, Germany). The trachea was intubated and ventilated (IPAP 14; EPAP 4; FiO2 21%) using a NIPPY 2 ventilator (RespiCare Ltd., Swords, Ireland). The lungs were cleared of debris by instilling 10 ml aliquots of 0.9% saline and suctioning. The electrospray catheter system was composed of an electrospray catheter, power pack and a syringe pump (Avectas). Prior to bronchoscopy electrospray delivery, a target endobronchial area was selected and marked with 3 dots to form a target using SPOT endoscopic marker (GI Supply, Camp Hill, PA, USA).

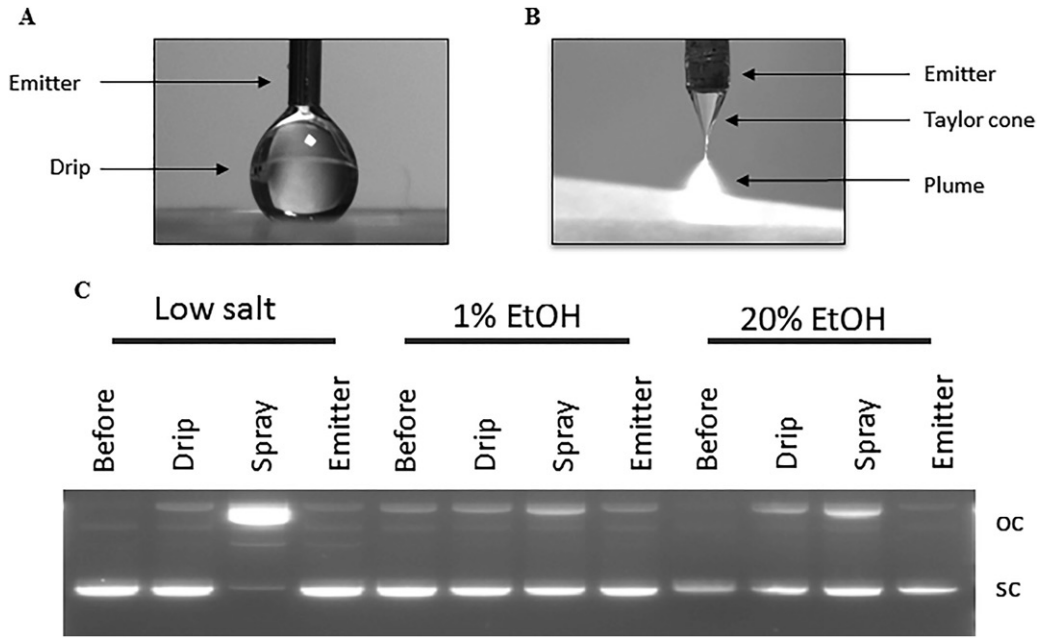

Figure 1. Effect of electrospray solution composition on plasmid DNA nicking. (A) Solution forms droplet in the absence of applied voltage. (B) Typical electrospray cone-jet mode with plume when voltage is applied. (C) Electrosprayed DNA solutions ('Spray') in low salt, 1% ethanol or 20% ethanol were analyzed by gel electrophoresis for the incidence of supercoiled (sc) and open circle (oc) structure. An increase in open circle DNA was evident in the electrosprayed low salt sample. Control samples were taken before solution was placed in emitter ('Before'), before voltage was applied to solution in emitter ('Drip') and from emitter after voltage removed ('Emitter'). Representative gel from three independent experiments is shown.

Statistics

Two-tailed, unpaired T-Tests were used for statistical analysis using Graphpad Prism version 7.03 for Windows (GraphPad Software, La Jolla, CA, USA).

Results

Effect of electrospray solution composition on plasmid DNA integrity

In their work, Zeles-Hahn et al. appear to have used various solutions containing 20 ng/µl GFP DNA without a solvent, the equivalent of a 25 gauge emitter, a flow rate of 200 µl/min and applied voltages of 3–7 kV in negative mode at a distance of 25 mm from ground.[9] They reported dripping, stable cone-jet and whipping cone electrospray profiles at −3 kV, −6 kV and −7 kV respectively. Boehringer et al. used a sucrose solution containing 100 ng/µl GFP DNA without a solvent, a 29 gauge emitter, flow rate of 20 µl/min, applied voltages of 3 kV in positive mode at distances of 3–6 mm.[10] For the present study, we aimed to further optimize electrospray parameters for transfection of airway tissue and to analyze the effect of these parameters on electrospray mode and DNA integrity. For these studies, we used positive mode electrospray as we found this more conducive than negative mode for the formation of stable sprays, similar to others who report that corona discharge is often observed during negative mode electrospray of aqueous solutions.[11] Control non-electrosprayed samples were taken either before loading into the electrospray system or generated by allowing solutions to drip through an emitter without the application of an electrical charge and collecting in a tissue culture plate from which they were retrieved for analysis (Figure 1A). When an electric charge was applied to the emitter, the DNA solution formed a classic Taylor cone and plume and these are referred to as 'Spray' samples (Figure 1B).

Covalently closed circular DNA will adopt a supercoiled topology. A single break on one strand of the molecule will result in the release of the supercoiled form into an open circle form. Another cleavage on the opposite strand will result in a linear DNA form. Further DNA nicks

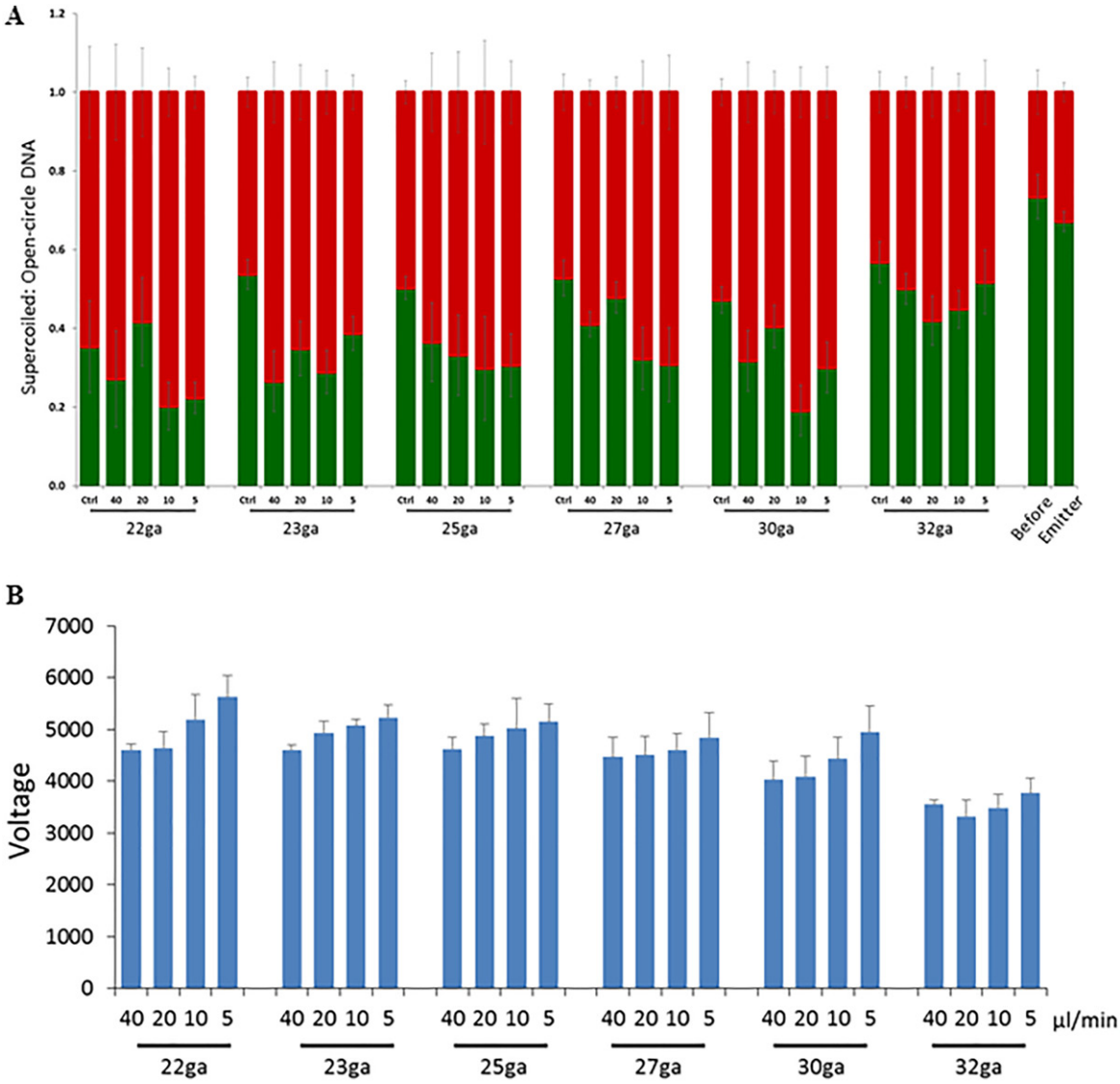

Figure 2. Effects of flow rate and emitter size on plasmid DNA nicking. (A) Plasmid DNA was electrosprayed in low salt solution through various emitter gauges at various flow rates. The ratio of supercoiled (sc) versus open circle (oc) structure was analyzed by gel electrophoresis and densitometry. Control samples were taken before solution was placed in emitter ('Before') and from emitter after voltage removed ('Emitter'). While there was no statistical difference between the conditions, there was a trend towards the 32 gauge emitter preserving the highest levels of supercoiled plasmid at all flow rates. (B) The voltage required to produce a stable electrospray was recorded and was found to decrease as the emitter guage increased. N = 3 independent experiments, ga = gauge.

will result in fragmentation of the DNA. Any of these events could lead to reduced transfection efficiency.

Three solutions containing low concentration salts or ethanol were selected and their effect on DNA integrity during electrospray was determined. DNA (pEGFP, 100 ng/µl) was resuspended in low salt solution, 1% ethanol/H_2O or 20% ethanol/H_2O. Solutions were delivered at flow rates of 5 µl/min for 5 min into empty tissue culture plates and retrieved for analysis. All solutions achieved plume electrospray mode, however voltages of approximately 4 kV were typically required for the low salt and 1% ethanol solutions compared with 3 kV for the 20% ethanol solution. We also observed that the low salt and 1% ethanol solutions had narrower plumes and were less stable over time compared with the 20% ethanol solution. There was an increase in open circle form of the plasmid in the sprayed low salt solution compared with the ethanol

solutions indicating increased single strand nicking (SSN) in the low salt solution (Figure 1C).

Effects of emitter gauge and flow rate

Emitter gauge and solution flow rates are two further parameters that affect Taylor cone formation and the ability to generate a stable electrospray. We therefore used the low salt solution to examine whether these two parameters were contributing to the nicking observed in the DNA plasmid.

Plasmid DNA in low salt solution was electrosprayed at four different flow rates (40 µl/min, 20 µl/min, 10 µl/min and 5 µl/min) through six different sized emitters (22, 23, 25, 27, 30, 32 gauge). For each emitter gauge tested, each flow rate was compared to the corresponding 40 ul/min drip control. While there was no statistically significant difference in the supercoiled: open circular ratio between the samples, there was a trend towards the 32 ga emitter preserving the highest levels of supercoiled plasmid at all flow rates (Figure 2A).

The voltage required to form a stable cone-jet electrospray for each emitter was noted and it was observed that higher voltages were required for the wide internal diameter emitters (22 ga) compared to the narrower internal diameter emitters (32 ga) (Figure 2B). These results suggest that although the larger internal diameter emitters caused more plasmid nicking, this was likely due to the higher voltages required to achieve a Taylor cone and plume electrospray.

Effect of high voltage

We next examined the effects of voltage on DNA nicking. For these experiments the resistance was kept constant and the voltage was increased in order to assess the effect of current on DNA nicking. The voltages examined were 3 kV, 4 kV, 5 kV and 6 kV. Flow rates of 60 and 30 µl/min and emitter gauges of 22 ga and 32 ga were tested. Plasmid DNA was electrosprayed in low salt solution.

Firstly, the effect of these parameters on spray mode was observed by eye (Table 1). At 3 kV the potential was not high enough to generate a spray at either flow rate or with either emitter gauge. At 4 kV, for both flow rates, a spray was generated with the 32 ga emitter but not the 22 ga emitter, which remained in drip mode. At 5 kV, for both flow rates, an unstable spray that alternated between drip and spray was observed with the 22ga emitter while a stable spray was generated with the 32ga emitter. At 6 kV the electrical potential was high enough to generate a continuous stable electrospray with both emitter sizes and both flow rates.

Table 1. Effect of electrospray parameters on spray mode.

Voltage	Flow rate	Needle gauge	Spray mode
3 kV	60	22	Drip
		32	Fast drip
	30	22	Drip
		23	Drip
4 kV	60	22	Drip
		32	Spray
	30	22	Drip
		32	Spray
5 kV	60	22	Drip/Spray
		32	Spray
	30	22	Drip/Spray
		32	Spray
6 kV	60	22	Spray
		32	Spray
	30	22	Spray
		32	Spray

DNA nicking was next examined at each voltage, flow rate and emitter gauge. Firstly, using a 32 ga emitter, an increase in plasmid nicking was observed with increasing voltage at both 60 and 15 µl/min flow rates, as indicated by an increase in the level of open-circle plasmid DNA (Figure 3A). When the flow rate was kept constant at 60 µl/min and the voltage was increased, there was no significant difference in supercoiled: open circular ratio between 22 ga or 32 ga emitters indicating that emitter gauge *per se* does not affect DNA integrity (Figure 3B). However, there was increased plasmid nicking as the voltage increased. There was a significant increase in plasmid nicking using the 22 ga emitter at 6 kV compared to 3 kV ($P = 0.0003$). There was also a significant increase in plasmid nicking using the 32ga emitter at 5 kV compared to 3 kV ($P = 0.002$). These results indicate that high voltage, or more specifically the higher current that this equates to, is the causative factor of DNA nicking during electrospray.

To determine the consequence of plasmid nicking on DNA expression, a plasmid encoding

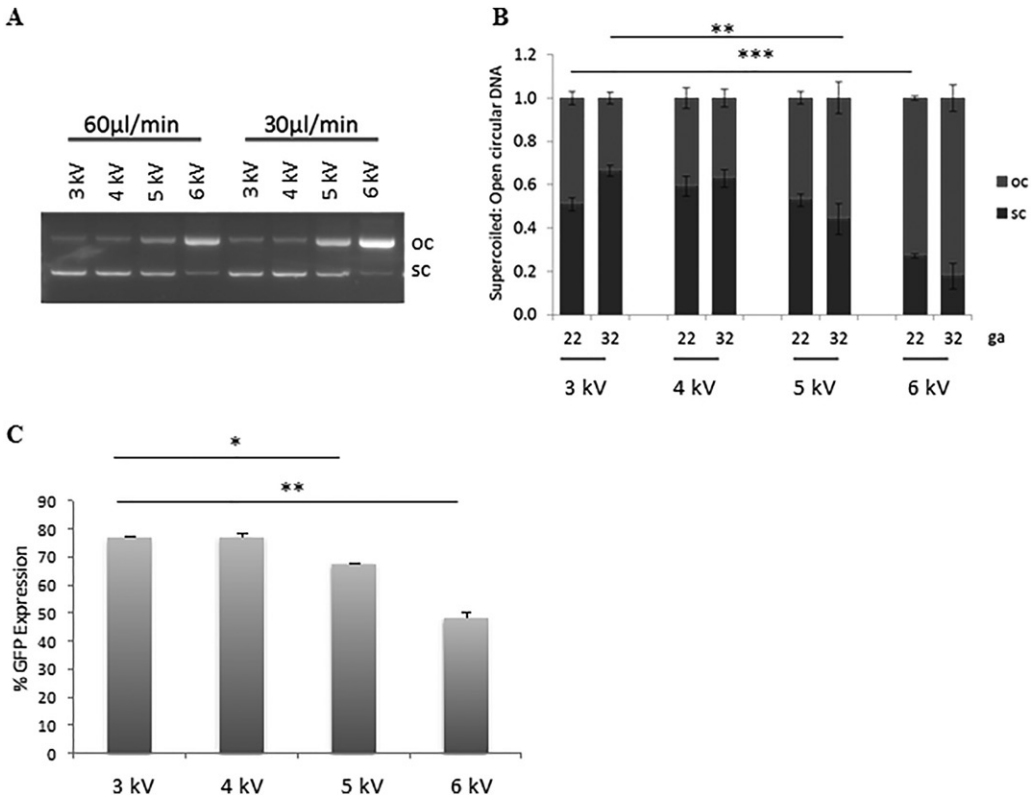

Figure 3. Effect of voltage on plasmid DNA nicking. (A) Plasmid DNA was electrosprayed in low salt solution through a 32 gauge emitter at 60 µl/min or 30 µl/min and DNA nicking was analyzed by gel electrophoresis. Increases voltage caused an increase in the incidence of open circle (oc) structure compared with supercoiled (sc). (B) Emitter gauge did not affect the incidence of open circle structure. (C) GFP DNA was electropsrayed at varying voltages (emitter size 32 ga at flow rate 60 µl/min) and transfected with Lipofectamine 2000 into A549 cells. The efficiency of GFP transfection was reduced with increasing voltage.

for green fluorescent protein (pGFP) was subjected to electrospray and subsequently transfected into A549 lung epithelial cells by lipofection. Equal concentrations of DNA were lipofected and the transfection efficiency was determined. Increased levels of DNA nicking correlated with decreased levels of GFP expression (Figure 3C). This indicates that single strand nicking of plasmid DNA induced by high voltage leads to open-circular confirmation and ultimately a reduction in downstream protein expression.

Electrospray delivery of plasmid DNA, mRNA and siRNA to cultured porcine tracheal explants

Having examined the effects of various electrospray parameters on integrity of nucleic acids, we then went on to assess the feasibility of delivery to airway tissue. Two *ex vivo* model systems were used: cultured porcine tracheal explants and whole porcine lung. Various reporter plasmids have been used in studies aimed at developing vectors for gene therapy for airway diseases. A secreted *Gaussia princeps* luciferase (GLuc) reporter gene has been shown to be a sensitive reporter in pre-clinical studies of gene transfer in cystic fibrosis models so this plasmid (pGLuc) was used for studies here.[12] Based on results above, the following parameters were deemed best for maintaining integrity of nucleic acid molecules: 20% ethanol/H_2O as delivery solution, 32 ga emitter and low flow rates.

Porcine tracheal explants were electrosprayed with 5000 ng pGLuc (833 ng/µl; flow rate 3 µl/min; duration 2 min) once each day for 3 consecutive days and tissues were then cultured for a further 48 hr (Figure 4A). 20 µl of culture media was sampled from each well and luciferase activity was quantified. There was a significant increase in luciferase activity ($P = 0.0002$) in the media of pGLuc electrosprayed tissue compared to the media from electrosprayed controls (Figure 4B).

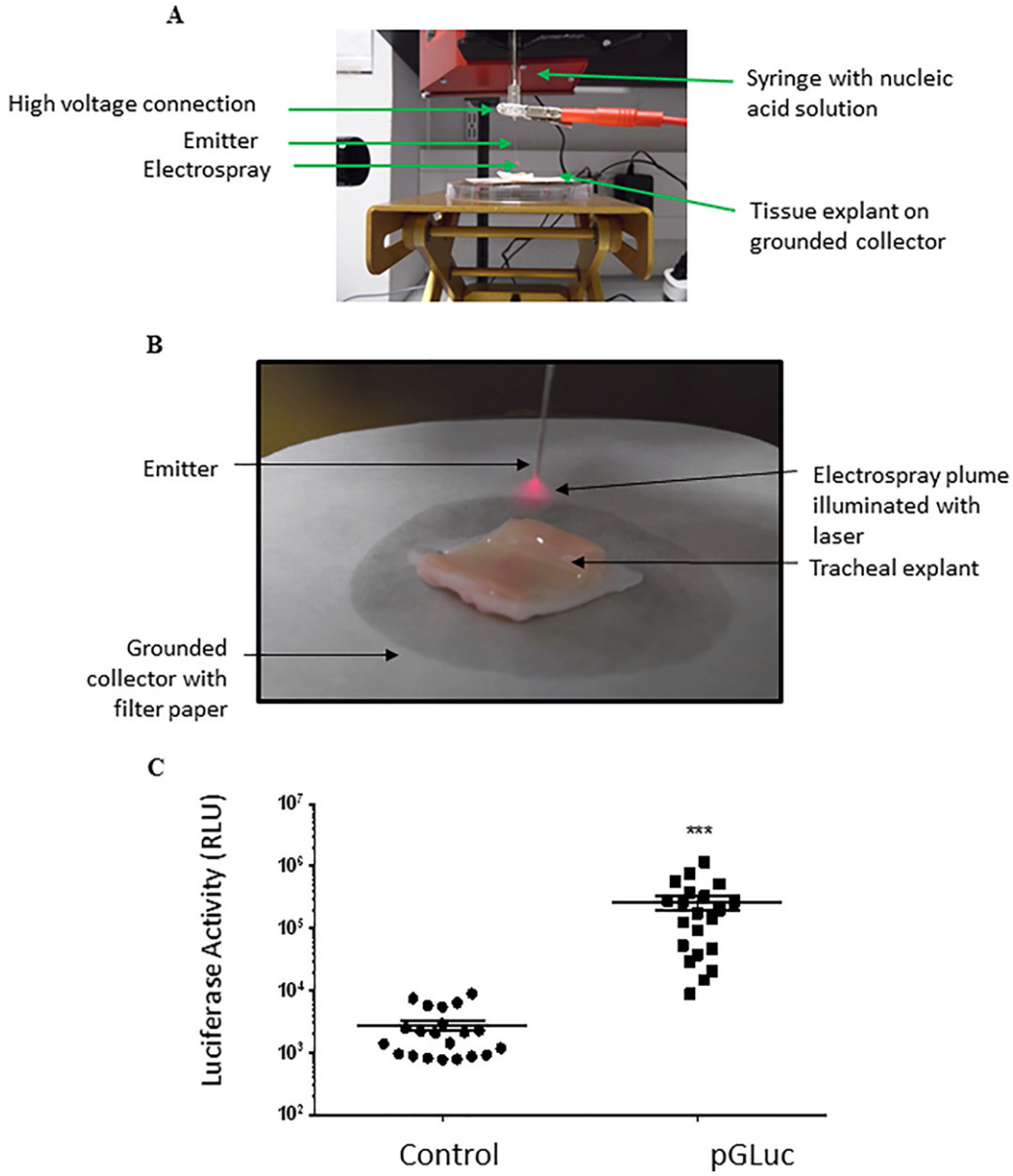

Figure 4. Electrospray delivery of plasmid DNA to porcine tracheal explants. (A) Elecrtospray set up. (B) Electrospray plume visualized with a laser during delivery to tissue demonstrating emitter, electrospray plume, tissue explant and grounded collector. (B) Quantification of Gaussia luciferase activity following pGLuc transfection compared to negative control (pEGFP). $n = 21$ independent experiments. ***$p < 0.001$.

We next evaluated electrospray delivery of RNA molecules. Delivery of mRNA encoding for luciferase was performed on porcine tracheal tissue. Luciferase mRNA (10 µg) was electrosprayed onto the trachea tissue (700 ng/µl; flow rate 4.8 µl/min; duration 3 min) and incubated for 48 hours. Delivery solution without mRNA was electrosprayed as a negative control. Quantification of luciferase activity showed a significant increase in luminescence expression ($P = 0.023$) in the mRNA electrosprayed samples compared to control explants (Figure 5A).

siRNA-FITC (20 µl 100 µM) was delivered to the trachea explant (100 µM; flow rate 10 µl/min; duration 2 min) and compared to a delivery solution-only negative control. Following delivery, the epithelial layer was microdissected and examined under a fluorescent microscope. FITC fluorescence was evident in tissue that were electrosprayed with siRNA but not in control tissue (Figure 5B). In addition, tracheal segments were cryosectioned in the transverse plane, allowing visualization of siRNA within the tissue (Figure 5C).

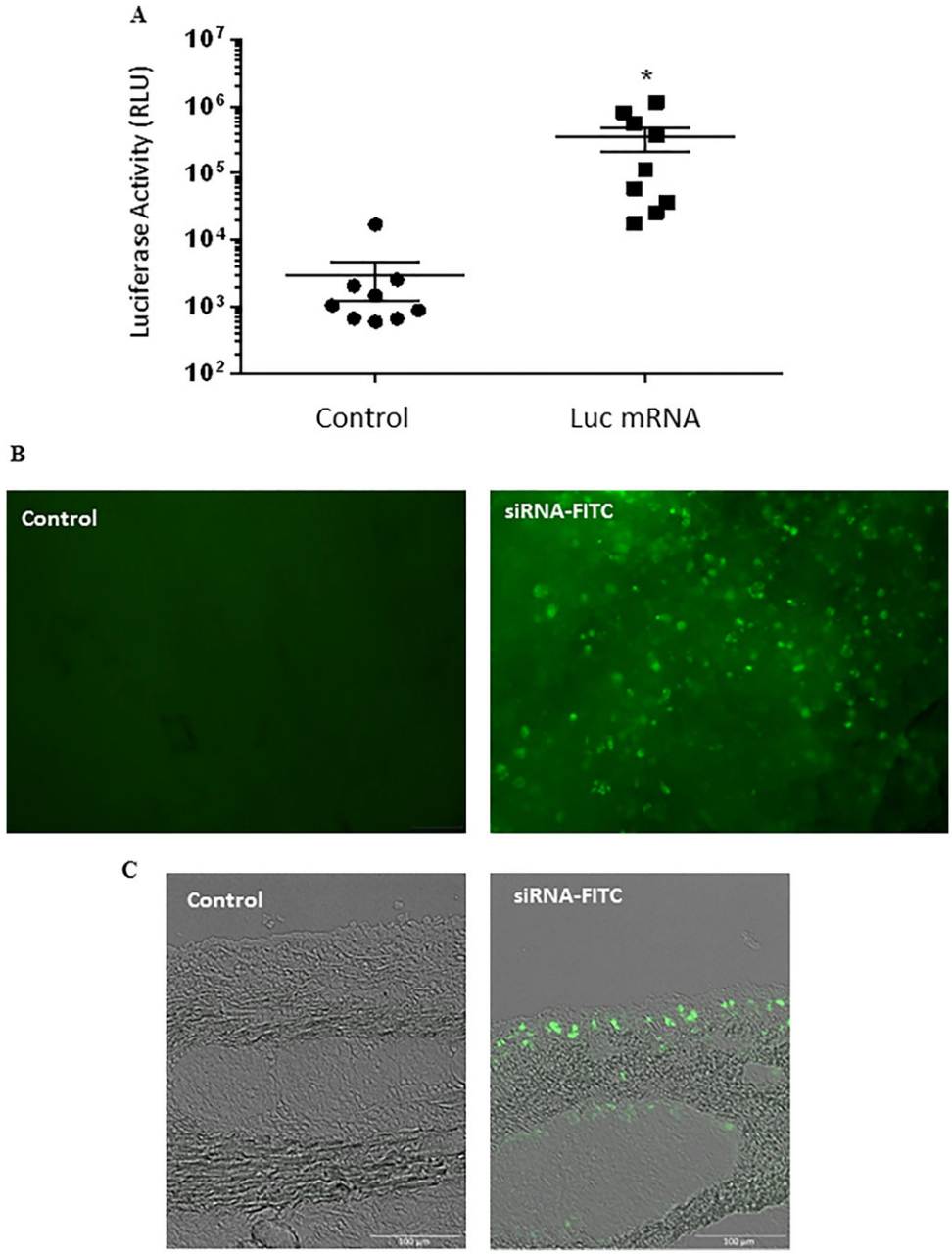

Figure 5. Electrospray delivery of RNA to porcine tracheal explants. (A) Quantification of luciferase activity following luciferase mRNA transfection compared to buffer-only control. $n = 9$ independent experiments. $^*p < 0.05$. Representative fluorescence microscopy images of FITC localization following siRNA-FITC delivery compared to buffer-only control are shown for (B) microdissected epithelial layer and (C) tracheal segments cryosectioned in the transverse plane, 20X magnification.

Bronchoscopic electrospray delivery of mRNA to whole porcine lung ex vivo

We next examined whether electrospray could be used to deliver nucleic acids to the airways within whole lungs. An apparatus was set up comprising and catheter, bronchoscope and electrospray equipment as before (Supplementary Figure 1). Dissected lungs were obtained and prior to bronchoscopic electrospray delivery, a target endobronchial area was selected and marked with 3 dots to form a target using SPOT endoscopic marker. The electrospray catheter was then inserted into the working port of a bronchoscope, positioned 15 mm from the pre-marked target and delivery solution containing mRNA was delivered to the center of the dots. 10 µl (3 µg) mRNA-GLuc (300 ng/µl; flow rate 100 µl/sec; duration 0.1 sec) was delivered to target areas

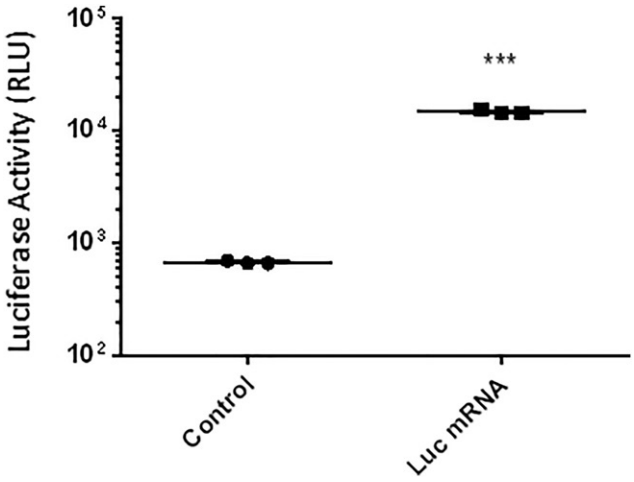

Figure 6. Bronchoscopic electrospray delivery of mRNA to whole porcine lungs *ex vivo*. Quantification of luciferase activity following luciferase mRNA transfection, compared to buffer-only control. $n = 3$. ***$P < 0.001$.

which were then resected, washed and cultured at air liquid interface. Luciferase activity was measured at 48 hr post-delivery. A significant ($P < 0.0001$) increase in luciferase activity was detected in luciferase mRNA-treated samples compared to controls (Figure 6).

Discussion

Nucleic acid based therapies have significant disease modifying potential. As such, there has been much interest in developing techniques that successfully deliver these molecules *in vivo*. Multiple viral and non-viral vectors have been utilized for this aim, but success has been limited to date. Challenges of cytotoxicity, immunogenicity and transfection inefficiency have hampered progress. Improved safety profiles with non-viral vectors have increased interest in these techniques. However, technologies to improve transfection efficacy are needed if non-viral vector therapies are to have a clinically meaningful effect. This study is the first, to our knowledge, to report the successful electrospray delivery of RNA or DNA molecules to lung tissue. Porcine lung was selected as a target for gene delivery in view of its extensive use in translational research and its close approximation of the human lung for bronchoscopic intervention.[13,14] Furthermore, the epithelial surface of the lung provides an accessible target for noninvasive (inhalation) and semi-invasive (bronchoscopy) drug delivery. Electrospray atomization has the potential to be utilized as a drug delivery platform for both modalities.

Electrospray ionization is a vector-free delivery system that has the potential to overcome multiple barriers associated with inefficient transfection. This technique has previously been reported to successfully transfect plasmid DNA *in vitro* and in mouse skin.[8,15,16] Here we report the successful electrospray *ex vivo* delivery of plasmid DNA, mRNA and siRNA molecules. In this study, protein expression following transfection was identified, indicating that biological integrity of nucleic acid solutions is maintained during the electrospray process.

Zeles-Hahn et al. failed to achieve transfection of luciferase DNA in the form of naked plasmid DNA, lipid/DNA complexes, polyethyleneimine/DNA complexes and poly L-lysine/DNA complexes into human airway epithelial cells.[9] They do not appear to have used a solvent and this may have necessitated high voltages in order to cause the solution to break up into a spray. Such voltages may damage the structure of the DNA plasmid and they report an increase in open circle and fragmented DNA at 6 kV and 7 kV.

The process of electrospray atomization involves the generation of an electric field between a charged solution and a grounded collector. As the electromagnetic field overcomes surface tension, the solution is dispersed into nano-sized particles that move towards a collector at high velocity. The characteristics of electrospray generated particles - size, charge, velocity and direction – may benefit delivery by potentially addressing factors that reduce the efficiency of nucleic acid delivery to tissue. Electrospray may create conditions where particles pass through a cell membrane by kinetic force, as happens with a 'gene gun'.[17] This may also be achieved by the interaction of charged particles with membrane-bound voltage gates or ion channels. In addition, the electric field associated with an electrospray may induce the transient formation of pores within the cell membrane, known as electroporation.[18]

Delivery of plasmid DNA to tissue can be challenging because the large size of these molecules can negatively impact upon cellular uptake. In addition, cellular uptake of DNA does not

guarantee protein expression as nuclear relocation must occur before protein encoding can commence. We hypothesized that the delivery of smaller molecules, such as mRNA, would increase the efficacy of protein expression by removing barriers associated with molecular size and ribosomal transcription. mRNA may also have therapeutic advantages over DNA, where factors such as transient protein expression may be an important consideration in drug development. In our study, we did not directly compare transfection efficiency of mRNA to plasmid DNA, however a 2 log increase in luciferase activity was seen with both plasmid DNA and mRNA compared to controls. Other groups have used GLuc plasmid DNA as a reporter for gene deliver to the lungs using lipid-mediated delivery to sheep lung tissue and electroporation-mediated delivery to mouse lungs.[12,19] Differences between models makes direct comparisons of delivery efficiencies difficult but demonstrate the utility of GLuc as a useful reporter for *ex vivo* and *in vivo* studies. Wider use of this tool in future studies should enable comparisons between various delivery methods.

Gene regulation, and therefore disease modulation, by siRNA molecules also have great potential for treating conditions such as cancer.[20] We have shown that fluorescent labeled siRNA molecules can be delivered to the epithelial and mucosal layers of lung tissue by electrospray. siRNA function was not assessed in the study and further studies are required to confirm knock down of target molecules using electrospray-mediated delivery of siRNA. If such studies prove successful, this therapeutic approach has potential to downregulate disease pathways during infection, inflammation or oncogenesis.[21–23]

This proof of concept study has not demonstrated functional consequences or therapeutic effects of nucleic acid delivery by electrospray. Neither has it been employed in a disease model. Further study will be required to target specific pathways in disease models. Assessing bronchoscopic delivery of reporter molecules *in vivo* is also a key future step.

In summary, in this study, we have shown that vector-free delivery of nucleic acids to lung tissue using electrospray is feasible. Furthermore, using an electrospray catheter, we have developed a technique that allows for bronchoscopic lung delivery, highlighting the potential of this technology to move from bench to bedside.

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Declaration of interest

This research is sponsored by Avectas Ltd. and may lead to the development of products which may be commercialized by Avectas Ltd., in which I have a business and/or financial interest. I have disclosed those interests fully to Taylor & Francis, and have in place an approved plan for managing any potential conflicts arising from this arrangement.

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References

[1] van Haasteren J, Hyde SC, Gill DR. Lessons learned from lung and liver in-vivo gene therapy: implications for the future. *Expert Opin Biol Ther*. 2018; 18(9):959–972.

[2] Duncan GA, Jung J, Hanes J, Suk JS. The mucus barrier to inhaled gene therapy. *Mol Ther*. 2016; 24(12):2043–2053.

[3] Hardee CL, Arévalo-Soliz LM, Hornstein BD, Zechiedrich L. Advances in non-viral DNA vectors for gene therapy. *Genes (Basel)*. 2017;8(2):65.

[4] Chan JGY, Wong J, Zhou QT, Leung SSY, Chan H-K. Advances in device and formulation technologies for pulmonary drug delivery. *AAPS PharmSciTech*. 2014;15(4):882–897.

[5] Gomes dos Reis L, Svolos M, Hartwig B, Windhab N, Young PM, Traini D. Inhaled gene delivery: a formulation and delivery approach. *Expert Opin Drug Deliv*. 2017;14(3):319–330.

[6] Jaworek A, Sobczyk AT. Electrospraying route to nanotechnology: an overview. *J Electrostat*. 2008; 66(3–4):197–219.

[7] Rosell-Llompart J, Fernández de la Mora J. Generation of monodisperse droplets 0.3 to 4 μm in diameter from electrified cone-jets of highly conducting and viscous liquids. *J Aerosol Sci*. 1994;25(6):1093–1119.

[8] Boehringer S, Ruzgys P, Tamo L, et al. . A new electrospray method for targeted gene delivery. *Sci Rep*. 2018;8(1):4031.

[9] Zeles-Hahn MG, Lentz YK, Anchordoquy TJ, Lengsfeld CS. Effect of electrostatic spray on human pulmonary epithelial cells. *J Electrostat*. 2011;69(1):67–77.

[10] McCrea Z, Arnanthigo Y, Cryan SA, O'Dea S. A novel methodology for bio-electrospraying mesenchymal stem cells that maintains differentiation, immunomodulatory and pro-reparative functions. *J Med Biol Eng*. 2018;38(3):497–513.

[11] McClory PJ, Håkansson K. Corona discharge suppression in negative ion mode nanoelectrospray ionization via trifluoroethanol addition. *Anal Chem*. 2017;89(19):10188–10193.

[12] Griesenbach U, Vicente CC, Roberts MJ, et al. Secreted Gaussia luciferase as a sensitive reporter gene for in vivo and ex vivo studies of airway gene transfer. *Biomaterials*. 2011;32(10):2614–2624.

[13] Rogers CS, Abraham WM, Brogden KA, et al. The porcine lung as a potential model for cystic fibrosis. *Am J Physiol Lung Cell Mol Physiol*. 2008;295(2): L240–L263.

[14] Judge EP, Hughes JML, Egan JJ, Maguire M, Molloy EL, O'Dea S. Anatomy and bronchoscopy of the porcine lung: a model for translational respiratory medicine. *Am J Respir Cell Mol Biol*. 2014;51(3):334–343.

[15] Ikemoto K, Sakata I, Sakai T. Collision of millimetre droplets induces DNA and protein transfection into cells. *Sci Rep*. 2012;2:1–5.

[16] Lee YH, Wu B, Zhuang WQ, Chen DR, Tang YJ. Nanoparticles facilitate gene delivery to microorganisms via an electrospray process. *J Microbiol Methods*. 2011;84(2):228–233.

[17] Zhang D, Das DB, Rielly CD. Potential of microneedle-assisted micro-particle delivery by gene guns: a review. *Drug Deliv*. 2013;7544:571–587.

[18] Lambricht L, Lopes A, Kos S, Sersa G, Préat V, Vandermeulen G. Clinical potential of electroporation for gene therapy and DNA vaccine delivery. *Expert Opin Drug Deliv*. 2015;5247:295–310.

[19] Dean DA, Machado-Aranda D, Blair-Parks K, Yeldandi AV, Young JL. Electroporation as a method for high-level nonviral gene transfer to the lung. *Gene Ther*. 2003;10(18):1608–1615.

[20] Golan T, Khvalevsky EZ, Hubert A, et al. RNAi therapy targeting KRAS in combination with chemotherapy for locally advanced pancreatic cancer patients. *Oncotarget* 2015;6:24560–24570.

[21] Zamora MR, Budev M, Rolfe M, et al. RNA interference therapy in lung transplant patients infected with respiratory syncytial virus. *Am J Respir Crit Care Med*. 2011;183(4):531–538.

[22] Lomas-Neira JL, Chung C-S, Wesche DE, Perl M, Ayala A. In vivo gene silencing (with siRNA) of pulmonary expression of MIP-2 versus KC results in divergent effects on hemorrhage-induced, neutrophil-mediated septic acute lung injury. *J Leukoc Biol*. 2005;77(6):846–853.

[23] Davis ME, Zuckerman JE, Choi CHJ, et al. Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles. *Nature*. 2010; 464(7291):1067–1070.