DEVELOPMENT OF LENTIVIRAL AIRWAY GENE THERAPY AEROSOL DELIVERY TECHNIQUES FOR CYSTIC FIBROSIS

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ABSTRACT

Lentiviral (LV) vectors show promise as a gene therapy vector for cystic fibrosis (CF). The cystic fibrosis airway gene therapy group (CFARG) based in Adelaide, Australia have developed a HIV-1 based LV vector that demonstrated expression of the corrective CFTR gene up to 12 months in the airways of CF mice. Using their two-step airway conditioning and bolus gene vector delivery technique, the CFARG have shown effective transduction in the airways of animal models such as sheep and marmosets. As this vector approaches clinical realisation there is a need to translate the bolus delivery regimen of this vector to an aerosol form. Aerosolisation would enable non-invasive and easily repeatable vector delivery, which could be used in future clinical trials.

This thesis examines the efficiency of different delivery devices for aerosolising the LV vector. Cell culture studies showed that LV vector aerosolised using a newly developed ultrasonic surface acoustic wave (SAW) nebuliser produced significantly lower levels of gene expression than bolus delivery. This led to examination of an intra-tracheal sprayer, the MADgic™ atomisation device, which demonstrated promising results on delivering the vector as a spray, in cell culture studies. However, use of this device in human clinical studies is invasive. Therefore, the efficacy of delivering LV vector as an aerosol through other nebulisers was investigated.

A baseline *in vivo* study was designed to aerosolise the LV vector into the lungs of mechanically ventilated mice using an Aeroneb®Pro vibrating mesh nebuliser with a flexiVent™ small animal ventilator. This was the first study to compare the levels of gene expression produced by a HIV-based LV vector delivered either as an aerosol or as a bolus dose into mouse lungs. Lower levels of gene expression were obtained in the trachea of

aerosol-treated animals compared to bolus-treated animals. However, the effect of LV aerosol delivery could not be determined in other conducting airways or the lung parenchyma, due to low power of the study produced by a substantial outlier producing a far larger than expected variability. The reason for the lowered gene expression observed in the trachea of mice treated with LV vector delivered as an aerosol through the Aeroneb®Pro-flexiVent™ ventilator apparatus was not conclusive.

Further experiments investigated the cause of the low levels of gene expression observed in the baseline *in vivo* study. Bench studies with dye solution revealed that the physical dose volume that reached the tip of the endotracheal (ET) tube following delivery through the ventilator circuit was only 2% of the initial dose volume, which likely explained the low levels of gene expression in trachea of aerosol-treated animals. The delivery parameters were therefore optimised to increase the aerosol output available at the end of the delivery circuit.

Subsequent cell culture studies examined the gene expression produced by the LV vector aerosolised with parameters used in the baseline *in vivo* study. The results demonstrated lowered gene expression produced by LV vector released at the end of the ventilator circuit compared to bolus delivery. To address this problem, different protective diluents were tested to try to preserve LV viability following aerosol delivery. Of these, FreeStyle™ medium produced higher levels of gene expression than our standard diluent, mouse serum in saline.

On further examination, the LV vector suspended in an optimal diluent combined with optimal aerosolisation parameters produced higher levels of gene expression compared to baseline *in vivo* delivery parameters. However, despite these improvements the levels of gene expression produced by LV aerosol delivery was still significantly lower than bolus delivery. Hence, these studies indicate that the Aeroneb®Pro nebuliser was not ideal for aerosolising

the LV vector in its current formulation.

Together the results presented in this thesis highlighted the problems associated with aerosolising a HIV-based LV vector using different nebulisers and through a small-animal ventilator circuit. Although the nebulisers investigated in this thesis were not efficient for aerosolising LV vector, an intra-tracheal sprayer suitable to deliver this vector for testing in larger animal models was identified. Future research should examine other newly introduced nebulisers to develop an optimal vector delivery protocol for use in CF airway gene therapy.

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ABBREVATIONS

^{99m}Tc-DTPA	^{99mt}technetium saline diethylene-triamine penta-acetic acid
Ad	Adenovirus
AAV	Adeno associated virus
AAD	Adaptive aerosol delivery
ANOVA	Analysis of variance
ASL	Airway surface liquid
BSA	Bovine serum albumin
CA	Capsid
cAMP	Cyclic adenosine 5'-monophosphate
$CaPO_4$	Calcium phosphate
$CaCl_2.2H_20$	Calcium chloride dehydrate
CF	Cystic fibrosis
Cl^-	Chloride ion
CFTR	Cystic fibrosis transmembrane conductance regulator
cDNA	Complementary deoxyribonucleic acid
CHO-K1	Chinese hamster ovary K1
CO_2	Carbon dioxide
cPPT	Polypurine tract in the central position
Ct	Cycle threshold
DC	Duty cycle
DMF	Dimethylformamide
DMEM	Dulbecco's modified Eagle's medium

DNA	Deoxyribo-nucleic acid
DPI	Dry powder inhaler
EboZ	Ebola virus
EDTA	Ethylenediaminetetraacetic acid
EIAV	Equine infectious anaemia virus
ENaC	Epithelial sodium channel
ET tube	Endo-tracheal tube
FCS	Fetal calf serum
FEV_1	Forced expiratory volume measured during the 1^{st} second
FIV	Feline immunodeficiency
F-Luc	Firefly luciferase
FreeStyle™ medium	FreeStyle™ 293 expression medium
GFP	Green fluorescent protein
HA	Haemagglutinin
HCO_3^-	Bicarbonate ions
HCl	Hydrochloric acid
Hd-Ad	Helper-dependent adenovirus
HEK-293T	Human embryonic kidney 293T cells
HIV	Human immunodeficiency virus
H & E	Haematoxylin and eosin
IDT	Interdigital transducer
IN	Integrase
I:E	Inspiration: Expiration

i.p.	intra-peritoneal
KCl	Potassium chloride
$K_3Fe(CN)_6$	Potassium ferricyanide
$K_4Fe(CN)_6$	Potassium ferrocyanide
LacZ	β-galactosidase
$LiNbO_3$	Lithium niobate
LPC	L-α-lysophosphatidylcholine
LTR	Long terminal repeats
Luc	Luciferase
LV	Lentivirus
MA	Matrix
MCC	Mucociliary clearance
$MgCl_2$	Magnesium chloride
$MgCl_2.6H_2O$	Magnesium chloride hexahydrate
mRNA	Messenger ribonucleic acid
MSD	Membrane spanning domain
MS	Mouse serum
MS/saline	Mouse serum in saline
MPSV	Myeloproliferative sarcoma virus
MV	Minute ventilation
Na^+	Sodium ion
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NC	Nucleocapsid

NBD	Nucleotide binding domain
NBF	Neutral buffered formalin
OGTR	Office of the gene technology regulator
ORF	Open reading frame
PBS	Phosphate buffered saline
PC1	Physical containment 1
PC2	Physical containment 2
PEEP	Positive-end expiratory pressure
PFA	Paraformaldehyde
pDNA	Plasmid deoxyribose-nucleic Acid
pen/strep	Penicillin/ Streptomycin
pMDI	Pressurised metered dose inhalers
PR	Protease
qRT-PCR	Quantitative real-time polymerase chain reaction
R-Luc	Renilla luciferase
RR	Respiratory rate
RNA	Ribonucleic acid
RT	Reverse transcriptase
SAW	Surface acoustic wave
Saf O	Safranin O
S.D.	Standard deviation
SeV	Sendai virus
SIV	Simian immunodeficiency virus
SO_2	Sulphur dioxide

V_T	Tidal volume
VSV-G	Vesicular stomatitis virus G glycoprotein
v/v	Volume/volume
w/v	Weight/volume
WPRE	Woodchuck post-transcriptional regulatory element

DECLARATION

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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1 INTRODUCTION

Cystic fibrosis (CF) is an early-fatal, autosomal recessive disorder prevalent in people with European ancestry[1]. It affects one in 2,800 live births in Australia, and one in 25 people are non-symptomatic carriers of the cystic fibrosis transmembrane conductance regulator (CFTR) gene mutation[1]. CF mainly affects organs with mucus secreting glands, such as the lungs, pancreas, gastro-intestinal tract, biliary tract, vas deferens, and sweat glands[2]. The cornerstone for CF treatment in the early half of the 19th century was pancreatic enzyme replacement[3]. Managing nutritional intake and early aggressive treatment of respiratory symptoms has improved life expectancy of CF patients to a median predicted age of ~37 years[4, 5]. Today, the leading cause of morbidity and mortality in CF patients is lung disease[6].

1.1 *CFTR* gene

The *CFTR* gene was first sequenced in 1989 by positional cloning and chromosome walking techniques[7-9]. This gene is present on chromosome 7q31.2 and spans approximately 190 kilo base (kb) pairs of genomic DNA, containing 27 exons[10]. This gene codes for a CFTR protein, consisting of 1,480 amino acids, with a mass of 168 kilo Daltons[10]. In healthy individuals, the CFTR protein functions as a cyclic adenosine 5'-monophosphate (cAMP) activated chloride ion channel that is present on the apical membrane of epithelial cells lining the airways, pancreas, intestines, reproductive organs, and exocrine glands[11, 12]. The CFTR protein consists of two halves, each with a membrane spanning domain (MSD1 and MSD2) made up of six alpha-helices. Each MSD is connected to a nucleotide binding domain (NBD1 and NBD2) and both halves of the CFTR protein are connected by the R domain[12] (Figure 1-1).

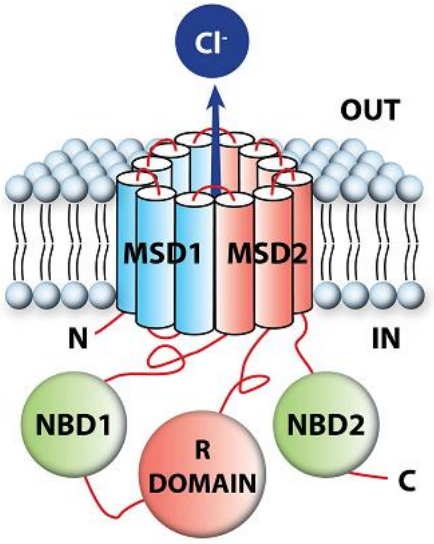

Figure 1-1: Structure of the CFTR protein showing two halves, each containing a MSD and NBD joined together by the R domain[12].

In healthy individuals, the CFTR channel is responsible for movement of chloride ions (Cl^-) and bicarbonate ions (HCO_3^-) from within the cell to airways[12]. CFTR also has direct and indirect control over other channels within epithelial cells, such as the epithelial sodium channel (ENaC). CFTR down regulates ENaC, which is responsible for cell uptake of sodium ions (Na^+)[13]. CFTR also controls other ion channels, such as the outwardly rectifying chloride channel, and calcium activated chloride channel. Thus, the CFTR channel affects overall molecular transport within the cell [14] and controls hydration of the epithelial surface[15].

In CF patients, absence of the CFTR protein or presence of a dysfunctional CFTR protein results in reduced chloride secretion and increased sodium and water absorption into tissues[11, 16]. This leads to dehydration of airway surface liquid (ASL) lining the respiratory tract, reduced mucociliary clearance (MCC), and accumulation of viscous mucus in the airways. This mucus obstruction triggers subsequent pathogenic infection, with bacteria including *Pseudomonas aeruginosa, Staphylococcus aureus,* and *Haemophilus influenzae,* which results in inflammation and reduction in lung function[11, 15, 17, 18]. This cycle of infection, inflammation,

and reduction in lung function ultimately results in respiratory failure, the leading cause of death in CF patients[19, 20] (Figure 1-2).

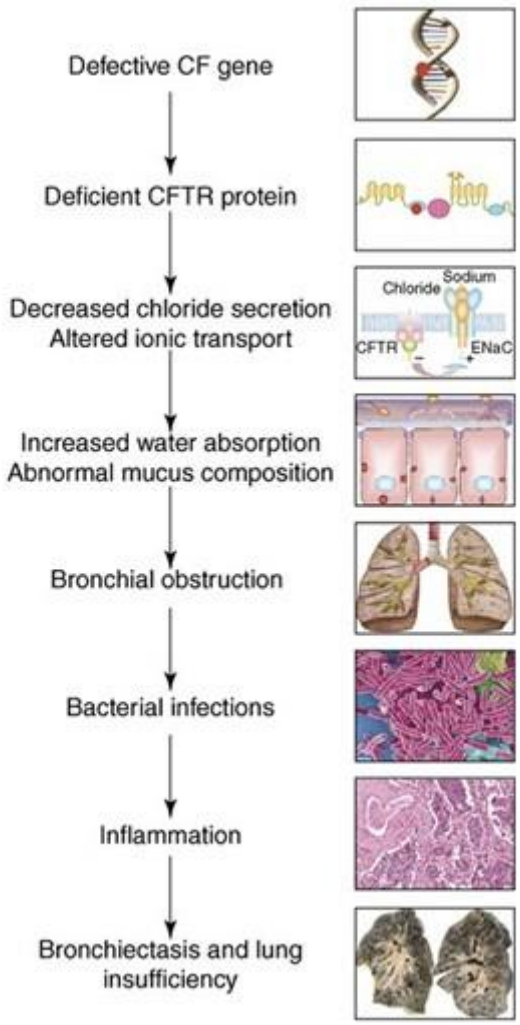

Figure 1-2: CF pathogenesis cascade, demonstrating that a defective CFTR gene leads to decreased chloride secretion and altered ionic conductance, bronchial obstruction, inflammation, and ultimately lung insufficiency[21].

Nearly 2000 *CFTR* gene mutations have been identified to date, and these can be categorised into six different classes[16, 22, 23]. In class I mutations CFTR protein synthesis is absent; in class II the protein is inadequately processed; class III demonstrates defective regulation of the CFTR protein; class IV shows reduced CFTR channel conductance; class V is characterised by reduced synthesis of the CFTR protein; and class VI presents decreased CFTR protein stability

[20, 22, 24]. Mutation classes I to III are more severe compared to classes IV to VI, as the latter classes retain some level of CFTR function[25].

1.2 Current treatments for CF

Current CF treatments target downstream symptoms of the defective *CFTR* gene, such as mucus accumulation, bacterial infection, inflammation, and bronchial hydration[26, 27]. Mucolytics, like α-dornase, are administered to reduce mucus viscosity and increase MCC[28]. Osmotic agents are also prescribed to hydrate the ASL and improve MCC in CF patient airways[26]. Commonly prescribed osmotic agents include hypertonic saline and mannitol[26]. Antibiotics, such as inhalable tobramycin powder, amikacin, levofloxacin, and colistin, are prescribed to target infection in CF airways[26]. Neutrophil mediated airway inflammation is prevalent in CF patients for which anti-inflammatory medicine, such as prednisone (steroids) and ibuprofen (non-steroidal), are recommended[28]. Additionally, patients undergo physiotherapy and airway clearance techniques to assist with sputum clearance[29]. Despite these treatments, lung disease remains the main cause of mortality in CF and lung transplant is required for end-stage patients with severe lung disease[30].

Therapeutic drugs specific to CF mutation classes include Ivacaftor (Kalydeco®) a CFTR 'potentiator', that improves CFTR channel gating and is designed to treat CF patients with class III mutation, such as the G551D and R117H gating mutations[31, 32]. CF patients with the G551D mutation treated with Ivacaftor have demonstrated an average 10% improvement in lung function[32]. However, patients with at least one gating mutation are rare and represent only 4% of CF patients[31, 33, 34]. Lumacaftor is a 'corrector' that improves processing and stability of CFTR with F508del, a class II mutation. *In vitro* studies using bronchial cells isolated from CF patients have shown that Lumacaftor improved maturation of CFTR F508del and

increased chloride secretion by four-fold. However, a combination of Lumacaftor/Ivacaftor (Orkambi™) improved lung function by only 3% in CF patients with F508del mutation[35]. Another drug combination used for CF patients is Tezacaftor-Ivacaftor (Symdeko™), which provides two correctors and one potentiator to the airways of patients[36]. Cell culture studies using this drug combination have shown restoration of >50% CFTR activity and a stage I trial has shown to improve lung function of CF patients with F508del mutation by 7%-12%[36]. Other modulators, such as VX-445 and VX-659 are also being examined for their effectiveness in improving lung function of CF patients[36, 37]. Despite improvement in pharmaceutical therapies targeted at improving CFTR function there is a need to investigate new treatment methods that correct the initial CF gene defect and provide long-term, therapeutic benefits to CF patients belonging to all *CFTR* mutation classes.

1.3 CF airway gene therapy

CF airway gene therapy is an alternative therapeutic option to overcome the underlying gene defect by either correcting the *CFTR* gene (gene-editing), or supplementing with corrected copies of the *CFTR* gene (gene-addition)[6, 38]. The principle of airway gene-addition therapy is to use a vector to deliver a correctly functioning copy of the *CFTR* gene to relevant cells of the respiratory epithelium, thereby restoring normal cellular homeostasis and lung function[29, 39].

Studies reported in the literature suggest that restoration of CFTR function in 6-10% of cells is sufficient to bring about phenotypic correction[40, 41]. The main targets for CF gene therapy are ciliated epithelial cells[39, 42], submucosal glands[42], and basal cells[43] in the larger and smaller conducting airways. Basal cells are the airway progenitor cells for ciliated epithelial and submucosal cells[43]. Furthermore, these basal cells are also responsible for maintenance and repair of the human airway[43]. Hence, transducing basal cells could provide long-term

correction of the CF gene defect as they can generate corrected daughter cells thus improving the therapeutic benefits[43]. Transducing these basal airways cells using an effective single dose-protocol reduces the need to re-administer the viral vector formulation to patient airways[43].

Another type of cell that can be targeted in future studies to produce effective correction in the airways is the ionocytes. Recently published studies using single-cell RNA sequencing methods have identified that this new cell type, present in the proximal airways, expresses about 60% of CFTR activity in mice and humans, but represent only 1% of the airway cells [44, 45]. This makes them ideal targets for future CF gene-editing and cell therapy[46]. Various viral and non-viral vectors have been developed to deliver the therapeutic gene to the airway epithelium[39], but efficient delivery of gene vectors to appropriate sites in the airway remains crucial for successful treatment[39].

1.4 Challenges for delivery to the lung

The lung was initially thought to be an easily accessible organ for gene vector delivery because the vector could reach airway epithelial cells by non-invasive methods, and the possibility of the vector targeting other organs was reduced[47]. However, challenges in delivering vector formulations to the lung have become apparent in airway gene therapy studies[16]. The lungs have a series of intracellular and extra-cellular barriers that pose a challenge for effective vector administration[16]. Gene therapy vectors have to overcome complex host mechanisms that fight invasion of foreign particles in the lung, including (1) a mucus layer that can bind the vector and remove it via MCC mechanisms[48], (2) a glycocalyx protein layer that can prevent binding of vector to its receptors[48], and (3) host immune responses to the vector, which can reduce effectiveness on repeated administration[48]. To overcome these barriers, it

is important to develop an optimal gene vector and an optimal vector delivery method.

1.5 Vector delivery methods

Gene vectors used for CF gene therapy have predominantly been delivered as a bolus dose to the airways of animals and CF patients[49-51]. These bolus delivery studies have reported small but significant physiological benefits[52, 53]. However, delivering the vector as bolus dose can be invasive as the patient is likely to require sedation and a bronchoscopy procedure[49, 54, 55]. Additionally, *in vivo* studies have shown that delivering viral vectors as a bolus dose produces patchy gene expression in the airways[54, 56, 57]. This patchy gene expression possibly results from uneven distribution of the vector or pooling of the bolus dose in certain airway regions[58] and uneven coverage of the airway surfaces[57]. Researchers have speculated that chronic inflammation and injury of lung tissue could also lead to patchy gene expression[59]; however, this gene expression pattern does not inhibit the expression of a functional *CFTR* gene in animal models[57]. Regardless of this, the inability to easily, rapidly, and uniformly, deliver a gene vector to the conducting airways led to a search for alternative delivery methods.

Aerosol delivery was considered an ideal delivery method as it is minimally-invasive, easily repeatable, and is likely to be a more clinically acceptable method of vector administration[54, 60]. Delivering the viral vector as an aerosol has been demonstrated to produce uniform vector distribution in the airways of some animal models[54]. However, these studies highlighted problems associated with aerosolising viral vectors. One major issue is reduced viability of some viral vectors, like adenovirus (Ad) and sendai virus (SeV), during transit through the nebuliser[58]. This lowered vector viability is probably due to shear stress applied on the vector during transit through the nebuliser[58]. The process of aerosolisation can produce a change in temperature, concentration, and pH of the liquid formulation[61-63], which can also affect the

biological viability of a viral vector[64, 65]. Hence, it is critical to use an appropriate nebuliser to aerosolise the viral vector to obtain a positive therapeutic outcome.

1.6 Aerosol delivery devices

Success of developing a viable aerosol gene therapy treatment is dependent on identifying a suitable vector and then delivering it using an appropriate aerosol delivery device[66]. There are many delivery devices available in clinics to aerosolise drug formulations to the airways of CF patients. A brief description of the devices, which have been used primarily in drug delivery studies and a few gene therapy studies, are described below.

1.6.1 Dry powder inhalers

Dry powder inhalers (DPIs) are a popular aerosol delivery device as they are small and easily transportable[67] (Figure 1-3). The capsule containing the drug is placed in a spinning chamber, where it is punctured by piercing buttons[68]. The drug is then broken down into finer particles by the inspiratory force of the patient. During inhalation the drug is filtered as it passes into the mouth piece[68]. These devices are available in a single capsule-based design or multi-dose units[68]. DPIs can deliver a larger dose volume of drugs to patient airways (approximately 10% and 37%) compared to the 7% dose volume delivered by pressurised metered dose inhalers (pMDIs; see Section 1.6.2)[68]. Drugs such as mannitol, colistin mixed with lactose-carrying particles, and thrombomycin, have been delivered using DPIs to the airways of CF patients[68]. However, DPIs also pose certain disadvantages. Many drugs are not available in a powdered form and hence cannot be used by DPIs[69]. Also, elderly patients with chronic lung disease find it difficult to apply sufficient inspiratory force to aerosolise the drug using DPIs[68]. The same may be true for some CF patients.

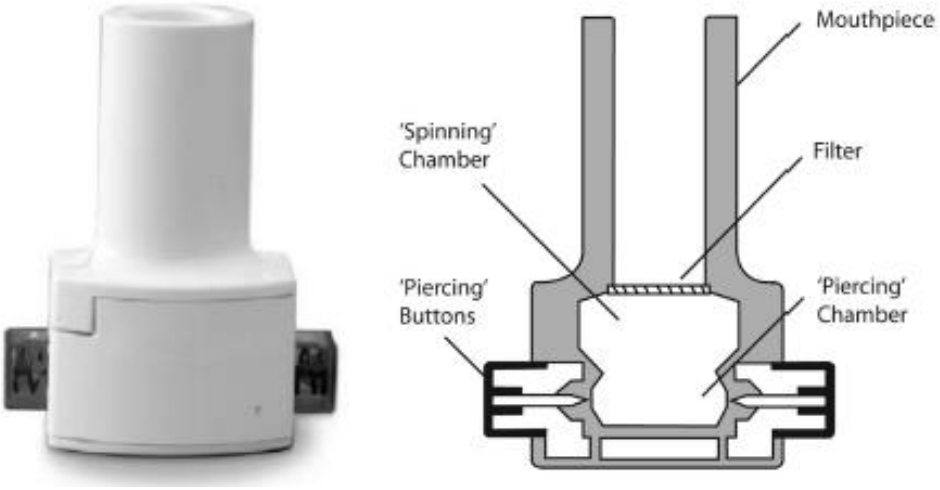

Figure 1-3: A dry powder inhaler with a single capsule-based design[69].

1.6.2 Pressurised metered-dose inhalers

Pressurised metered-dose inhalers (pMDIs) were introduced in 1956 at Riker laboratories and became popular due to small size, portability, and efficiency (Figure 1-4 A). The pMDI consists of three main components: a canister containing the drug dissolved in liquid gas propellant; a metering valve, which delivers a known amount of the drug; and a spray actuator[68]. When the patient presses the canister, a mixture of propellant and drug formulation is propelled into the expansion chamber where it is sheared by the actuator nozzle to produce aerosols[68] (Figure 1-4 B). Despite the popularity of these inhalers, they have several disadvantages such as high particle delivery velocity, deposition of majority of the dose volume at the oropharynx, and the patient must learn the technique of coordinated actuation and inspiration[67]. These inhalers can only deliver a small quantity of the drug which was the main disadvantage in the case of drugs used for the treatment of CF. The pMDI is commonly used to deliver small quantities of drugs, such as beta-agonists, anticholinergic agents, and inhaled corticosteroids[67].

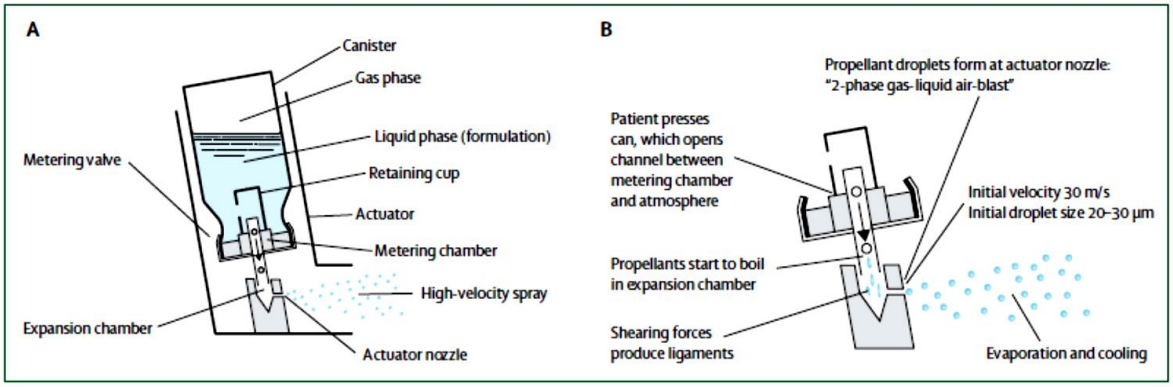

Figure 1-4: (A) Parts of pMDI and (B) formation of aerosol when the formulation is propelled into the expansion chamber[68].

1.6.3 Jet nebulisers

Jet nebulisers are devices that evolved along with the advent of DPIs and pMDIs. Jet nebulisers gained popularity in inhalation therapy as they can aerosolise a large volume of liquid medication[70]. The jet nebuliser works by releasing aerosols when a jet of compressed air is pushed through a small tube to disperse a thin film of liquid[67] (Figure 1-5). The size of the primary aerosols generated are ~100 µm, which is not suitable for inhalation[71]. Hence baffles are installed within the jet nebuliser to break down larger aerosols into smaller ones (< 5 µm) for deposition in the bronchial and alveolar region of the lung[67, 71]. These nebulisers are efficient in delivering highly viscous formulations, such as dextrose solution[72]. However, one drawback of this nebuliser is that it increases drug concentration in the reservoir and has a large residual volume (1 to 1.5 ml)[67]. Moreover, conventional jet nebulisers operate continuously and result in substantial loss of drug formulation during exhalation[67]. This led to the invention of the breath-actuated jet nebuliser[67].

The breath-actuated nebulisers (also known as breath-enhanced nebulisers) are a type of jet nebuliser that deploys a valve system to control aerosol release during the inspiratory phase[67]. PARI LC PLUS® (PARI Gmbh, Stamberg, Germany), PARI LC SPRINT® (PARI Gmbh,

Stamberg, Germany), LC STAR® (PARI Gmbh, Stamberg, Germany), and Ventstream® (Respironics respiratory drug delivery, New Jersey) are popular devices that incorporate this mechanism[67]. There have also been various modifications to jet nebulisers such as adaptive aerosol delivery (AAD), which adapts to the breathing pattern of individuals and delivers the drug in pulses during the first half of the patient's inspiratory phase[70]. Devices like AeroEclipse® (Trudell medical international, Canada), HaloLite® (Respironics respiratory drug delivery, New Jersey), and ProDose® (Respironics respiratory drug delivery, New Jersey), incorporate this design[67]. Jet nebulisers are used to aerosolise various drugs, such as antibiotics, mucolytics, and recombinant products, such as rhDNaSe. In CF, the jet nebuliser is used to deliver drugs like Pulmozyme® (Dornase alpha) and Tobramycin[72].

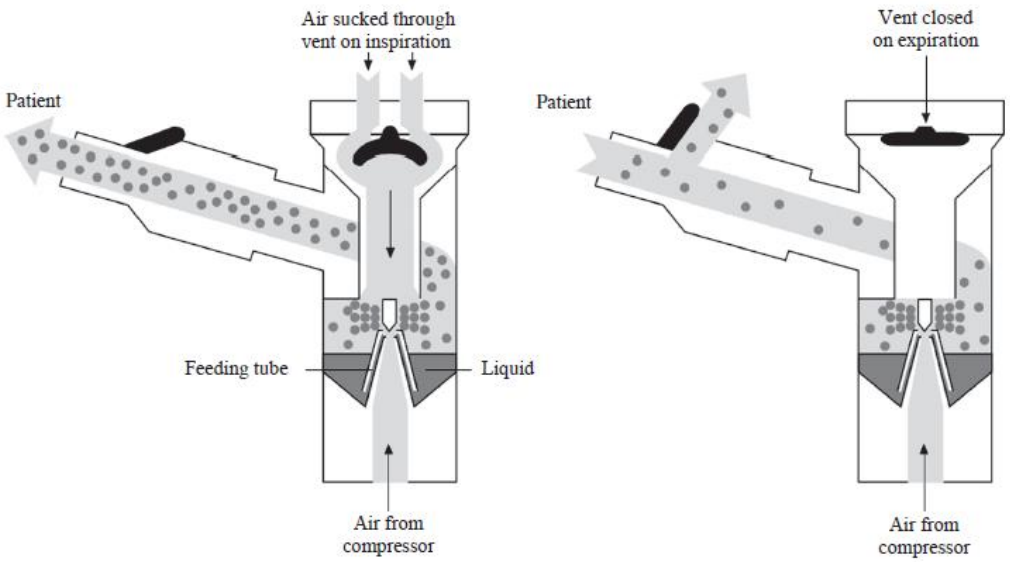

Figure 1-5: Mechanism of aerosolisation in a jet nebuliser[72].

1.6.4 Vibrating mesh nebulisers

Recent advances in technology led to introduction of the vibrating mesh nebuliser, which addresses some problems associated with earlier nebulisers[67]. Vibrating mesh nebulisers use "micro-pump technology" to generate aerosols[73]. These nebulisers have a mesh plate

containing up to 1,000 dome shaped apertures and a vibrating element[73] (Figure 1-6). When an electric current is applied the mesh moves up and down by a few micrometres, with the force of displacement generating aerosols[73]. Aerosols delivered by the vibrating mesh nebuliser are 1 -5 µm in diameter, which is optimal for deposition of aerosols in the bronchial airway and lung parenchyma[73]. Vibrating mesh nebulisers have several advantages, such as being easily portable, noise-less, and single-pass, i.e. these nebulisers do not recycle the formulation within the system[67]. Compared to jet nebulisers, vibrating mesh nebulisers produce high output rates, can aerosolise small volumes of drug formulation and have almost no residual volume[72]. Furthermore, they can also be breath actuated to release aerosols during inspiration only[74]. However, one disadvantage of vibrating mesh nebulisers is that they cannot efficiently aerosolise viscous formulations. Commercially available nebulisers incorporating this design are the Aeroneb® Pro and Aeroneb® Go (Aerogen Inc., Ireland), eFlow® rapid (PARI Gmbh, Stamberg, Germany), and Omron MicroAir® (Omron Healthcare, Japan). The eFlow® rapid nebulisers have been used to aerosolise aztreonam lysinate, an antibiotic against *Pseudomonas aeruginosa* infection in the airways of CF patients[75]. Additional information about the Aeroneb®Pro nebuliser is presented in Chapter 3.

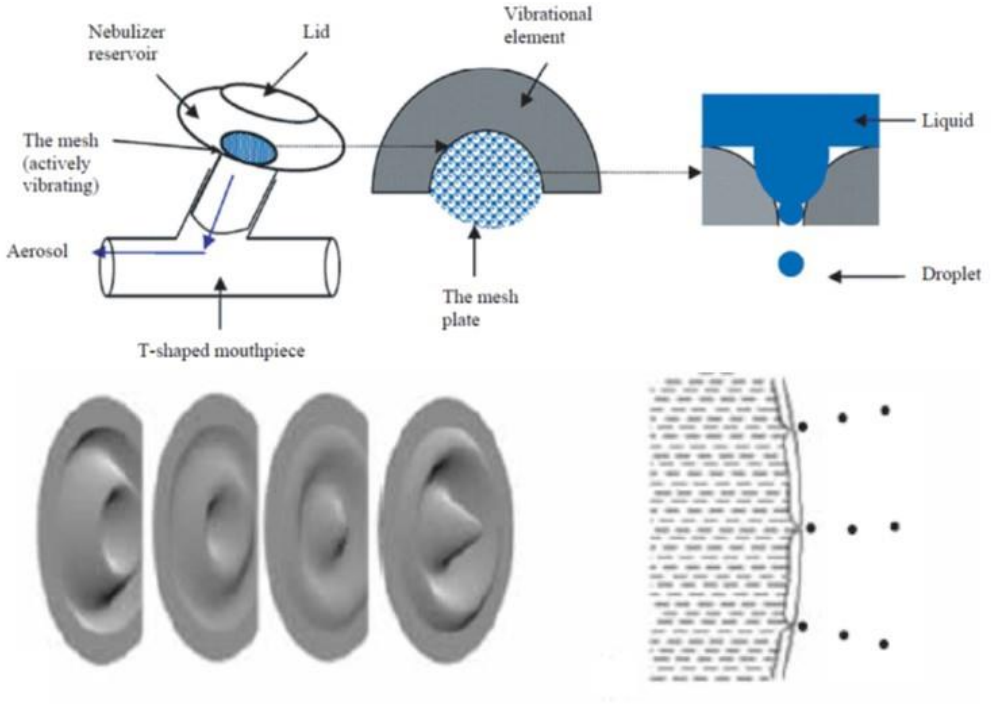

Figure 1-6: Vibrating mesh nebuliser showing the mesh plate with tapered holes and production of aerosols[72, 75].

1.6.5 Ultrasonic nebulisers

The ultrasonic nebuliser is another novel device with the potential to deliver large volumes of drug formulations[76]. Ultrasonic nebulisers are small, portable, noise-less, and do not require compressed air to deliver aerosols[77]. These nebulisers contain a piezoelectric crystal that vibrates at high frequencies (up to 3 MHz) on the liquid surface to generate aerosols[67, 77, 78]. However, the major disadvantage of these nebulisers is the heat generated during aerosol delivery, which could denature biological formulations[67]. Also, traditional ultrasonic nebulisers are not suitable for delivering suspensions and protein formulations[67].

A new type of ultrasonic nebuliser, called the surface acoustic wave (SAW) nebuliser, was recently developed. This nebuliser uses surface acoustic waves generated when a sinusoidal electric field is applied to an interdigital transducer (IDT) electrode[79] (Figure 1-7). The waves then propagate, at nanometre amplitude and MHz to GHz-order frequencies, through a

piezoelectric lithium niobate ($LiNbO_3$) substrate[79]. Upon reaching the edge of the substrate the waves are refracted into the adjoining liquid medium, generating aerosols[79]. This nebuliser can effectively aerosolise plasmid DNA (pDNA)[79] and stem cells[80].

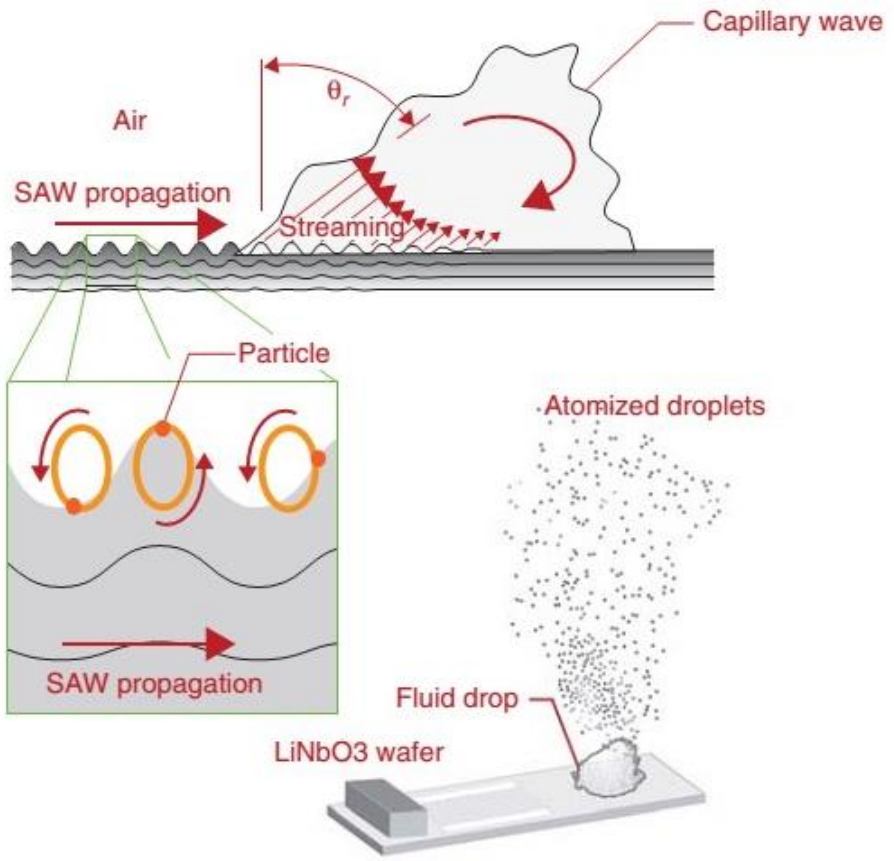

Figure 1-7: Propagation of SAWs through $LiNbO_3$ substrate which is then refracted into the liquid formulation to generate aerosols[78].

1.6.6 Intra-tracheal sprayers

Intra-tracheal sprayers or nebulisation catheters are used to deliver fluid formulation as a spray to the airways of patients and animal models [81, 82] (Figure 1-8). These devices typically have a barrel in which the formulation to be aerosolised is loaded. The device tip is then guided to the delivery location within the airway tree with the help of a bronchoscope[83]. Constant air pressure (30 to 40 psi) applied to the bottom of the syringe results in the

formulation passing through the sprayer nozzle at the tip to produce a plume of aerosol spray[83]. Although placement of this device into the airway is invasive and requires sedation and endo-tracheal intubation of subjects[84], it does enable targeted delivery of formulation to a particular region of the lung with a high degree of efficiency and control[83]. Intra-tracheal sprayers deliver aerosols of a large size (25 to 30 µm) to subject airways[85]. The majority of these larger sized aerosols released from intra-tracheal sprayers deposit in the tracheo-bronchial region of rats[86]. The Aeroprobe™ intra-corporeal nebulising catheter (Trudell Medical Corporation, London, Ontario, Canada) and Microsprayer® (PennCentury, Inc., Wyndmoor, PA) are two intra-tracheal sprayers commonly used to deliver drug formulations to animals[83, 85, 87]. These devices have demonstrated effective delivery of shear sensitive formulation, such as fibroblast cells[85], and liposomes[83]. Production of both the Microsprayer® and AeroProbe™ catheter has now halted (PennCentury™ closed for business in 2015, and Trudell discontinued the AeroProbe™ in its product line) and new sprayers like the MADgic™ atomisation device (Teleflex, Ireland) have been developed.

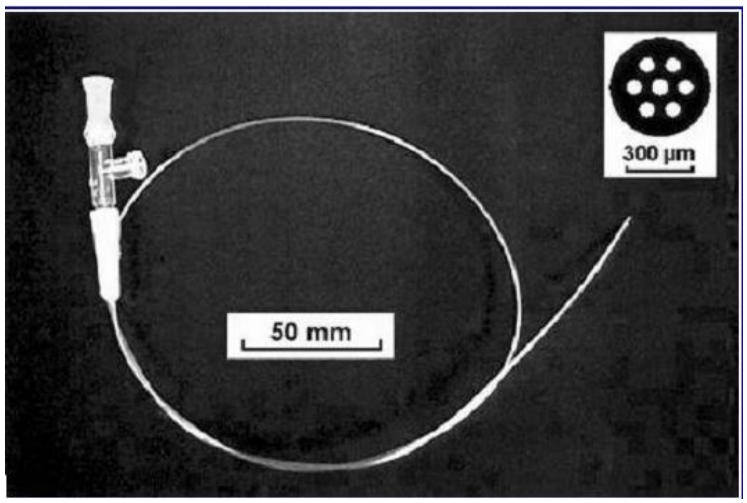

Figure 1-8: Aeroprobe™ intracorporeal nebulising catheter showing the sprayer nozzle at the tip[86].

1.7 Viral vectors for CF gene therapy

Since the discovery of the *CFTR* gene in 1989, there has been extensive research to develop an optimal viral vector that delivers the therapeutic *CFTR* gene to the lungs of the CF patients.

1.7.1 Adenoviral vectors

Gene therapy studies in the 1990's were carried out using recombinant adenoviral (Ad) vectors. Ad is a type of non-enveloped virus containing double-stranded DNA, and has a complex icosahedral capsid[88]. The Ad vector could transduce non-dividing cells and could accommodate the 4.5 kb human CFTR complementary DNA (cDNA) in its genome[89].

Initial gene therapy studies examined the effectiveness of delivering the Ad vector as an aerosol in animal models. Katkin et al. aerosolised Ad vector carrying a β-galactosidase (*LacZ*) reporter gene to the airways of cotton rats using a raindrop jet nebuliser (Puritan-Bennett Corporation) and showed uniform gene expression[54] (Figure 1-9 A). In comparison, patchy gene expression was observed when the same vector was delivered as an intra-tracheal bolus instillation (Figure 1-9 B)[54]. They also showed that approximately 22% of initial dose of the viable vector was recovered following delivery through the jet nebuliser[54]. Later studies used other jet nebulisers to deliver Ad vector aerosols to the lungs of larger animal models, such as rhesus macaques and baboons[90, 91]. These studies demonstrated effective transduction of animal airways along with dose dependent inflammation[90, 91]. However, a reduction in vector viability following delivery through an Optineb® jet nebuliser (Air Liquide, France) (approximately 43% of initial dose) and reduced dose volume (approximately 45% of initial volume) reaching the airways of the rhesus macaques was also reported[91]. The results obtained from aerosol delivery of Ad vector in animal models led to clinical studies in CF patients. Bellon et al. were the first to deliver aerosols of Ad-CFTR vectors to the lungs of CF

patients along with nasal instillations[92]. Expression of CFTR DNA was observed for up to 21 days, and they also detected the presence of CFTR mRNA in patient bronchial brushings up to 15 days with no evidence of acute inflammation in patient lungs[92]. The outcomes of other pre-clinical and clinical studies delivering Ad vector as an aerosol are given in Table 1-1.

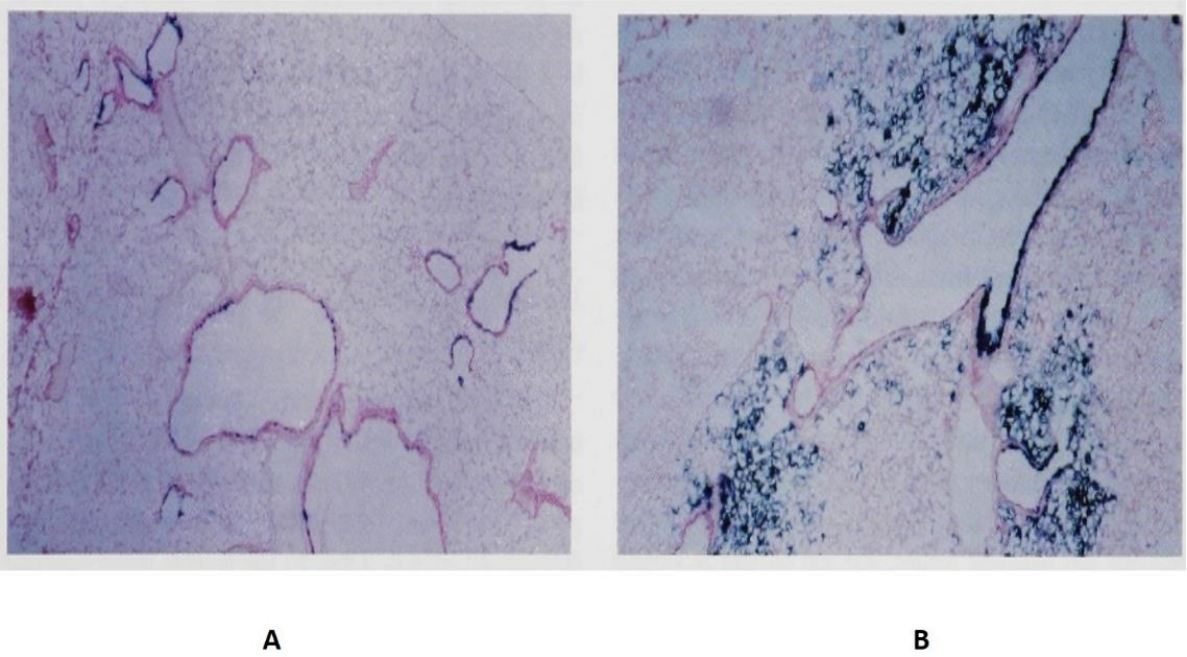

Figure 1-9: Study conducted by Katkin et al. showing lung sections of cotton rat exposed to Ad-LacZ vector delivered as an (A) aerosol and (B) bolus dose delivery[54].

Overall, the recombinant Ad vector utilised in CF gene therapy studies provided useful information on vector development and administration strategies. Gene therapy studies using this vector demonstrated a small but clinically insignificant correction of the CF gene defect[93]. The lowered clinical efficacy observed using the Ad vector was caused by inability of the vector to access receptors on the basolateral surface[94]. Furthermore, this vector (1) was non-integrating and therefore only produced transient gene expression, (2) produced dose dependent lung inflammation, (3) demonstrated reduced viability following delivery through the nebuliser, and (4) exhibited a reduced efficacy on vector re-administration[95]. These features led to the search for other viral vectors for CF airway gene therapy.

Table 1-1: Outcome of pre-clinical and clinical studies delivering Ad vector as an aerosol.

Study	Aerosol delivery device	Outcome
Pre-clinical Studies: **1) Sene C et al.[91], 1995**	Optineb® nebuliser (breath-activated jet nebuliser)	Delivered Ad vector as an aerosol to the lungs of Rhesus monkeys. The researchers detected CFTR mRNA up to 13 days following delivery of Ad vector; however, pneumonia was observed in a few animals in the study.
2) Jobe et al.[96], 1996	Low-flow prototype gas jet aerosoliser (Baxter labs)	Lungs of rabbits treated with aerosols of Ad-luciferase (Luc) demonstrated 30% gene expression in whole lung, while bolus delivery produced 72% gene expression.
3) McDonald, RJ et al.[97], 1997	Mini Heart breath actuated jet nebuliser (Westmed, Arizona, US)	Delivered aerosols of the Ad-*CFTR* to the airways of rhesus monkeys, detected the presence of CFTR mRNA up to 21 days following vector treatment. The researchers also showed evidence of bronchointerstitial pneumonia 7 days after vector delivery.

Study	Aerosol delivery device	Outcome
4) Lerondel, S et al.[90], 2001	Sidestream®/Optineb® (breath-activated jet nebuliser)	Showed homogenous distribution of aerosolised Ad-CFTR vector coupled with a radio-isotope in the lungs of baboons. They showed that only 42.5% of the initial dose deposited in the animal airway and the biological viability of the aerosolised vector was 45% of the initial titre.
Clinical Studies: **1) Harvey et al.[98], 1999**	Bronchial sprayer Olympus PW-6P washing pipe; (Olympus America Inc, NY)	Delivered a spray of Ad vector to CF patients and detected CFTR mRNA in the lungs of the patients lasting less than 30 days and observed mild inflammation from bronchial brushings. Intermediate cDNA gene expression was observed after second vector administration; however, no expression was observed after third dose of the vector.
2) Joseph, PM et al.[93], 2001 **Perricone MA et al.[99], 2001**	Mini Heart breath-actuated jet nebuliser	The change in pulmonary function (measured as FEV_1) was small and variable in both treatment groups and FEV_1 levels returned to baseline, 28 days post-treatment. Transient fever was experienced in patients receiving bolus dose, but no such symptoms observed in patients in the aerosol group.

1.7.2 Helper-dependent adenoviral vectors

Helper-dependent adenoviral (Hd-Ad) vectors were developed to reduce the immune response produced by Ad vectors[100]. All viral coding regions of the Ad vector were deleted to produce Hd-Ad, hence giving it the name "gutted vector"[100, 101]. Hd-Ad vectors demonstrated enhanced gene expression compared to Ad vectors[102]. Researchers examined the efficiency of delivering this vector as a spray into the airways of animal models. Kohler et al. sprayed Hd-Ad vector into the lungs of rabbits using an intra-tracheal AeroProbe™ catheter[81]. The vector was formulated in 0.1% L-α-lysophosphatidylcholine (LPC), a mild surfactant naturally present in the lung[38]. LPC was used to open tight junctions between epithelial cells and allow viral vector access to its receptors on the basolateral surface[51, 81]. The researchers demonstrated transduction in 66% of tracheal epithelial cells and observed a mild fever and patchy pneumonia in vector treated animals[81]. Cao et al. sprayed Hd-Ad vector suspended in 0.01% LPC into the airways of pigs using an Aeroprobe™ catheter[103]. They reported uniform gene expression in major and minor bronchial airways and respiratory bronchioles in the left lungs[103]. However, no gene expression was observed in the right lung which was used as a negative control[103]. Furthermore, there was no inflammation following aerosol delivery of the Hd-Ad vector in pig airways[103]. Despite the positive results obtained from this study, inflammation and acute toxicity remains the major problem associated with Hd-Ad vector[104]. Furthermore, problems producing this vector on a large scale continue to delay its clinical translation[104].

1.7.3 Adeno-associated viral vectors

Another vector extensively used for CF gene therapy treatment development is the recombinant adeno-associated viral (AAV) vectors[105]. AAV is a non-enveloped virus with an icosahedral capsid[105] enclosing a single DNA strand made up of 4,700 nucleotides[39].

Recombinant AAV is non-integrating and has an episomal nuclear configuration after transduction, unlike its wild type that integrates into the human genome[88]. There are many AAV vector serotypes. The first generation AAV serotype and AAV2 serotype were used in initial gene therapy studies[106, 107]. While newer serotypes like AAV1, AAV5, and AAV9 have been developed to improve efficacy[108].

The effectiveness of delivering AAV as an aerosol was examined in three clinical trials. Aitken et al. aerosolised first generation AAV-CFTR vector using a Pari LC Plus™ nebuliser into the lungs of 12 CF patients exhibiting mild lung disease[107]. The vector dose was found to be evenly distributed in the bronchial airways and was tolerated well in CF patients[107]. Bronchial brushing from patients revealed copies of the CFTR DNA up to 30 days, using real-time polymerase chain reaction (PCR)[107]. Following this, Moss et al. conducted a trial in 2004 in which three aerosol doses of either AAV2-CFTR vector or placebo (randomised 3:1) were delivered to the lungs of 37 CF patients with mild lung disease, at 30-day intervals. Repeated administration of the vector was well tolerated in CF patients[106]. Encouraging improvements in FEV_1 levels of >10% from baseline were observed in CF patients receiving the aerosolised vector dose compared to the placebo group at 30 days following treatment ($p=0.04$)[106]. Moss et al. in 2007 conducted a larger trial with 102 CF patients having mild to moderate lung disease[109]. The patients received either two aerosol doses of AAV2-CFTR vector or a placebo dose, administered 30 days apart[109]. This study showed that repeated administration of aerosolised AAV2-CFTR vector was safe and well tolerated in CF patients[109]. However, no significant difference in FEV_1 was observed between the aerosol treatment and placebo groups[109]. In a review, Guggino et al. speculated that the different outcomes between the two trials conducted by Moss et al. can be attributed to reducing the number of doses

administered from three doses to two[108], and/or including CF patients with mild lung disease. Also, the larger clinical trial (Moss et al. 2007) did not quantify the levels of CFTR DNA in patient airways thus making it difficult to compare the effectiveness of gene therapy across the two trials[108]. On closer examination of the Pari LC plus™ jet nebuliser used in all three clinical trials [106, 107, 109], Leung et al. observed that only 47% of the initial dose was delivered to patient airways[110]. They also reported an increase in vector concentration within the nebuliser as a result of evaporative losses; however, this nebuliser did not reduce the viability of the aerosolised vector[110].

Animal studies have examined the effectiveness of delivering the AAV vector through intra-tracheal sprayers. Beck et al. showed that saline radiolabelled with ^{99mt}technetium saline diethylene-triamine penta-acetic acid (^{99m}Tc-DTPA) delivered using the MicroSprayer® resulted in higher levels of dose deposition in the lungs of rhesus macaques compared to the Pari-LC Plus™ jet nebuliser[111]. The authors sprayed AAV2-green fluorescent protein (GFP) vector mixed with ^{99m}Tc-DTPA saline into the lungs of macaques using the MicroSprayer® and demonstrated a deposition of 50.1% and 30.3% of the initial dose in the right and left lungs, respectively[111]. In the same study, Beck et al. showed that there was no reduction in vector viability after being sprayed with the Microsprayer®[111]. In contrast, Guggino et al. demonstrated a reduction in vector viability (reduced by 27% to 35%) following delivery through the Microsprayer®[112]. Four other studies used the MicroSprayer® to deliver a coarse spray of AAV vector into the lungs of animals (Table 1-2). The effect of delivering this vector as an aerosol through a vibrating mesh nebuliser was also examined. Guggino et al. showed that an Aeroneb® solo nebuliser (Aerogen Inc., Ireland) delivered only 1.2% of the initial vector dose to the lungs of rhesus macaques[112]. These aerosol delivery studies demonstrated the

challenges of delivering AAV vector as an aerosol and also highlighted the importance of pairing vector formulation with appropriate delivery devices.

Newer AAV serotypes have been developed to better target the airway cells[108], but despite these advances the inability of the AAV vector to integrate into the host genome remains the major disadvantage[113]. This makes it challenging or impossible to provide long-term gene expression from a single dose[113]. Hence, there is a need to investigate the effectiveness of other viral vectors in producing long-term gene expression.

Table 1-2: Pre-clinical and clinical trials delivering AAV vector as an aerosol.

Study	Aerosol delivery device	Outcome
Pre-clinical studies: **1) Fischer, AC et al.[114], 2003**	MicroSprayer®	Repeated dosing of recombinant AAV2-*GFP* vector aerosols demonstrated effective gene transfer without inflammatory responses in rhesus macaques. GFP-DNA and mRNA were detected from the lungs of treated animals up to 8 weeks after vector treatment. The researchers demonstrated 50.3% deposition of initial dose of aerosolised vector in the airways of the animals.

Study	Aerosol delivery device	Outcome
2) Fischer, AC et.[115], 2007	MicroSprayer®	Aerosolised either AAV5 -CFTR vector or AAV5-GFP reporter gene to the lungs of rhesus macaques. This study detected CFTR DNA and mRNA in animal dosed with AAV5-CFTR and GFP DNA expression was observed in the airways of AAV5-*GFP* treated animals. The expression of the reporter gene was also confirmed by confocal microscopy.
3) Flotte et al.[116], 2010	MicroSprayer®	Conducted a dual reporter study using firefly luciferase (F-Luc) and Renilla luciferase (R-Luc) to compare the efficiency of AAV1 and AAV5 vectors, in the airways of chimpanzees. Luciferase imaging studies showed that expression of the AAV1 vector was found to be 20-fold higher than the AAV5 vector at 90 days.

Study	Aerosol delivery device	Outcome
4) Steines et al.[117], 2016	AeroProbe™ catheter	A modified AAV vector derived from the AAV2 serotype with 5-point mutation was used in this study. This modified vector was called the AAV2H22 vector and it had an evolved capsid. This vector was sprayed into the lungs of CF pigs. Correction of the CF gene defect was observed from excised nasal tissue two weeks following vector administration to the nose and lungs.
5) Guggino et al, 2017[112]	MicroSprayer®	Two groups of rhesus macaques were treated with a spray of either AAV1-F-Luc or AAV5-R-Luc vector. No significant difference in luciferase activity was observed in the lung samples of the two treatment groups, collected at day 45. However, further analysis showed that the genomes copies of AAV1 vector was 10 times more prevalent than AAV5 vector.

1.7.4 Sendai viral vectors

Another gene therapy vector that has been examined is the Sendai viral (SeV) vector. The SeV belongs to the family of Paramyxoviruses, an enveloped virus with a negative RNA strand[118]. Griesenbach et al. showed that viability of the SeV vector was reduced to 1% of its initial level following delivery through the Pari LC Plus® jet nebuliser, which was probably due to shear stress produced during aerosol delivery[58]. In contrast, spraying the vector through an AeroProbe™ catheter resulted in a viability of 49% of the initial level[58]. Thus, they chose to use the AeroProbe™ catheter to deliver the SeV vector to sheep airways[58], and reported uniform gene expression in the lungs of animals sprayed with the vector, compared with patchy gene expression in the lungs of bolus-treated animals[58]. One disadvantage of this vector is that it produces transient gene expression and repeated administration of the vector proved to be ineffective, rendering it unsuitable for CF gene therapy[58]. This led to investigation of other viral vectors that could provide stable, long-term gene expression for CF airway disease.

1.7.5 Lentiviral vectors

Lentiviral (LV) vectors are enveloped RNA viruses which belong to the family of Retroviridae[88]. LV vectors have been derived from a variety of viruses, such as the feline immunodeficiency virus (FIV), simian immunodeficiency virus (SIV), human immunodeficiency virus (HIV), and equine infectious anaemia virus (EIAV)[119]. They have the ability to package up to 10 kb DNA thus allowing them to carry the full CFTR cDNA[105]. Furthermore, they can transduce both dividing and non-dividing cells[88], and contain the integrase enzyme which helps integrate the vector DNA into the host genome[105]. The LV pre-integration complex gets imported into the nucleus independent of mitosis, which allows it to transduce terminally differentiated cells, such as ciliated epithelial cells of the airways, which are the main targets for airway CF gene

therapy[105]. Furthermore, the LV vector can transduce the progenitor cells/stem cells of the airway, which could facilitate long-term gene expression[43].

Aerosol delivery of LV has been challenging due to its fragile nature, as observed during vector production[120, 121]. Hence, only a few studies have examined the effectiveness of aerosolising LV vectors. Cooney et al. sprayed an FIV vector pseudotyped with baculovirus envelope protein GP64 and carrying the *CFTR* gene into the lungs of CF pigs using a MADgic™ atomisation device[87]. They showed partial restoration of the CF gene defect from excised lung, tracheal, and bronchial tissue two weeks post-treatment[87]. They also showed a significant increase in the amount of CFTR mRNA present in the treated animals compared to untreated animals[87].

To date, only one (lung cancer) study has delivered a HIV-1 derived LV vector as an aerosol into mouse airways using a patented nebuliser[122]. However, that study did not examine the efficiency of the nebuliser for delivering the HIV-1 vector to animal airways, the distribution of the vector in the lungs, or the vector viability following aerosol delivery. Hence, there is a need to investigate the efficiency of delivering these types of vectors as an aerosol.

The studies presented in this thesis examine the effectiveness of aerosolising a HIV-1 derived LV vector to optimise an efficient aerosol delivery protocol for future use in pre-clinical and clinical studies.

1.7.5.1 Genome of the HIV-1 virus

The wild type HIV-1 virus is made up of 15 proteins and two RNA strands[123] (Figure 1-10). The HIV genome is 9 kb long and codes for nine open reading frames (ORF) [123]. Three of these frames code for the Gag, Pol, and Env proteins, which are common to all retroviruses[123]. The structural component of the core is made up of four Gag proteins; the matrix (MA), capsid

(CA), nucleocapsid (NC), and p6. The envelope is made up of two Env proteins: surface protein (SU) (also known as gp120) and transmembrane protein (TM) (also known as gp41)[123]. The enzymatic proteins present within the virus are integrase (IN), protease (PR), and reverse transcriptase (RT)[123]. The HIV-1 virus has six accessory proteins: Vif, Vpu, and Vpr that control the rate of production of vector particles, the essential gene regulatory functions are controlled by the Tat and Rev accessory proteins[123], and Nef enhances the pathogenicity of the virus[124].

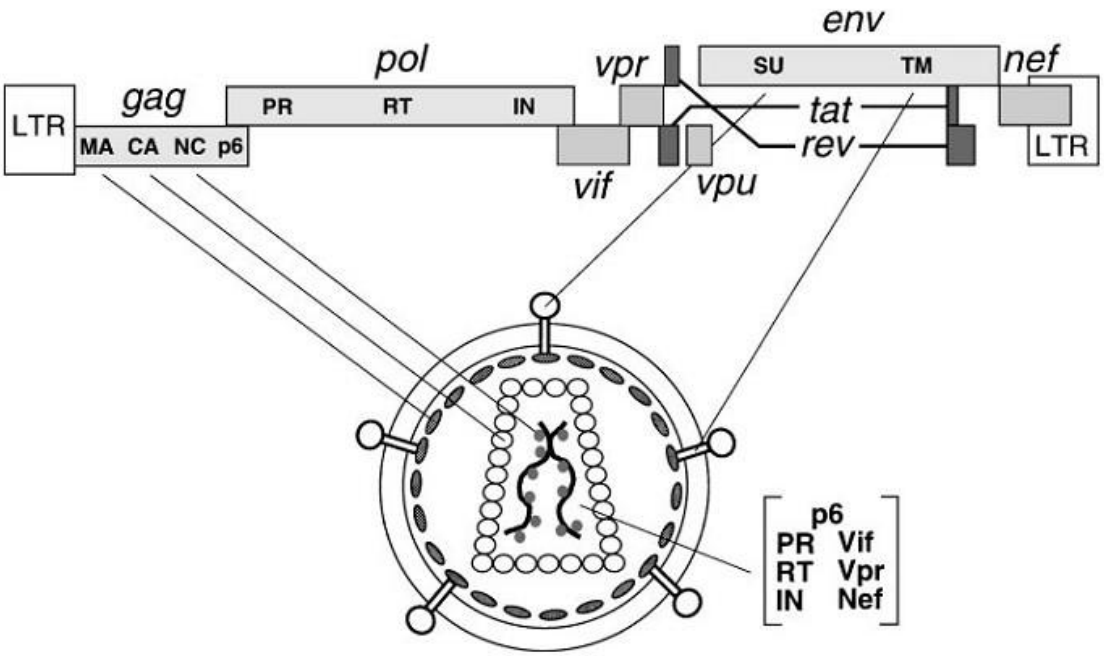

Figure 1-10: Structure and organisation of RNA genome of HIV-1 virus[123].

1.7.5.2 Structure of HIV-1 virion

A mature HIV-1 virion is spherical and approximately 100 nm in diameter[124]. The envelope of the virion is made up of a lipid bilayer, which is composed of the Env proteins gp120 and gp41[124]. The envelope also has 72 knobs made up of trimers of Env proteins[124]. Trimers of the gp120 protein are anchored to the membrane by trimers of the gp41 protein. The envelope encloses the outer capsid membrane that is made up of the MA protein[124]. Within this lies

the conical capsid that is formed by inner capsid protein p24. The inner capsid is attached to the outer capsid through a linking protein[124]. The inner capsid encloses two identical RNA strands and other enzymatic proteins, such as IN, PR, and RT[124].

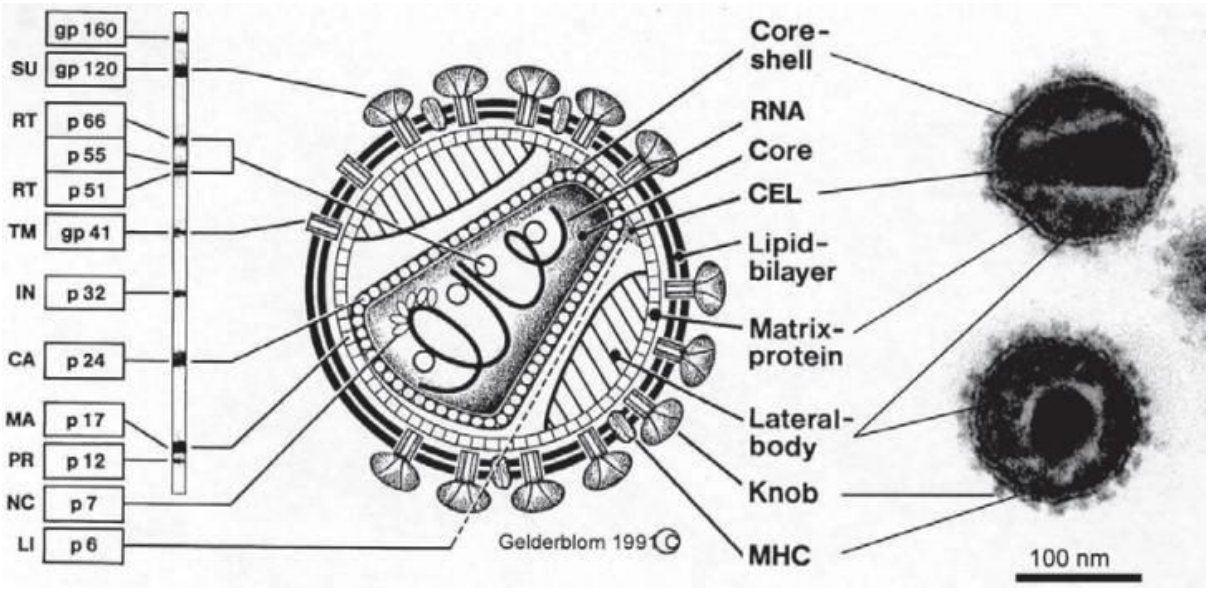

Figure 1-11: Structure of the HIV-1 virion showing two RNA strands enclosed in a conical capsid (core shell) that is then surrounded by the envelope (lipid bilayer)[124].

1.7.5.3 HIV-1 replication

Replication of the wild type HIV-1 virus begins by binding of gp120 to the CD4 receptors present on the surface of the host cell[124]. This leads to fusion of viral and cell membranes, which then releases the viral capsid into the cytoplasm of the host cell and entry of the HIV RNA and various enzymes into the cell[124]. A single RNA strand is then reverse transcribed by the RT into cDNA[124]. The DNA-dependent DNA polymerase activity of the RT then converts the cDNA into a double stranded pro-viral DNA[124]. The pro-viral DNA along with IN (known as the pre-integration complex) is then taken up into the nucleus of the host through the nuclear pore[124]. The pro-viral DNA is then randomly inserted into the host cell genome by IN, and is later transcribed along with the host genome to produce viral proteins[124].

1.7.5.4 HIV-1 viral vector

Self-inactivating HIV-1 vectors were created by deleting viral genes and regions that promote transcriptional activity of the virus[125]. The regions coding for accessory viral proteins that increase the pathogenicity of the vector, such as Vif, Vpu, Vpr, and Nef, are not required for production of the vector and were deleted [126]. The packaging component consists of G*ag, Pol, Tat,* and *Rev* genes, the transfer vector, and the envelope vector[125]. Additionally, a polypurine tract located in the central position (cPPT) and post-transcriptional regulatory elements of the woodchuck post-transcriptional regulatory element (WPRE) have been introduced to help facilitate nuclear translocation of the pre-integration complex and improve levels of transgene expression, respectively[125].

The wild type pro-viral DNA contains the 5' long terminal repeats (LTR), the trans-element that codes viral proteins, and the 3' LTR[127]. The 5'LTR and the 3'LTR are known as *cis* elements. They code for a transcriptional promoter, a primer binding site for DNA synthesis during reverse transcription, a signal for packaging genomic RNA, a polyadenine sequence, and other sequences required for reverse transcription[127]. To render the wild type HIV-1 virus replication defective the *cis* element of the virus is replaced with a heterologous promoter, and a heterologous polyadenine tail that is then packaged with the transgene[126]. To further improve the safety of the vector and minimise chances of homologous recombination, sequences coding for viral proteins and enzymes are separated onto different helper plasmids[128, 129].

The wild type HIV-1 vector does not have a tropism for the cells of the lung. However, tissue tropism can be broadened using different envelopes or pseudotypes, such as vesicular stomatitis virus G glycoprotein (VSV-G), baculovirus GP64, ebola virus (EboZ), or influenza

virus haemagglutinin (HA)[130]. Receptors for the GP64, EboZ, and HA pseudotypes lie primarily on the apical surface of airway epithelia, whereas receptors for the VSV-G pseudotype are present on the baso-lateral surface[130]. VSV-G is a commonly used pseudotype, which has demonstrated effective transduction of airway epithelial cells[131]. As the VSV-G receptors reside on the baso-lateral membrane, airway conditioning treatments, such as LPC[132], sulphur dioxide (SO_2)[133], or sodium caprate[134], are required to provide access to the receptors for optimal effectiveness. Allowing viral vectors access to the basolateral surface should also lead to transduction of epithelial progenitor cells, which would normally not be accessible from the apical surface[43].Furtheremore, VSV-G vectors are also resistant to the shear forces present during vector production[135].

The studies conducted in this thesis used a HIV-1 derived LV vector carrying a (nuclear localised) *LacZ* reporter gene pseudotyped with an VSV-G envelope. This LV vector was prepared using codon-optimised Gag, Pol, Tat, and Rev plasmids[136, 137] (Chapter 2). The LacZ nuclear localisation signal allows easy identification of transduced cells following X-Gal histological processing for the β-Galactosidase protein[136, 138].

1.7.5.5 Bolus delivery studies using HIV-1 derived vector

HIV-1 derived vectors have shown promising results being delivered as a fluid bolus in the airways of animal models. The Cystic Fibrosis Airway Research Group (CFARG, Adelaide) have developed a HIV-1 derived LV vector pseudotyped with a VSV-G glycoprotein envelope[132], as well as a two-step liquid bolus gene delivery method, in which animal airways were conditioned with LPC followed by the LV vector dose one hour later[51, 132, 139]. Limberis et al. instilled a bolus dose of LPC conditioning followed by a bolus dose of HIV-CFTR vector onto the nasal airways of CF mice and demonstrated electrophysiological correction of CF gene

defect up to 110 days[139]. In a long-term study, Cmielewski et al. demonstrated correction of the CF gene defect up to 12 months in the nasal airways of CF mice using the same vector and delivery protocol[132]. Reporter gene expression has also been observed in airways of larger animal models, such as sheep[140], ferrets[56], and marmosets[57], treated with a bolus dose of the HIV vector. Animals treated with a bolus dose of the vector demonstrated patchy gene expression in the trachea, conducting airways, and alveolar tissue[57, 140], which could be due to uneven distribution of the vector and pooling of the bolus dose in airways, as well as a mismatch between regions treated with the LPC conditioning and the LV vector dose [57, 58]. Additionally, these bolus studies showed that the HIV vector transduced ciliated cells and basal cells present in airway epithelium of the conducting airways[56, 57, 140]. A recent study by Farrow et al. confirmed that basal cells present in the respiratory epithelium could be transduced with a HIV vector and that the transgene was passed onto future daughter cells[43]. These promising results suggest that the HIV vector is a rational and feasible candidate for future gene therapy trials. Hence, there is a need to optimise a non-invasive aerosol delivery protocol that may effectively deliver this vector to the airways of CF patients in future clinical trials.

1.8 Aerosol delivery of a HIV-1 derived vector

HIV-1 derived vectors are fragile and susceptible to shear-stress, as observed during vector production[121, 141, 142], and exhibit reduced viability with changes in temperature, pH, and drying[64, 141, 143]. HIV-1 vectors are stable at a pH of 7 and their half-life decreases at temperatures above 4°C[64]. Hence, a nebuliser that produces minimal change in pH, temperature, and can effectively aerosolise shear-sensitive drug formulations, would be optimal for aerosolising the HIV-1 vector. Based on the analysis in Section 1.6, the most well

suited nebuliser is likely to be the Aeroneb®Pro vibrating mesh nebuliser, which can effectively aerosolise shear-sensitive biological formulations, such as liposomes and nanoparticles[83, 144]. This vibrating mesh nebuliser can aerosolise very small (µl) volumes of liquid formulation and can therefore be used to deliver small volumes of aerosols to airways of smaller animal models, such as mice and rats[74, 145]. This nebuliser is operated by an electronic controller that allows timed delivery of aerosols during the inspiratory phase of the subject[74], reducing the wastage of expensive vector formulations.

The flexiVent™ small animal ventilator (Scireq Scientific Respiratory Equipment Inc., Canada) has been commonly used in conjunction with the Aeroneb®Pro nebuliser to deliver aerosols to the lungs of ventilated mice[146-148]. This ventilator could also be used to monitor physiological lung function of mechanically ventilated mice[149]. Hence, this thesis investigated the effectiveness of aerosolising the HIV-1 based LV vector using an Aeroneb®Pro operated in conjunction with the flexiVent™ ventilator into the lungs of mice.

1.9 Aims

The overall aim of this program of work was to develop an optimised aerosol delivery method for HIV-1 derived LV vectors. This thesis has the following aims:

(1) Determine the efficiency of the Aeroneb®Pro nebuliser for aerosolising a HIV vector pseudotyped with the VSV-G envelope into the lungs of mice, during mechanical ventilation with a flexiVent™ ventilator. Compare the distribution of the LV vector delivered as an aerosol or bolus dose in lungs of mice.

(2) Quantify the volume of aerosols reaching the mouse trachea, i.e. how much aerosol is released at the end of the Aeroneb®Pro-flexiVent™ ventilator circuit compared to bolus delivery.

(3) Quantify the viability of the aerosolised LV vector at the point at which it enters the mouse trachea, i.e. the viability of the LV vector released at the end of the Aeroneb®Pro-flexiVent™ ventilator circuit.

(4) Optimise the delivery parameters to develop an effective LV aerosol delivery protocol that could be used in further pre-clinical studies.

This sequence of experimental aims was designed to establish a baseline difference between the standard LV delivery protocol[150] .The subsequent aims were designed to conclusively identify the causes of reduced gene expression resulting from LV aerosolisation, and develop methods to overcome them. This was achieved using one study designed to improve the physical quantity of aerosol reaching the lung, and a second study aiming to improve the biological quality of the LV vector reaching the lung. A series of *in vitro* methods were used instead of in *vivo s*tudies, as this allowed many optimisation tests to be performed, reduced the time taken to obtain useful results, minimised the quantity of LV vector required, prevented the use of a very large number of animals, and enabled the studies to be completed using the funding available for the project. Cell culture studies were then used to validate the improvements produced by the optimal parameters identified from the bench studies. Due to the relatively modest improvements in delivery efficiency an additional animal experiment was not warranted at the completion of the described studies.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and suppliers

α-Lactose monohydrate	Sigma Aldrich (USA) Cat# L2643
0.9% sodium chloride (Saline)	Baxter Healthcare (USA) Cat# AHF7124
Agarose, type C, gelling 40°-43°C	Bio-Rad (USA) Cat# 1613102
Anti-Sedan (5 mg/ml)	Pfizer (NZ) Cat# 107264-8
Bacto™ agar	Becton, Dickinson (USA) Cat# 214010
Bacto™-tryptone	Becton, Dickinson (USA) Cat# 211705
Bacto™-yeast extract	Becton, Dickinson (USA) Cat# 212750
Bovine serum albumin (BSA)	Sigma Aldrich (USA) Cat# A7906
Calcium chloride dehydrate ($CaCl_2.2H_2O$)	Sigma Aldrich (USA) Cat# C8106
Dimethylformamide (DMF)	Sigma Aldrich (USA) Cat# D4551
Domitor	Pfizer (NZ) Cat# 107332-8
DNA molecular marker	GeneWorks (AUS) Cat# DMW-1
DPX	Leica Biosystems (Germany) Cat# 046430011
Dulbecco's modified eagle medium (DMEM)	Sigma Aldrich (USA) Cat# D5796
Ethylenediaminetetraacetic acid (EDTA)	Sigma Aldrich (USA) Cat# EDS
Eosin Y	Australian Biostain (AUS) Cat# AEPA
Ethanol	Chem Supply (AUS) Cat# EA043
Fetal calf serum (FCS)	Bovogen (AUS) Cat# SFBS
Dye solution (yellow)	Queens (AUS) Cat# QYE50
FreeStyle™ medium	Thermo Fisher Scientific (US) Cat# 12338018

GelRed® nucleic acid gel stain	Biotium (USA) Cat# 41003
Gentamycin (40 mg/ml)	Pfizer (NZ) Cat# 08060180
Glacial acetic acid	Chem Supply (AUS) Cat# AA009
Glutaraldehyde, grade II (25%)	Sigma Aldrich (USA) Cat# G6257
Glycerol	Chem Supply (AUS) Cat# GA010
Hams F12media	Thermo Fischer Scientific (AUS) Cat# 11765-5411765-054
Haematoxylin (Mayer's)	ProSciTech (AUS) Cat# AMH
HEPES	Sigma Aldrich (USA) Cat# H3375
Hydrochloric acid (HCl,32%)	RCI Labscan (Thailand) Cat# RP1104
Indian ink	Windsor and Newton (UK) Cat# 1005754
Ketamine (100 mg/ml)	Ceva (AUS) Cat# E55920B
L-Glutamine	Life Technologies (USA) Cat# 21051-024
L-α-Lysophosphatidylcholine (LPC)	Sigma Aldrich (USA) Cat# 4129
Magnesium chloride hexahydrate ($MgCl_2.6H_2O$)	Sigma Aldrich (USA) Cat# M2670
Mouse serum	Life Technologies (USA) Cat# 016501
Neutral buffered formalin (NBF)	Fronine (AUS) Cat# JJ0185
OptiPro™ SFM	Life Technologies (USA) Cat# 12309-019
Paraformaldehyde (PFA)	Sigma Aldrich (USA) Cat# P6148
PBS without calcium and magnesium	Sigma Aldrich (USA) Cat# D8537
Penicillin G (5000 U/ml)/Streptomycin (pen/strep) (5mg/ml)	Life Technologies (USA) Cat# 15140-122
Phenol chloroform isoamyl alcohol	Sigma Aldrich (USA) Cat# 77618
Polybrene (Hexadimethrine bromide)	Sigma Aldrich (USA) Cat # 107689
Potassium chloride (KCl)	Sigma Aldrich (USA) Cat# P9541

Potassium ferricyanide [$K_3Fe(CN)_6$]	Sigma Aldrich (USA) Cat# D8537
Potassium ferrocyanide [$K_4Fe(CN)_6$]	Sigma Aldrich (USA) Cat# P3289
Schiffs reagent	Australian Biostain (AUS) Cat# ASC
Sodium chloride (NaCl)	Sigma Aldrich (USA) Cat# S3014
Sodium hydroxide (NaOH)	Sigma Aldrich (USA) Cat# S5881
TE buffer	Usb Corp (USA) Cat# 75834
Trypan blue	Bio-Rad (USA) Cat# 145-0013
Trypsin	Life Technologies (USA) Cat# 12604
Virkon	Med-con (Aus) Cat# 500607
Water for irrigation	Baxter Healthcare (USA) Cat# AHF7114
X-gal	Progen Industries (AUS) Cat# 200-0191
Xylene	Scharlau (Spain) Cat# X100572500

2.1.2 Consumable and suppliers

6-well flat bottom cell culture plate	Sigma Aldrich (USA) Cat# CLS3516-50
12-well flat bottom cell culture plate	Sigma Aldrich (USA) Cat# CLS3513
24-well flat bottom cell culture plate	Costar (Corning Scientific, USA) Cat# 3524
96-well flat bottom cell culture plate	Costar (Corning Scientific, USA) Cat# 3590
Circular cell culture plates (150 mm x 25 mm)	Costar (Corning Scientific, USA) Cat# 430599
Haemocytometer	ProSciTech (AUS) Cat# SVZ4NIOU
Histology cassette	ProSciTech (AUS) Cat# RCH40-G
Gel loading tips	Quality Scientific Plastics (USA) Cat# 010-Q
Micro-loader tips	Eppendorf (Germany) Cat# 5242956003
Microscope coverglass slips	ProSciTech (AUS) Cat# G414
Microscope slides	Menzel Glaser (Germany) Cat# AD00000112E

Mustang Q acrodiscs	Pall Corporation (USA) Cat# MSTG25Q8
Polypropylene centrifuge tubes	Beckman Coulter (USA) Cat# 331374
T-75 flask	Greiner Lab (Germany) Cat# 658175
Typan blue	Bio-Rad (USA) Cat# 145-00143
Square cell culture plates (224 mm x 244 mm)	Costar, Corning Scientific (USA) Cat# 4301110

2.1.3 Bacterial strains and media

Broth	1.5% (w/v) Tryptone, 1% (w/v) Yeast extract, 0.5% (w/v) NaCl in distilled water
E. coli	E. Coli (Sure cells)
LB agar	1% (w/v) Tryptone, 0.5% (w/v) Yeast extract, 0.5% (w/v) NaCl, 1 M NaOH in distilled water

2.1.4 Cell lines

HEK-293T cells	American Type Culture, CRL 11268
CHO-K1 cells	American Type Culture, CCL 61
NIH3T3 cells	American Type Culture, CRL 1658

2.1.5 DNA plasmids

GagPol	pHCMVgagpolmllstwhv
Rev	pHCMVRevmlwhvpre
Tat	pcDNA3Tat101ml
VSV-G	pHCMV-G
LacZ	pHIV-MPSV-nlsLacZ (Figure 8-6)

2.1.6 Plasmid kit and buffers

Agarose gel	1% (w/v) agarose in TBE buffer
Endofree Plasmid Mega Kit	Qiagen (Germany) Cat# 12391

2x HeBS	0.28 M NaCl,0.05 M Hepes,1.5 mM Na_2HPO_4 pH 7.04
TBE buffer	Bio-Rad (US) Cat# 161-0770

2.1.7 Quantitative real-time PCR assay

2.1.7.1 PCR kits

TaqMan universal PCR master mix	Life Technologies (USA) Cat# 4304437
TaqMan MGB probe (50,000 pmol)	Thermo Fisher Scientific (AUS) Cat# 4316032
Wizard SV genomic DNA system	Promega (USA) Cat# A2361
20x assay mix	18 µM forward primer, 18 µM reverse primer, 5 µM probe in TE buffer

2.1.7.2 Primers and probes

Gag forward primer	AGC TAG AAC GAT TCG CAG TTG AT
Gag reverse primer	CCA GTA TTT GTC TAC AGC CTT CTG A
Gag probe	6FAM-CCT GGC CTG TTA GAA AC-NFQ
mTransferrin forward primer	AAG CAG CCA AAT TAG CAT GTT GAC
mTransferrin reverse primer	CGT CTG ATT CTC TGT TTA GCT GAC A
mTransferrin probe	6FAM-CTG GCC TGA GCT CCT-NFQ
NLS-LacZ 3' primer	GCC ACT TCT TGA TGG ACC ACT T
NLS-LacZ 5' primer	CCG CCA CCG ACA TCA TCT
NLS-LacZ probe	FAM-CAC GCG GGC GTA CAT-NFQ

2.1.8 LV LacZ titre assay

Pre-X-gal	35 mM [$K_3Fe(CN)_6$], 35 mM [$K_4Fe(CN)_6$], 1 mM $MgCl_2$ in PBS
X-gal	40 mg/ml X-gal in DMF

2.1.9 Animal models

C57Bl/6 mice	Laboratory Animal Services, University of Adelaide, SA (AUS)

2.1.9.1 Anaesthesia

Anti-sedan reversal	0.5 mg/ml Atipamazole (Pfizer, NZ) in sterile water
Domitor: Ketamine mix	0.1 mg/ml Medetomidine (Pfizer, NZ), 7.6 mg/ml Ketamine (Ceva, AUS) in sterile water

2.1.10 Processing of mouse head, trachea and lung

10% NBF	10% (v/v) Formalin, 0.22 M NaH_2PO_4, 0.45 M NaH_2PO_4 in distilled water
Carnoy's fixative	60% (v/v) Ethanol, 30% (v/v) Chloroform, 10% (v/v) Glacial acetic acid
Decalcification solution	7% (v/v) HCl in 1.5% EDTA (w/v) in distilled water
PFA/Glutaraldehyde	2% (w/v) PFA, 0.5% (v/v) Glutaraldehyde in PBS
Pre-X-gal	5 mM [$K_3Fe(CN)_6$], 5 mM [$K_4Fe(CN)_6$], 1 mM $MgCl_2$ in PBS
X-gal	20 mg/ml X-gal in DMF

2.1.11 Equipment

Aeroneb®Pro nebuliser	Aerogen Inc., (Ireland)
Centrifuge	Beckman Coulter (USA) Allegra™25R Centrifuge, TS-5.1-500 rotor
Cell culture incubator	New Brunswick, Eppendorf (Germany) Galaxy 170 R
ET tube	BT Insyte™ (Aus) 20-gauge intra-venous catheter
Compound-light microscope	Nikon (Japan) Eclipse E400
Gel electrophoresis tank	Bio-Rad (AUS) Mini-Sub GT 7X10
Microplate reader	Bio- Rad (AUS) iMark™ absorbance reader
Microtome	Leica (Germany) RM2235
Spectrophotometer	Amersham Biosciences (UK) Ultra Spec 2100 Pro

Shaker	Ratek (AUS) Orbital mixer incubator
Stereo-microscope	Nikon (Japan) SMZ1500
qRT-PCR	Bio-Rad (USA) CFX™ Connect PCR machine
Ultra-centrifuge	Beckman Coulter (USA), SW-60 Ti rotor, Optima L-100XP
Ventilator (flexiVent™)	Scireq Scientific Respiratory Equipment Inc. (Canada)

2.1.12 Software

CFX™ Manager	Bio-Rad (USA) Cat# 1855201
Flexiware 7.2	Scireq Scientific Respiratory Equipment Inc. (Canada)
G* Power 3.2	Universität Düsseldorf (Germany)
NIS-element	Nikon (Japan) Cat# MQS33000
Prism 8	GraphPad (USA)

2.2 Methods: *In vitro*

2.2.1 Plasmid preparation

2.2.1.1 Large scale plasmid production

All plasmids used in this study were prepared using the Endo-free Plasmid Mega-Kit as per the manufacturer's instructions.

2.2.1.2 Agarose gel electrophoresis

Qualitative assessment of plasmid DNA was performed using restriction digests followed by agarose gel electrophoresis. Plasmid DNA was digested using appropriate restriction enzymes and visualised using 1.2-1.8% agarose gel in 1x TE buffer, with gel red nucleic acid stain. The gel was submerged in 1x TE buffer and run at 100 volts, 400 mA and 100 watts. DNA fragments produced on digesting plasmid DNA were compared to a standard DNA molecular weight marker *SPP1/EcoR1*.

2.2.1.3 Spectrophotometry

Plasmid DNA was quantitated using a spectrophotometer at an absorbance of 260 nm.

2.2.2 Cell culture

All cell culture work was performed in a class II biosafety cabinet in a PC2 laboratory using appropriate aseptic techniques.

2.2.2.1 Cell culture initiation

HEK-293T and NIH-3T3 cells were cultured using DMEM containing 10% (v/v) FCS. CHO-K1 cells were cultured using Hams F12 medium containing 10% (v/v) FCS. A vial of frozen cells (stored in liquid nitrogen at -80°C) was thawed using a warm water bath at 37°C and added to 9 ml of appropriate pre-warmed cell culture media in a 10 ml sterile centrifuge tube. The cell suspension was centrifuged at 5,000 RPM at 4°C for 10 minutes. Supernatant was discarded, and the cell pellet was suspended in 5 ml of cell culture media. 10 µl of cell suspension was diluted by 50% (v/v) with trypan blue to determine viable cell count using a haemocytometer. The cell suspension was later transferred to a T-75 flask containing 14 ml of pre-warmed media and placed in a humidified incubator at 37°C supplied with 5% carbon dioxide (CO_2) and allowed to expand.

2.2.2.2 Cell culture expansion

2.2.2.2.1 HEK-293T and NIH-3T3 cells

Cells were sub-cultured for further experiments and for regular cell culture maintenance. DMEM present in a confluent T-75 flask was aspirated and the cell layer was rinsed with phosphate buffered saline (PBS) to ensure removal of remaining media. To detach the adherent layer of cells, 4 ml of 10% (v/v) trypsin diluted in PBS was added to the T-75 flask and incubated for 2 minutes at room temperature. Equal amounts of DMEM were then added to neutralise trypsin, and cells were pipetted to obtain a single cell suspension as observed

using a light microscope. Cells were passaged with media, 1:2 (v/v) to be confluent in 24 hours and 1:4 (v/v) to be confluent in 48 hours. Flasks were incubated at 37°C and 5% CO_2 for further adherence and cell growth.

2.2.2.2.2 CHO-K1 cells

CHO-K1 cells were sub-cultured using the previously mentioned protocol with minor modifications. Hams F12 cell culture media was used to maintain and sub-culture CHO-K1 cells. To detach adherent layer of CHO-K1 cells, trypsin was added to the flask and incubated for 7 minutes. Cells were passaged with Hams F12 media, 1: 3 (v/v) to be confluent in 24 hours and 1:6 (v/v) to be confluent in 48 hours.

2.2.3 Lentiviral vector production

2.2.3.1 Cell culture

HEK-293T cells were sub-cultured and expanded into 8 x T-75 flasks over two days. Cells from eight confluent T-75 flasks were sub-cultured and seeded onto eight round plates (150 mm) and were then cultured overnight at 37°C and 5% CO_2. Round plates with confluent cells were harvested and cell concentration was determined using a haemocytometer. The cell suspension was diluted with DMEM containing 10% FCS (v/v), penicillin and streptomycin (pen/strep) 1:100 (v/v), to obtain a final cell concentration of 0.375 x 10^6 cells/ml. Cells were seeded onto 8 x 245 mm square plate by pipetting 110 ml of cell suspension per plate. These plates were incubated for 20-24 hours at 37°C and 5% CO_2.

2.2.3.2 Lentiviral vector production

LV vector containing the *LacZ* reporter gene was produced by transfecting HEK-293T cells with a five-plasmid system using calcium phosphate co-precipitation[136]. A DNA/calcium chloride ($CaCl_2$) mix containing 320 µl of 2.5 M $CaCl_2$ added to 3.2 ml of water was prepared. Plasmids

were added to this mixture in the ratio (per 245 mm square plate): 170 µg of pHIV-MPSVnls-LacZ (Figure 8-6), 3.16 µg of pcDNA3Tat, 3.16 µg of phCMVRev, 2 µg of phCMVgagpol, and 7.9 µg of pVSV-G, and the mix was gently vortexed. An aliquot of 3.2 ml of 2 x HeBS buffer was vortexed and an equal volume of DNA/$CaCl_2$ mixture was added drop-wise over 5 to 10 seconds. This mixture was vortexed for an additional 20 to 25 seconds and allowed to stand at room temperature for a further 90 seconds. This DNA-$CaPO_4$ precipitate was gently added to the 245 mm square dish containing HEK-293T cells using a pipette and was allowed to stand at room temperature for 30 seconds. An even distribution of DNA-$CaPO_4$ precipitate was attained by gently swirling the plate. This procedure was repeated for all 245 mm square plates, which were then incubated for 8 hours at 37°C and 5% CO_2. A media change was performed using OptiPro serum-free medium, supplemented with 4 mM glutamine and pen/strep 1:100 (v/v). Plates were then incubated for a further 48 hours.

2.2.3.3 Lentiviral vector purification and concentration

LV vector obtained from supernatant of transfected HEK-293T cells was filtered and concentrated by ultracentrifugation. Supernatant of the 8 x 245 mm square plates was decanted into a 1 L bottle, which was supplemented with 0.1% (w/v) BSA. This procedure was carried out in a bio-safety cabinet and the 1 L bottle was transferred to a sterile bench for further purification. The ultrafiltration system was setup using 0.45 µm filter (polyethersulfone membrane) connected to two Mustang Q Acrodisc® filters[151, 152], as shown in Figure 2-1.

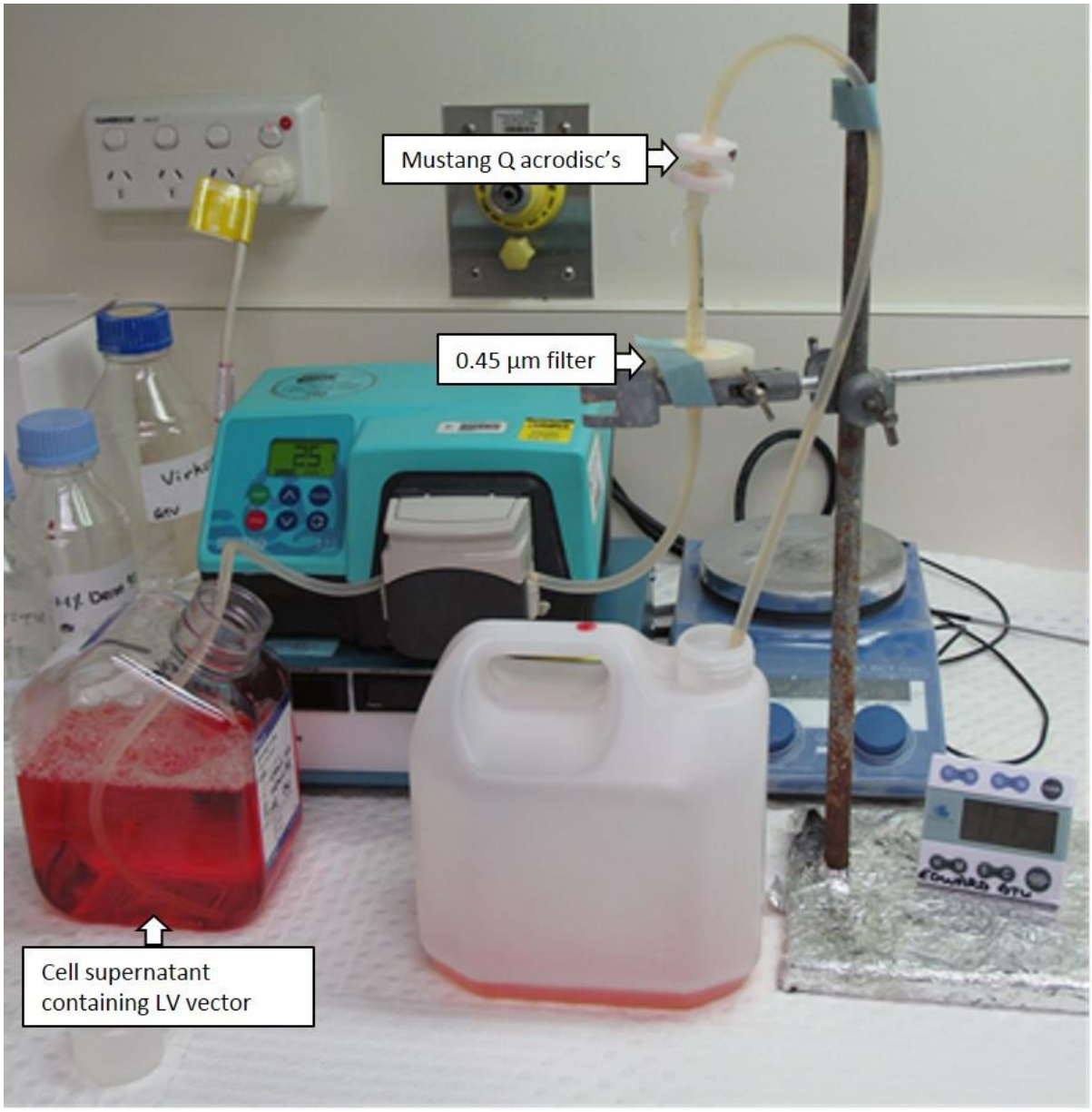

Figure 2-1: Ultrafiltration system to harvest LV-LacZ vector from supernatant.

Supernatant was pumped through the filtration system at a flow rate of 10 ml/min. On completion, the filtration system was flushed with 100 ml of PBS/0.1% BSA (w/v). The LV-LacZ vector was eluted from the Mustang Q Acrodisc® filters using 4 ml of 1.5 M NaCl into equal volume of 2% heat inactivated mouse serum diluted in water[152]. For further concentration, the elution was divided equally into two polypropylene ultracentrifuge tubes and ultracentrifuged at 20,000 RPM (SW 60 Ti rotor, Beckman Coulter) for 90 mins at 4 °C. Pellets containing the LV-LacZ vector were suspended into appropriate volumes of diluent and

aliquoted to avoid freeze-thaw. The vials were stored at -80°C.

2.2.4 Viral titre determination

Titre of LV-LacZ vector was obtained by quantitative real-time PCR (qRT-PCR) analysis of pro-viral genomic DNA (gDNA), obtained by transducing NIH-3T3 cells.

2.2.4.1 Preparation of pro-viral qDNA

NIH-3T3 cells suspended in DMEM supplemented with 10% FCS were seeded at a concentration of 0.05 x 10^6 cells/well of a 24-well plate and incubated at 37°C at 5% CO_2 for 3 hours. Media on the 24-well plate was replaced with DMEM medium supplemented with 10% FCS, 4 µg/ml polybrene, and 2 µg/ml gentamycin. Cells were transfected with 1:1,000 (v/v) diluted LV vector and incubated for 24 hours. A media change for the 24-well plate was carried out using DMEM supplemented with 10% FCS and gentamycin. Cells were incubated at 37°C at 5% CO_2 until confluent then split 1: 4 (v/v). Cells were maintained for 30 days using standard sub-culturing methods.

After 30 days, the media was aspirated and cells were washed with PBS. This was followed by 0.5 ml of 10% trypsin/PBS (v/v) for 2 minutes, which was then neutralised using equal volumes of 1% FCS/PBS solution (v/v). The cell suspension was centrifuged at 2,000 RPM for 5 minutes at 4°C. The supernatant was aspirated, and the pellet was suspended in 3 ml of PBS. This process of rinsing cell pellets in PBS was repeated three times in total. Pro-viral gDNA was isolated from the cell suspension using the Wizard® genomic DNA purification kit as per manufacturer protocol, and was then stored at -20°C for qRT-PCR.

2.2.4.2 qRT-PCR analysis

The pro-viral gDNA extract was analysed using qRT-PCR to determine titre of the LV-LacZ vector. For every sample two assays were carried out: one to detect presence of the gag

sequence and the other to identify the transferrin sequence. A non-template control was also included by using water in place of the gDNA sample. Each reaction contained 1 µl of 20 x assay mix (gag or transferrin), 10 µl of 2 x TaqMan universal PCR master mix, 4 µl of water, and 5 µl of gDNA sample. All samples were analysed in triplicate, which included a non-template control and standard. Reactions were performed using the following cycling conditions: 50°C for 2 min, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. At the end of each cycle the fluorescence emitted by the reaction was read and an amplification plot was constructed using the CFX™ software. The cycle threshold (Ct) for each sample was calculated using the same software. The LV titre (infectious units/ml) was determined using the following formula:

ΔCt = Ct of gag – Ct of mTransferrin for a sample

ΔΔCt = ΔCt – 1 (or average of standard)

$1/ 2^{\Delta\Delta Ct}$ = copy number per cell

$$\text{Titre} = \frac{\text{number of cells initially plated x copy number per cell x (1000/ volume in µl)}}{\text{dilution factor}}$$

= infectious units/ml

2.2.5 Lentiviral aerosol viability assay

A β-galactosidase assay was used to quantify the level of gene expression produced by aerosolised LV-LacZ vector released at the end of a nebuliser-ventilator circuit (Chapter 4, Figure 4-1). A T-75 flask of confluent CHO-K1 cells was split using Hams F12 media supplemented with 10% FCS and pen/strep 1:100 (v/v), and cells were seeded at a concentration of 0.05 x 10^6 cells/well onto a 24-well plate and incubated for 3 hours. Prior to LV transfection, media in wells was replaced with Hams F12 media supplemented with 10% FCS, pen/strep 1:100 (v/v), 4 µg/ml of polybrene, and 2 µg/ml of gentamycin. A 20 µl aliquot

of LV-LacZ vector diluted 1:10 (v/v) in an appropriate diluent was aerosolised using an Aeroneb®Pro nebuliser connected to a flexiVent™ ventilator (Figure 2-2). The Aeroneb®Pro nebuliser used in this study was expected to produce aerosols of 3.6 µm diameter, as determined by the manufacturer. The ventilator circuit consisted of: a Scireq in-line Aeroneb®Pro mount with an internal diameter of 0.8 mm that lies directly below the point of aerosol delivery, connector tube, Y-piece, endotracheal (ET) tube with an internal diameter of 1.1 mm, and an expiratory limb. The tip of the ET tube was immersed in 500 µl of supplemented Hams F12 cell culture media contained in a 1.5 ml screw capped tube, with a loosened lid.

Aerosols of LV-LacZ vector released at the tip of the ET tube were collected in supplemented cell culture medium, which was later assayed. The ventilator circuit was cleaned between samples using the following protocol: aerosol delivery of 50 µl Virkon for 30 seconds, followed by three aerosol doses of 100 µl water, for 45 seconds each. The ventilator circuit was then disassembled and dried.

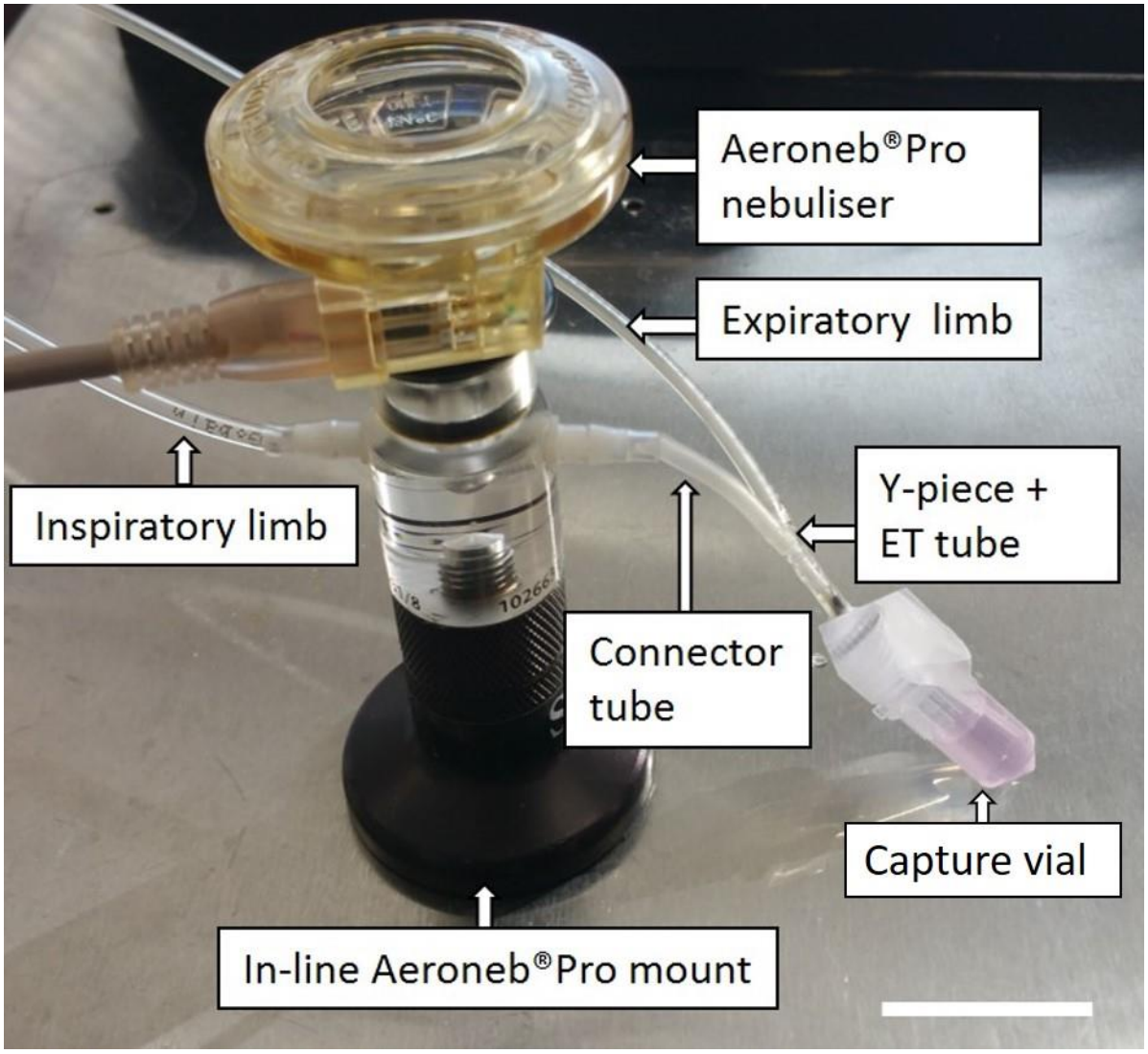

Figure 2-2: Aeroneb®Pro nebuliser and parts of flexiVent™ ventilator circuit (scale bar=2.5 cm).

The viability of the LV-LacZ vector released from the outlet of nebuliser (not connected to the ventilator circuit) was also quantified. The LV-LacZ vector of the same dilution was aerosolised using the Aeroneb®Pro nebuliser not connected to the ventilator circuit into wells of a 12-well plate containing 500 µl of supplemented Hams F12 cell culture medium. Media containing aerosols of LV-LacZ vector was later assayed. For a positive bolus control, LV-LacZ vector of the same dilution was delivered as a bolus dose to a separate well containing 500 µl supplemented Hams F12 cell culture medium.

Equal volumes of samples from each group were used to transduce CHO-K1 cells on a 24-well

plate (200 µl/well). Transfected cells were incubated at 37°C and 5% CO_2. A media change was carried out after 24 hours with pre-warmed Hams F12 media supplemented with 10% FCS, pen/strep 1:100 (v/v) and was incubated for a further 48 hours. Cells were rinsed with PBS and fixed with 0.05% glutaraldehyde in PBS for 15 minutes on a shaker at room temperature. The fixative was aspirated, and cells were washed three times with 1 mM $MgCl_2$ in PBS for 10 minutes each. The $MgCl_2$ was then aspirated and 1: 40 dilution (v/v) of X-gal: pre-X-gal solution (Section 2.1.7) was added onto the monolayer of cells and allowed to incubate overnight at 37°C. Cells were rinsed twice with PBS and stored in 80% glycerol. Images of each well of the 24-well plates were captured at 100x magnification using a stereo-microscope. The number of transduced cells in each well was quantitated by image analysis (Appendix, Section 8.2), using a custom-written MATLAB script (The Mathworks, Natick, USA).

2.2.6 Colorimetric assay to assess physical dose volume of aerosolised dye solution

The physical dose volume of aerosolised dye solution deposited in different ventilator circuit components and released at the tip of the ET tube (located at the end of the ventilator circuit) was quantified using the following protocol. A 20 µl aliquot of yellow food dye diluted 50% (v/v) with water was aerosolised through the Aeroneb®Pro nebuliser connected to the flexiVent™ ventilator circuit using appropriate ventilation parameters (Chapter 4). The volume of dye solution was constant for all experiments, unless otherwise mentioned. Aerosolised dye solution was collected at the tip of the ET tube, which was immersed in 500 µl of water contained in a 1.5 ml screw-cap tube with a loosened lid. The ventilator circuit was disassembled, and each ventilator circuit component was washed with 500 µl of water, which were collected in separate microcentrifuge tubes to be assayed later.

The physical dose volume of aerosols released at the outlet of the Aeroneb®Pro nebuliser not

connected to the ventilator circuit was also quantified. The nebuliser (not connected to ventilator circuit) was mounted on one well of a 12-well plate containing 500 µl of water. Dye solution was then aerosolised using the nebuliser with appropriate delivery parameters (Chapter 4). Aerosols released by the nebuliser were captured by water contained in the well to be assayed later.

As a bolus control, dye solution was delivered using a pipette into 500 µl of water. A 200 µl aliquot of sample collected from each group was then loaded into a clear 96-well plate. Two replicates were used per sample for all studies.

Dye absorbance was measured at 510 nm using a Microplate absorbance reader. A standard curve was established by measuring the absorbance of known concentrations of dye solutions (where 100% represented 20 µl of dye pipetted into 500 µl of water, the starting volume of dye solution). The standard curve was used to quantify the dose volume of aerosols released at the outlet the Aeroneb®Pro nebuliser alone, end of the ventilator circuit, deposited within circuit components, and bolus delivery.

2.3 Methods: *In vivo*

Mice were used as cost-effective *in vivo* models as only a very small starting volume of the expensive vector formulation is required to determine the effectiveness of different delivery regimen. LV vector was delivered to the lungs of mice as either a liquid bolus or an aerosol.

2.3.1 Animal care and management

All animal experiments were approved by the University of Adelaide Animal Ethics Committee and Women's and Children's Hospitals (WCH) Animal Ethics Committee. All experiments used female C57BL/6 mice housed at the WCH animal house physical containment 1 (PC1) facility.

Following vector delivery mice were transferred to the WCH physical containment 2 (PC2) facility, as per Office of the Gene Technology Regulator (OGTR) guidelines. All experiments involving LV delivery to mice were performed in a class II biosafety cabinet under PC2 conditions. Animals were monitored and weighed daily for one week post-LV treatment with additional monitoring as required.

2.3.2 Airway pre-treatment preparation

PBS tablets without calcium and magnesium were dissolved in MilliQ water (1 tablet/ 100 ml water) and sterile filtered using 0.45 µm filter. PBS solution was aliquoted and stored at -20°C for further use. Various concentrations of LPC were diluted in PBS solution and dissolved by sonication to avoid bubble formation. LPC was stored at 4^{o}C and used within 4 weeks.

2.3.3 Lentiviral vector delivery

C57BL/6 mice of 8-10 weeks of age were anesthetised with 10 µl/g body weight of medetomidine (Domitor) (0.1 mg/ml) and Ketamine (7.6 mg/ml) formulation delivered as an intra-peritoneal (i.p) injection. They were then suspended by upper incisors on a vertical support and intubated using 20-gauge polyethylene ET tube guided into the trachea by a fibre-optic cable within the tube. The fibre-optic cable was then withdrawn. Mice were placed in a supine position, and 10 µl of 0.1% LPC conditioning solution was delivered as a single bolus dose using a micropipette fitted with a microloader tip via the ET tube, administered smoothly over 5 seconds. Mice were placed on a heating pad while anaesthetised to maintain optimal body temperature.

2.3.3.1 Bolus lung instillation

One hour after LPC conditioning, 20 µl of LV-LacZ vector was delivered as a single bolus dose via the ET tube, administered smoothly over 10 seconds. The ET tube was removed, and mice

were given an i.p injection 2 µl/g bodyweight of atipamezole (Anti-Sedan) (0.5 mg/ml) to reverse anaesthesia.

2.3.3.2 Aerosol lung instillation

One hour after LPC conditioning, the mice were connected to the Aeroneb®Pro-flexiVent™ ventilator circuit via the ET tube as shown in Figure 2-3. This system was designed to minimise the distance between the Aeroneb®Pro and the ET tube, but keep the flexiVent™ system outside the biosafety cabinet. The mice were mechanically ventilated for two minutes using the following ventilation parameters: tidal volume (V_T) 10 ml/kg, respiratory rate (RR) 120 breaths/minute, duty cycle (DC) 1 (i.e. operated at a 100% during the inspiratory phase only), positive end expiratory pressure (PEEP) set at 3 cmH_20, and inspiratory: expiratory ratio (I: E) ratio ~ 1:1 (0.233 s: 0.266 s). The mice were closely observed to ensure breathing was synchronised to mechanical ventilation and for breathing difficulties throughout the experiment.

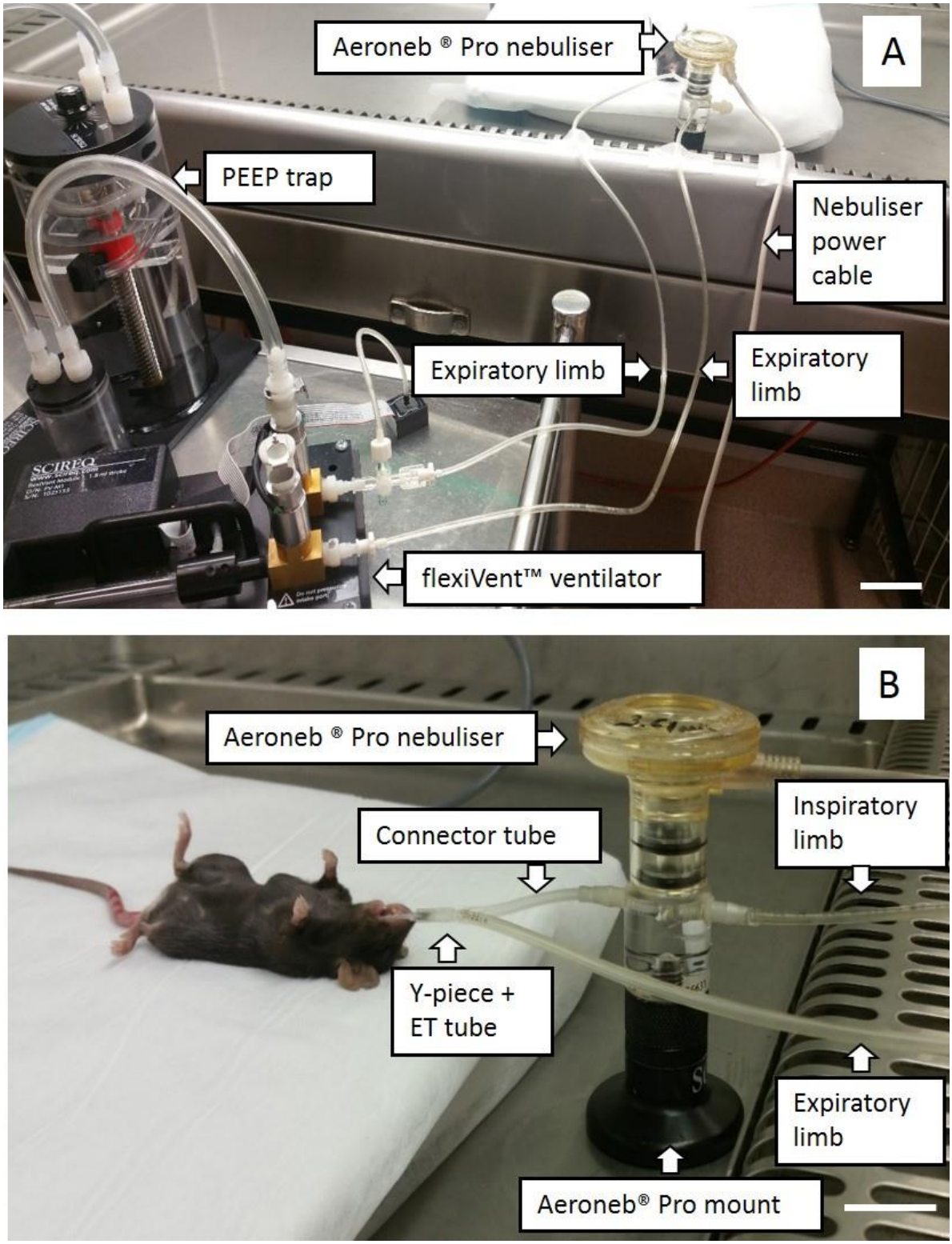

Figure 2-3: Aerosols of LV-LacZ vector: (A) the Aeroneb®Pro nebuliser connected in-line with the flexiVent™ ventilator (scale bar = 5 cm) and (B) mouse connected to the Aeroneb®Pro-flexiVent™ ventilator circuit via the ET tube (scale bar = 2.5 cm).

One hour after receiving the LPC conditioning, a 20 µl aliquot of LV-LacZ vector formulation was loaded onto the vibrating mesh of Aeroneb®Pro nebuliser and aerosolised. After delivery,

the mice were disconnected from the Aeroneb®Pro-flexiVent™ ventilator circuit and the ET tube was removed. All components of the ventilator circuit were thoroughly washed and dried prior to treatment of the next mouse. Anaesthesia was reversed as described in Section 2.3.3.1.

2.3.4 Assessment of *LacZ* gene expression

LacZ gene expression was detected by X-gal staining the trachea and lungs of mice treated with the LV vector. One week after LV-LacZ treatment, mice were humanely killed by CO_2 asphyxiation. Mice were placed in a supine position and cardiac puncture was performed. The chest and abdomen were swabbed with 70% ethanol; an incision was made from the abdomen to the chin. Rings of the trachea were exposed by separating the oesophagus and associated connective tissue. A blunt cannula was connected to a drip apparatus containing fixative (2% (w/v) PFA, 0.5% (v/v) glutaraldehyde in PBS, Section 2.1.10). The trachea was pierced using a 23-gauge needle and the blunt cannula was then inserted into the trachea and secured in place using silk sutures. The peritoneal cavity was then pierced to allow the lungs to collapse and the drip-line was opened to inflation fix the lungs.

Associated tissues (the oesophagus, ribs, and heart) were carefully separated, and the lungs were removed and placed in fixative at 4°C for 2 hours. The lungs were rinsed twice in 1 mM $MgCl_2$ at 4°C for 15 minutes and stained overnight (X-gal solution diluted 1:20 with pre-X-gal solution, see 2.1.10) at 37°C. The staining solution was removed, lungs were washed in 0.9% saline, and fixed in 10% neutral buffered formalin (NBF) for 22 hours. The lungs were stored in 70% ethanol and *en face* pictures of the whole lung were taken using a stereo-microscope. The lung tissue was separated into the trachea, left lung, and right lung. The left lung was cut horizontally into two equal halves, while the four lobes of the right lung were separated. *En*

face images of dissected lungs were recorded to visualise the *LacZ* gene expression. Longitudinal sections of the trachea and whole lobes of lungs were placed in histology cassettes and sent to Histology Services at Adelaide Medical School (University of Adelaide, SA) for embedding in paraffin. For every tissue sample, two sections of 4 µm thickness were taken using a microtome. One was stained with haematoxylin and eosin (H & E) to visualise morphology and the other counter stained with Safranin O (Saf O) to quantify the number of LV-LacZ transduced cells.

To measure the number of transduced cells in the tracheal section, images of the section were captured at 40x magnification using a compound-light microscope. The length of the trachea (represented as mm) was measured using NIS-element software. The number of transduced cells present within 4 mm of the trachea was counted from the Saf O stained sections at 400x magnification.

Similarly, an image of sectioned lobes of the lung were captured using the stereo-microscope at 75x and the area of tissue was measured using NIS-element software. The number of transduced cells present in lung parenchyma and airways within the entire lobe was then counted under the light microscope at 400x magnification. Results were expressed as number of transduced cells per length of the tracheal sections and per area for lobes of the lungs.

2.4 Statistical analysis

Results obtained were represented as mean and standard deviation of mean (Mean ± S.D), with n representing sample size of the group. G-Power version 3.1 was used to calculate the sample size of the study with statistical significance set at, p=0.05 and power=0.80. Statistical analysis was carried out using GraphPad Prism (version 8). Parametric or non-parametric tests

were carried out based on distribution of data. Student's T-test (for data with equal variance) or Welch's T-test (for data with unequal variance) were used to compare between two treatment groups and one-way analysis of variance (ANOVA) was used to compare across multiple treatment groups.

3 AEROSOL DELIVERY OF LENTIVIRAL VECTOR TO MOUSE AIRWAYS

3.1 Introduction

Lentiviruses (LV) are a leading vector of choice for CF gene therapy as they can transduce dividing and non-dividing cells[51, 132, 140], integrate into host cell genomes[132], carry the large *CFTR* gene[132], and are more efficient than some other viral vectors[153]. As mentioned in the introduction, the CFARG has developed a HIV-1 derived LV vector pseudotyped with a VSV-G glycoprotein envelope[132]. The two-step bolus vector delivery method developed by this group includes an LPC conditioning followed by the LV vector dose one hour later, to the airways of mice[51, 132, 139]. Studies by Cmielewski et al. showed that delivering a bolus of 15 µl 0.1% LPC conditioning solution followed by 20 µl bolus of LV-LacZ vector (8.8×10^9 gRNA copies/ml) to mouse airways and observed an average of 65.9 transduced cells per mm of conducting airway (including the trachea and the bronchial airways)[150]. In another study, Liu et al. delivered a bolus dose of 15 µl of 0.1% of LPC and a 30 µl bolus dose of LV-LacZ vector (9.36x 10^7 TU/ml) to mice airways[140]. This study showed lower levels of gene transduction in the trachea (two transduced cells per mm^2) and the bronchial airways (one transduced cell per mm^2) compared to the study by Cmielewski et al. Although the titre of these two studies cannot be compared directly it could be a contributing factor. These published studies clearly suggest that it is difficult to transduce mouse bronchial airways, even when the LV is delivered as a liquid. The two-step bolus vector delivery method was also used to deliver LV vector carrying a reporter gene to the airways of larger animal models, such as ferrets[56], marmosets[57], and sheep[140]. Gene expression associated with bolus delivery was patchy in major airways and lung parenchyma possibly caused by pooling of the vector dose at those

regions [57, 139]. Katkin et al. observed similar patchy gene expression in the lungs of cotton rats treated with a bolus dose of Ad vector, while uniform gene expression was observed in lungs of aerosol-treated animals[54], suggesting that a more uniform spatial distribution could be obtained by aerosolising the vector.

Aerosol delivery is attractive due to its non-invasive nature, relative ease of access to the airways, and its potential for uniform drug deposition. In comparison to other delivery routes, aerosol delivery offers immediate availability of drugs or agents at airway surfaces, which could trigger rapid onset of action and provide a positive clinical experience[154]. Aerosol vector delivery protocols have been well established for viral vectors, such as Ad[90, 91], AAV[106, 111, 112, 114], and SeV[58, 155]. Current LV delivery research focuses on developing an optimal aerosol delivery method, which has been challenging due to the more fragile nature of the LV vector in comparison to other viral vectors[156]. In a recent study, Cooney et al. successfully delivered a spray of FIV derived LV vector to airways of newborn pigs using an intra-tracheal spray device called the MADgic™ atomisation device[87]. Cell culture experiments in this thesis also demonstrated that this device was effective in delivering a coarse spray of the HIV-1 vector (Appendix, Section 8.1.4.2), but the device has some limitations (Introduction, Section 1.6.6). The HIV-1 vector has also been delivered as an aerosol in lung cancer studies[157], however the efficacy of aerosolising this vector has not been reported.

A major challenge reported in the above-mentioned studies was lowered viability of certain viral vectors, such as Ad, and SeV, following aerosol delivery[54, 58]. This lowered vector viability was likely due to shear stress applied on the vector during the process of aerosolisation[58, 158]. Thus, it is important to find a compatible nebuliser that can retain efficacy of the viral vector on aerosol delivery[66]. Conventional nebulisers, such as jet-nebulisers, produce high shear

stress due to generation of an air-liquid interphase and recycling of the aerosol formulation multiple times within the nebuliser before release[159]. Ultrasonic nebulisers are known to damage the aerosolised formulation due to heat generated during the process of aerosolisation[159]. A newly developed ultrasonic nebuliser called the SAW nebuliser has shown to effectively aerosolise sensitive biological formulations[80]. However, this thesis showed that the SAW nebuliser was not effective for aerosolising the HIV-1 vector (Appendix, Section 8.1.4.1)

In comparison, newer delivery systems, like vibrating mesh nebulisers are a single pass device developed to reduce shear stress produced by recycling the aerosolised formulation within the nebuliser[159]. Vibrating mesh nebulisers have been effective in delivering drug formulations sensitive to shear stress and temperature[159, 160], such as liposomal salbutamol sulphate[161], and biodegradable nanoparticles[144]. Hertel et al. demonstrated conservation and stability of a protein formulation SM 101, a soluble human FcγRIIB receptor, following delivery through an Akita^{2}Apixneb™ vibrating mesh nebuliser (PARI, Gmbh, Stamberg, Germany)[159]. In another study, Kleeman showed that aerosolising iloprost containing liposomes through an Aeroneb®Pro vibrating mesh nebuliser produced low physical damage compared to a jet nebuliser and an ultrasonic nebuliser[162]. Changes in the formulation after aerosolisation through vibrating mesh nebulisers has also been investigated. MacLoughlin et al. showed that formulation temperature increased by a maximum of 7°C at an ambient temperature of 18°C following three consecutive passes through the Aeroneb®Pro nebuliser, each lasting 24 minutes[74]. However, the authors mentioned that this increase in temperature did not exceed 37°C, the temperature at which many biological formulations, such as gene therapy vectors, DNA, and proteins, are denatured[74]. Moreover, vibrating mesh nebulisers also have a high

output rate, shorter nebulisation time, and almost no dose retained in the device following aerosol delivery compared to jet nebulisers[72, 144, 163]. Together, these features enhance suitability of the vibrating mesh nebuliser to aerosolise difficult to produce and expensive viral vectors.

The Aeroneb®Pro vibrating mesh nebuliser was chosen for all remaining studies presented in this thesis as is it approved for use in humans and has been well-established in clinics[164, 165]. This nebuliser may be used in-line to deliver aerosols to lungs of mechanically ventilated patients[166, 167] and is also easily adapted to deliver aerosols to the lungs of smaller animals, like rodents mechanically ventilated with the flexiVent™ ventilator[146]. Rodents have a low V_T and RR, which results in a very short inspiratory time[74]. The time taken by the Aeroneb®Pro nebuliser to begin generating aerosols is 2 ms, compared to 80 ms for a jet nebuliser and 150 ms for an ultrasonic nebuliser[74]. MacLoughlin et al. demonstrated that this ability of the Aeroneb®Pro nebuliser enabled them to deliver the maximum volume of aerosols during the inspiratory cycle of the rat[74]. The Aeroneb®Pro nebuliser has been successful in delivering vector formulation to the airways of rodents. MacLoughlin et al. used this nebuliser to aerosolise AAV vector to the airways of rats and demonstrated robust gene expression in rat lung[168].

3.1.1 Hypothesis and aims

The hypothesis of this experiment was that the Aeroneb®Pro vibrating mesh nebuliser would be effective in aerosolising a HIV-1 derived LV vector to the lungs of mice, due to its proven ability to aerosolise other viral vectors[168] and shear sensitive drug formulations[161].

The aim of this *in vivo* study was to quantify the baseline level of gene expression produced by aerosolising the LV gene vector into mouse lungs using an Aeroneb®Pro nebuliser operated

in conjunction with the flexiVent™ small animal ventilator and compare it to the level of gene expression obtained by delivering the LV vector as a bolus dose. This was the only complete *in vivo* study presented in this thesis, where the animals were divided into an aerosol group and a bolus delivery group, with n=12 in each group.

3.2 Methods

3.2.1 Animals

Animals used in this study were 8 to 10 week-old C57BL/6 female mice, weighing approximately 22 grams. All animals were cared for and managed, as described in Section 2.3.1.

3.2.2 Gene vector

The HIV-1 gene vector used in this study was pseudotyped with the VSV-G envelope, contained a nuclear-localised *LacZ* transgene driven by the myeloproliferative sarcoma virus (MPSV) promoter, and was produced by transfecting HEK-293T cells using a 5-plasmid system by calcium phosphate co-precipitation (Section 2.2.3). Pellets obtained at the end of the vector preparation process were suspended in a standard carrier fluid (or diluent) of 0.1% mouse serum in 0.9% saline (MS/saline). Four batches of LV-LacZ vector were prepared for this study and were pooled prior to vector administration. The LV-LacZ vector titre of individual vector batches was determined using qPCR methods (Section 2.2.4).

3.2.3 Aerosol airways vector delivery studies

Gene expression produced by the LV-LacZ vector in the lungs of mice following delivery as an aerosol was compared to that produced by vector delivered as a bolus dose (n = 12/group).

3.2.3.1 Airway conditioning and gene vector lung dosing

Mice in both groups (aerosol dose and bolus dose) were anaesthetised and intubated with a

20-gauge intravenous catheter as an ET tube. The airways of both groups were then conditioned with 10 µl of 0.1% LPC delivered as a bolus dose via the ET tube, one hour prior to vector delivery (Section 2.3.3). LPC is a mild surfactant and being of detergent-like nature at high concentrations it can damage the respiratory epithelium and cause pulmonary edema[169, 170]. Cmielewski et al. optimised the bolus dose of LPC conditioning solution (0.1%) required to efficiently transduce the lungs of mice, without much damage[150]. The present study did not deliver LPC as an aerosol because the optimal dosing protocol required to produce efficient gene transduction using this delivery regimen is yet to be determined and was not within the scope of this thesis. Delivering LPC as an aerosol could distribute it uniformly throughout the lungs, potentially damaging the alveolar tissue, and thus would need to be carefully optimised.

3.2.3.2 Vector administration

Intubated mice in the liquid bolus delivery group were treated with 20 µl of LV-LacZ vector pipetted through the ET tube into the trachea and lungs (Section 2.3.3.1). Intubated mice in the aerosol treatment group were connected to the Aeroneb®Pro-flexiVent™ ventilator circuit via the ET tube (Figure 2-3). The ventilator parameters used were: V_T 10 ml/kg, RR 120 breaths/minute, DC 1 (i.e. operated at a 100% during the inspiratory phase only), PEEP set at 3 cmH_20, and I: E ratio ~ 1:1 (0.233 s: 0.266 s). A 20 µl aliquot of LV-LacZ vector was aerosolised over 20 to 40 second through the nebuliser-ventilator circuit. After every aerosol delivery the ventilator circuit was cleaned (Section 2.3.3.2).

After LV treatment, anaesthesia was reversed, and mice were monitored post-operatively (Section 2.3.1). Mice were humanely killed by CO_2 asphyxiation one week later. Each mouse trachea, bronchial airways and lungs were dissected, stained with X-gal solution, and

processed for histological analysis (Section 2.3.4). The number of LacZ expressing cells present in trachea and lungs of mice were assessed.

3.3 Results

The four LV-LacZ vector batches prepared for this study had titres of 8.19 x 10^8 TU/ml, 1.04 x 10^9 TU/ml, 3.76 x 10^7 TU/ml, and 6.57 x 10^8 TU/ml. Equal volumes of the four LV vector batches were pooled prior to vector administration (average titre 6.38 x 10^8 TU/ml). The LPC conditioning and LV vector dose were tolerated well by all mice in both treatment groups. The general behaviour of mice, including demeanour, feeding, respiratory motion, and weight, was within normal limits post-treatment.

The lungs treated with a bolus dose of the vector demonstrated *LacZ* gene expression in the trachea, which extended into the bronchial airways and upper lobes of the left and right lungs (indicated by black arrows, Figure 3-1 A). Gene expression observed in the trachea of bolus-treated animals was along an axial line and was more intense than that seen in aerosol-treated animals (Figure 3-2). The *en face* images of bolus-treated animals revealed intense and patchy gene expression at the carina (Figure 3-1 A and B), although no gene expression was seen in bronchial airways themselves. This pattern of gene expression in tracheal and bronchial airways was seen in 10 out of 12 bolus-treated animals. Furthermore, a cross-section of the left lungs in 5 of 12 animals belonging to the bolus treatment group revealed diffuse *LacZ* gene expression in major airways (indicated by black arrow, Figure 3-1 C and D) and patchy gene expression in the parenchyma (indicated by blue arrow, Figure 3-1 C and D).

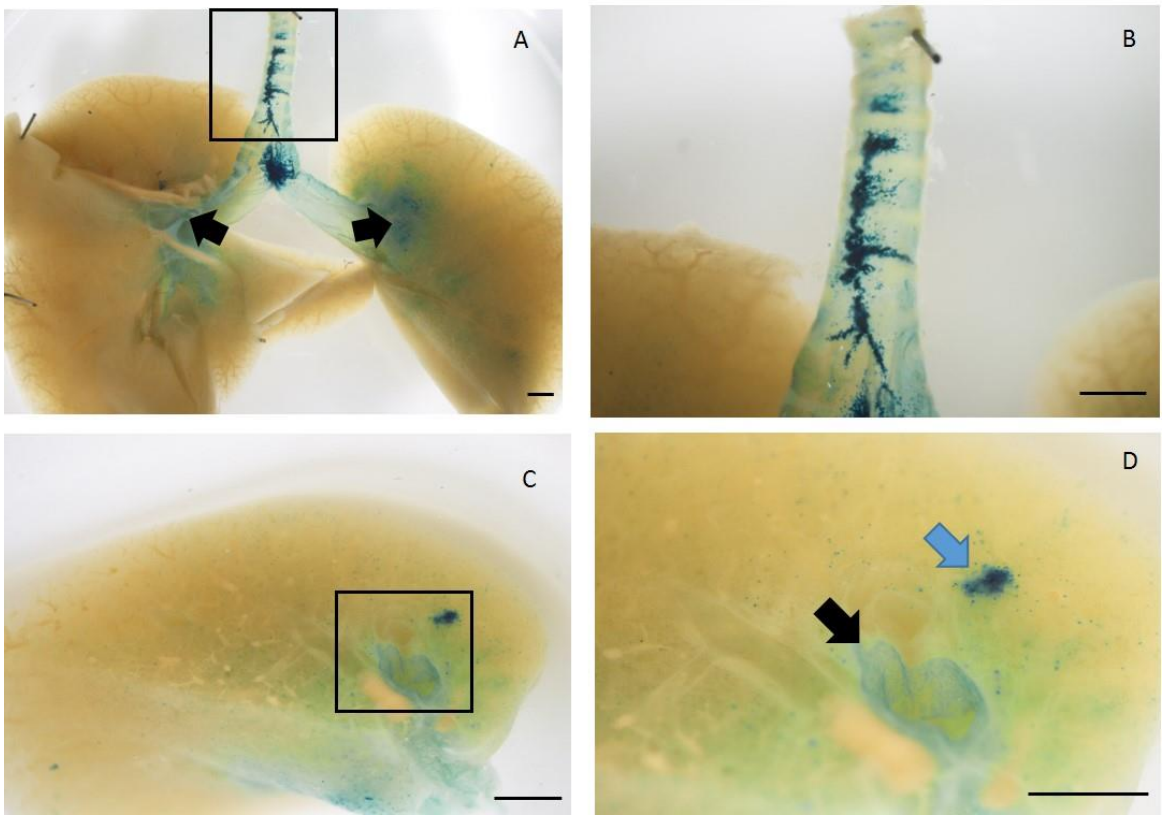

Figure 3-1: Bolus delivery of LV-LacZ vector: gene expression (blue-stained regions) observed from the en face images of (A) mouse lung showing LacZ gene expression in the upper lobes of the left and right lungs (black arrows), (B) magnified view of the trachea, (C) cross-section of left lung, and (D) magnified view of the sectioned lobe showing diffuse LacZ gene expression in the major airways (black arrow) and patchy gene expression in the parenchyma (blue arrow) (scale bar = 1 mm).

En face examination of the tracheas from mice treated with aerosolised LV vector (Figure 3-2 A and B) revealed that *LacZ* gene expression was primarily limited to regions near the site of dosing, with a few transduced cells observed in the more distal bronchial airways. Gene expression was not observed in the lung parenchyma of mice belonging to the aerosol treatment group.

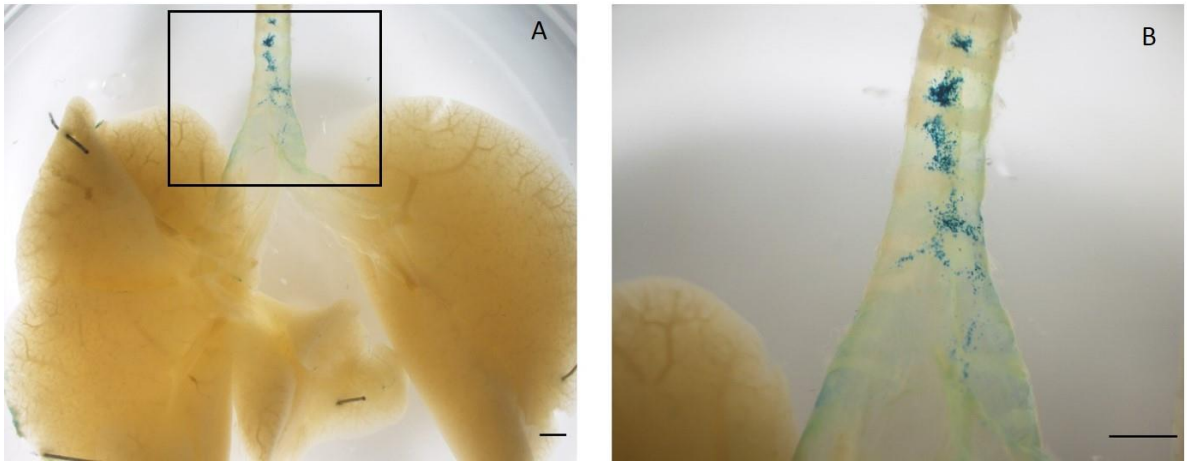

Figure 3-2: (A) En face images of X-gal stained mouse lungs following aerosol delivery of LV-LacZ vector LacZ expression (blue-stained regions) is limited to site of delivery in the trachea (B) magnified view of the same trachea (scale bar = 1 mm).

Transduction in of one of twelve animals in the aerosol treatment group exhibited very high levels of LacZ transduced cells. The *en face* images of this mouse showed *LacZ* gene expression in the trachea, bronchial airways, and throughout the lobes of the left and right lungs (Figure 3-3 A). Cross sections of the left lung revealed intense and uniform transduction along the major airways (Figure 3-3 B).

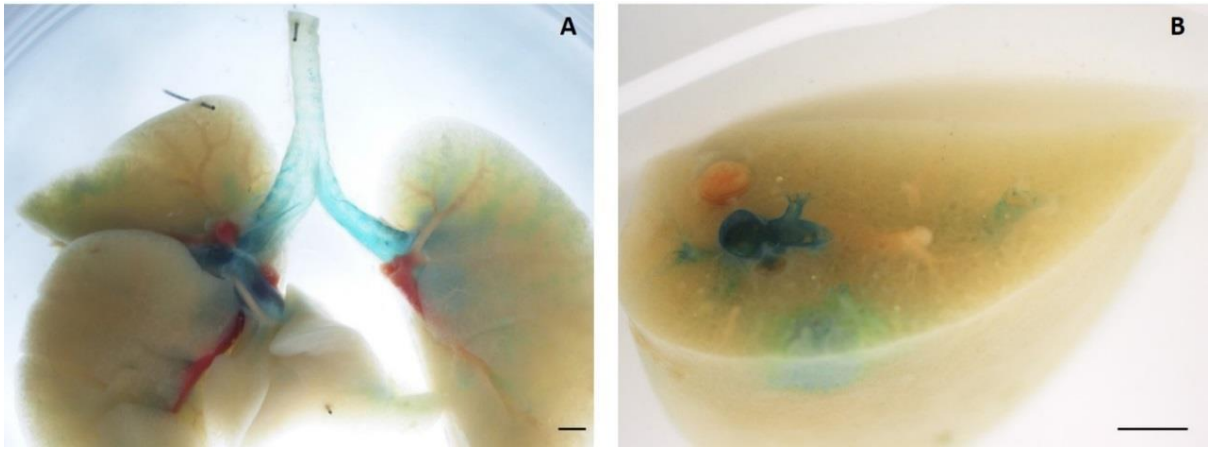

Figure 3-3 : Aerosol delivery of LV-LacZ vector: gene expression observed in the outlier animal (A) en face image of the whole lung (B) cross section of the left lung (scale bar = 1 mm).

Both aerosol and bolus delivery regimens primarily targeted ciliated cells of the conducting airways. Histological analysis of all tracheas revealed that the mean number of LacZ-

transduced cells in the bolus delivery group was nine-fold higher than in the aerosol delivery group (Figure 3-4). In addition, Figure 3-4 shows high variability in the number of transduced cells seen in the trachea of bolus treatment group.

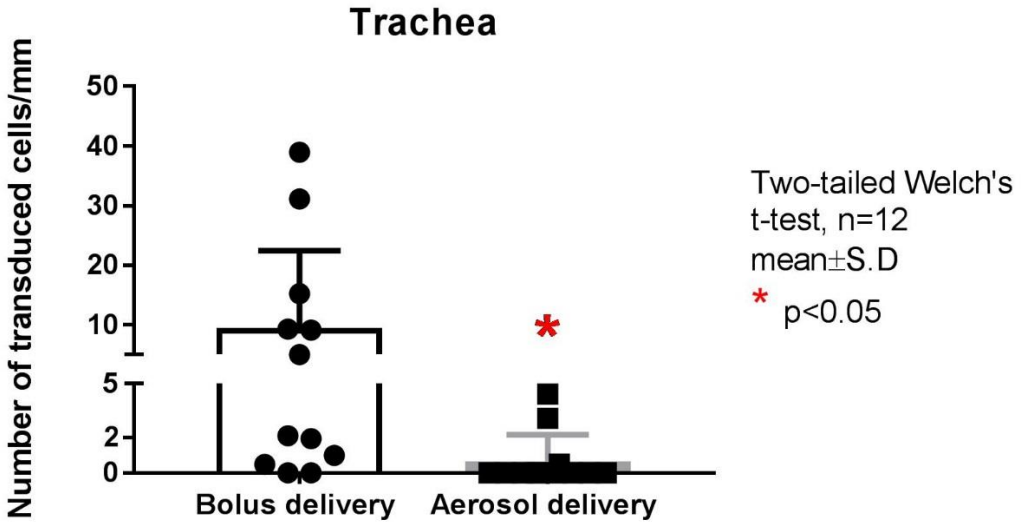

Figure 3-4: LacZ gene expression observed in the trachea on delivering the vector as a bolus or as an aerosol dose.

There were very few LacZ-transduced cells in the bronchial airways of animals from both treatment groups (data not shown). This result suggests that it was difficult to transduce mouse bronchial airways using either delivery methods employed in this study.

A histological analysis of the left lungs revealed no significant difference in the number of transduced cells between the two treatment groups (Figure 3-5).

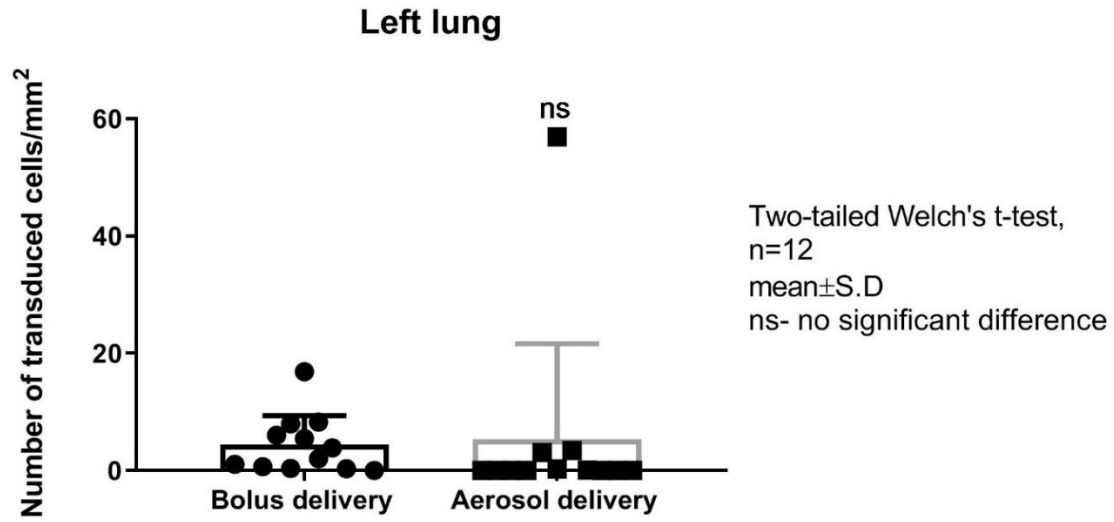

Figure 3-5: Number of transduced cells observed in the left lung of mice from each treatment group.

Similar to the bronchial airways, the number of LacZ-transduced cells observed in the right lung of mice in both treatment groups was very low (Figure 3-6). Thus, other than the observation of poor gene expression, this study was limited in its capacity to draw conclusive results from *LacZ* gene expression observed in the right lung of mice.

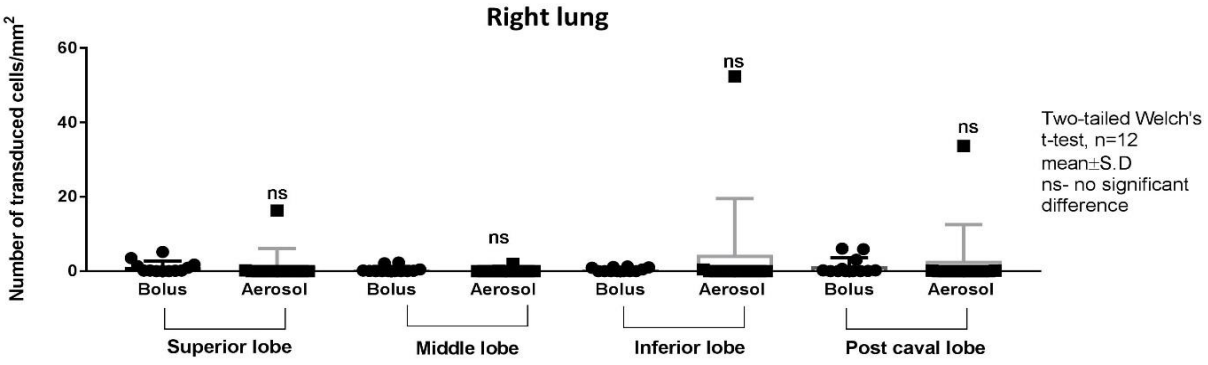

Figure 3-6: LacZ gene expression in the right lung produced by delivering the LV vector either as an aerosol or a bolus dose.

LacZ-transduced ciliated, non-ciliated, and basal cells were observed in the trachea of mice belonging to the bolus treatment group (Figure 3-7). In the aerosol treatment groups, only infrequent LacZ-transduced ciliated cells were observed.

Figure 3-7: H & E stained sections of a trachea from the bolus delivery group showing LacZ-transduced non-ciliated cells (dash arrow), ciliated cells (double-lined arrow), and basal cells (solid arrow) (scale bar =10 µm). The LacZ staining was strong enough to bleed out of the cell nuclear region.

In the bolus treatment group, LacZ-transduced ciliated cells were seen in conducting airways of the left lung (image not shown), along with transduced type 1 and type 2 pneumocytes (Figure 3-8 A). A small number of transduced macrophages were also observed in the alveolar region (Figure 3-8 B).

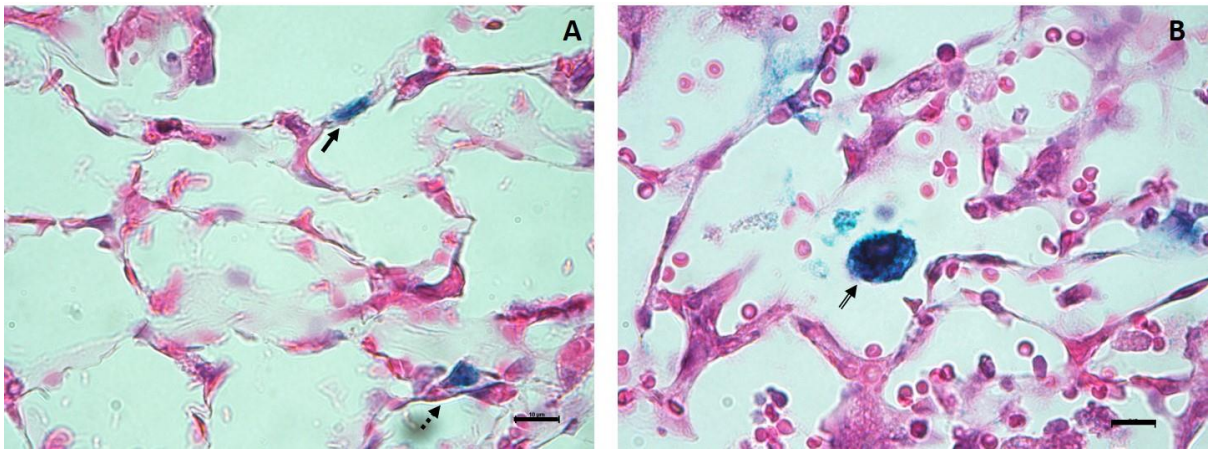

Figure 3-8: H & E stained sections of the alveolar region present in the left lung of mice from the bolus delivery group, showing (A) a LacZ-transduced type 1 pneumocytes having squamous morphology (solid arrow), type 2 pneumocytes having cuboidal morphology (dotted arrow) and (B) a LacZ transduced macrophage at the centre (double-lined arrow) (scale bar =10 µm).

One animal that was transduced in the aerosol treatment group demonstrated gene expression in a majority of cells present in the airways of the left and right lungs (Figure 3-9 A), and included LacZ-transduced ciliated, goblet and basal cells (Figure 3-9 B).

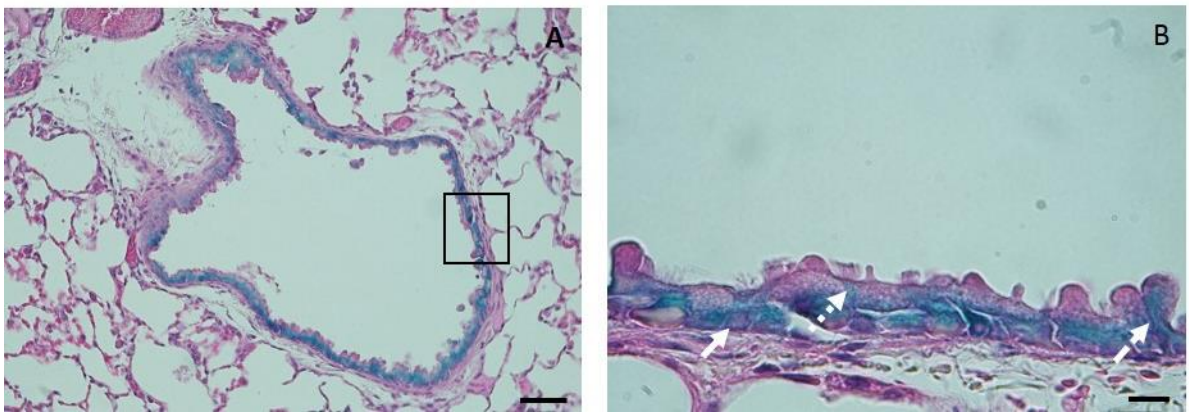

Figure 3-9: Outlier animal from aerosol delivery group showing (A) transduced cells in the airways (scale bar = 50 µm) and (B) magnified view of the airway showing LacZ-transduced basal (solid arrow), ciliated (dotted arrow) and goblet cells (dash arrow) (scale bar = 10 µm).

3.4 Discussion

Developing an effective vector delivery regimen is crucial for CF airway gene therapies. Aerosol vector delivery may be ideal for use in future CF gene therapy trials, as it is convenient and is thought to be more likely to distribute the vector uniformly in the lungs of animals compared to bolus delivery[54]. To my knowledge, this proof of concept *in vivo* study is the first to test the efficiency of aerosolising a HIV-1 based LV vector using an Aeroneb®Pro nebuliser in conjunction with a flexiVent™ ventilator circuit to the airways of mice.

Viral vectors can be administered as an aerosol to the lungs of animal models via the nasal[97] or tracheal route[112], or via (whole-body box) inhalation delivery[54]. In this study, the LV vector was delivered by the tracheal route using an ET tube, as this provides immediate availability of the LV formulation to the lung, and avoids loss of dose on the body surface (with whole animal exposure chambers) or in the nose or throat as can occur with other delivery routes[171, 172]. The intra-tracheal route has also been recognised as an optimal delivery method and has been used to deliver formulations such as sub-unit vaccines[173], cationic lipoplexes[174], and polymeric microspheres[175] to mouse lungs.

Results reported in the present study did not support the hypothesis that the Aeroneb®Pro nebuliser was effective in delivering a HIV-based LV vector to the lungs of mice when mechanically ventilated with the flexiVent™ ventilator. Histological analysis of the trachea revealed that levels of gene expression in the bolus-treatment group were nine-fold higher than the aerosol treatment group. The proximal conducting airway region in mice is the location of ciliated epithelial cells and the recently identified ionocytes that express approximately 60% of the CFTR mRNA in the lung[44]. Both the aerosol and bolus delivery regimens presented in the current study could transduce these cells, although this remains unproven and needs to be investigated in future studies. The proportion of ionocytes present in the mouse airway is very low (~1% of the airway cells)[44], but they are clearly a very important population of cells in CF. This means that future studies should also examine whether a vector specifically designed to target ionocytes would improve outcomes in CF.

High variability in the number of transduced cells, in the trachea, was observed in the bolus treatment group. In a previous study, Liu et al. found similar levels of variability in gene expression in the lungs of mice treated with a bolus dose of the same LV vector[140]. This variability in gene expression could be a result of different ET tube insertion depths or techniques, different respiratory characteristics of each mouse, or unpredictable loss of vector dose due to capillary action along the ET tube. The *en face* images of animals in both treatment groups also revealed intense LacZ staining along an axial line in the trachea, likely as a result of the flow path of the vector dose or physical disturbance of respiratory epithelium caused by inserting and withdrawing the ET tube. Disturbance of the epithelium by a physical process (e.g. ET tube insertion/withdrawal) or chemical agent (e.g. LPC conditioning solutions) are both known to result in higher levels of gene expression due to increased access of the

vector to receptors on the basolateral surface[51, 176].

An effective CF gene therapy must target the epithelial cells of the conducting airways, and in particular the small distal airways. This is the location of the ciliated epithelial cells and basal stem cells[177, 178],that express the highest levels of the CFTR protein in a healthy adult [179,]and also the region where the disease first manifests in CF patients[88]. However, results reported in this study suggest that the bronchial airways of the mice could not be transduced by either delivery regimen: the *en face* images of animals in both treatment groups showed no *LacZ* gene transduction in either region, and histological analysis revealed very low levels of gene expression that prevented conclusive findings. It was found that the experiment was not sufficiently powered to identify significant differences in this region. A one-tailed t-test comparing the number of LacZ transduced in the bronchial airways of the aerosol and bolus delivery group (A-priori effect size = 1.02, α=0.05, power =0.80) showed that a minimum of n = 123 animals per group was required to show a significant difference between the aerosol and bolus treatment groups in the bronchial airways. The large sample size estimate resulted from the low levels of gene expression observed in the bronchial airways, combined with the high variability observed within each group. Performing a study with such a large sample size is clearly not practical. Hence the subsequent chapters focus on optimising the vector delivery methods using *in vitro* techniques.

The low levels of gene expression observed in both aerosol and bolus treatment groups at the bronchial airways could be due to low residence time and/or low physical dose volume of the LPC conditioning agent or LV vector at these regions. Nonetheless, these results were similar to findings of Liu et al[140], who showed that only two transduced cells/mm^2 were present in conducting airways and lungs of mice treated with a bolus dose of 15 µl of 0.1% LPC and 30

µl of the same HIV-1 vector (9.36×10^7 TU/ml) used in the present study[140]. Although a lower volume of 0.1% LPC conditioning solution (10 µl) and LV vector (20 µl bolus dose, average titre 6.38×10^8 TU/ml) was used in the present study, the similarity in the results to that of Liu et al. could be because the titre of the LV vector used in the present study was higher compared to Liu et al. and improvement in bolus delivery methods[150]. In contrast, Cmielewski et al. demonstrated higher levels of gene expression in the conducting airways of mice-treated with a bolus dose of the same HIV-1 vector[150]. They measured an average of 65.9 LacZ transduced cells per mm of conducting airway following a bolus dose of 15 µl of 0.1% LPC conditioning solution and 20 µl LV-LacZ vector (8.8×10^9 gRNA copies/ml)[150]. As mentioned in the Introduction (Section 3.1), this elevated level of gene expression could have resulted from improvements in the method of administering the bolus dose of LPC conditioning solution and LV vector[150]. Thus, the results presented in this Chapter suggest that delivering LV vector as an aerosol was not likely to produce uniform and wide-spread transduction from the trachea to the smaller airways of mice. Together these results highlight the difficulties of efficiently transducing conducting airway tissue, a challenge faced by the entire CF gene therapy community.

Further observation of *en face* images of bolus-treated animals showed intense *LacZ* gene expression in the upper lobes of the left and right lungs. Cross sections of the left lung revealed patchy transduction in the parenchyma of bolus-treated animals. This patchy pattern of gene transduction was also observed in airways of sheep and marmosets treated with a bolus dose of the LV vector[57, 140]. This transduction pattern could be caused by any or all of the following: pooling of bolus vector dose in the lungs; incomplete coverage of the area due to small vector volume; specificity of the LV vector for a certain cell type; or mismatch

between the LPC and LV-treated areas[57, 58].

In the present study, histological analysis of the left lungs showed no significant differences in the number of transduced cells between the two treatment groups (Figure 3-5), which was likely due to high variability observed in the aerosol treatment group. This high variability was caused by one outlier animal with higher than expected gene expression levels (Figure 3-3). Interestingly, the elevated level of gene expression observed in the outlier animal was higher than that observed in the bolus treatment group, which could be caused by dosing error. Delivering a larger volume of LV vector or LPC conditioning solution to the airways, such as twice the required dose, could explain the unexpected levels of gene expression observed in this animal. Additionally, insufficient cleaning of the ventilator circuit component could have resulted in viable LV particles being retained in the components from the previous treatment and therefore a higher dose of vector was delivered to the lungs of the animal. Also, the elevated levels of gene expression could have been caused from an inherent and unexplained variation of the respiratory characteristic of that mouse. Although the level of gene expression observed in this outlier animal was greater than bolus dose, it demonstrates what might be achievable in future experiments provided optimal aerosol delivery parameters can be identified.

Histological analysis of the right lung showed lower levels of gene expression in both treatment groups, which could be due to lung and airway morphology, and the dosing method. In mice the right lung is divided into four lobes while the left lung has a single lobe. Aerosol and fluid deposition studies have shown that the morphology of the lungs (including the width of the airways and branching angle) heavily influences dose deposition[180]. The bolus delivery study published by Liu et al. showed contrasting results to the present study, by

demonstrating efficient gene expression in lobes of both the right and left lungs of mice[140]. These contrasting results may be due to a subtle difference in the bolus delivery protocol used by Liu et al. compared to the present study. Liu et al. suspended the mouse by its dorsal incisors and the mouse's dorsal side was supported against an inclined board during LPC and LV vector delivery[140]. In contrast, in the present study mice in both treatment groups were placed in a supine position during LPC and vector delivery. It is possible that this difference in animal position during delivery could have influenced the volume delivered to specific regions of the lung (particularly for the liquid group) and could be the reason for the absence of gene expression in the right lung. However, additional studies would be needed to confirm this hypothesis.

In summary, significant difference in levels of LV gene expression between the aerosol group and bolus treatment group was only identified at the tracheal region of mice airway. Three mechanisms may be responsible for the lower transduction levels seen in the trachea of the aerosol-treated animals compared to the bolus treated animals. Firstly, this could be caused by a lower physical volume of the vector reaching the trachea and lungs of animals in the aerosol group. This lowered dose volume could be caused by unoptimised aerosol delivery parameters and the design of the Aeroneb®Pro-flexiVent™ ventilator circuit in the present study. Delivering the vector as a bolus dose is less complicated as the dose is delivered directly to the ET tube via a fine polyethylene pipette, maximising the volume of fluid reaching the airways. The volume of aerosolised formulation that reaches the tip of the ET tube and the effect of aerosol delivery parameters on delivery efficiency of the Aeroneb®Pro-flexiVent™ ventilator circuit are examined in Chapter 4.

The second cause for lowered gene expression in the trachea of mice treated with LV aerosols

could be loss of vector viability due to partial damage of the LV vector during transit through the nebuliser and component of the ventilator circuit. Although, the Aeroneb®Pro nebuliser has been proven to efficiently aerosolise shear sensitive nanoparticle[144] and liposomes[161], it is still possible that LV vector viability was affected by the process of aerosolisation in the Aeroneb®Pro, the turbulent flow through the Scireq in-line Aeroneb®Pro nebuliser mount, and the transit through the inspiratory tubing and ET tube. Chapter 5 examines the viability of LV aerosol delivered through the Aeroneb®Pro nebuliser and that expelled at the end of the Aeroneb®Pro-flexiVent™ ventilator circuit.

The third cause of the lowered levels of gene expression observed in the aerosol treatment group could be the mismatch between the areas treated with a bolus dose of LPC conditioning agent and the aerosolised LV vector. Delivering LPC as an aerosol might enable uniform distribution throughout the lung that might better overlap with regions later treated with aerosolised LV vector, resulting in uniform transduction. However, as explained earlier delivering a higher concentration of aerosolised LPC could result in pulmonary oedema (Section 3.2.3.1). In this study LPC was deliberately delivered as a liquid rather than as an aerosol so that only one single variable associated with gene delivery, i.e. the method of delivering the LV vector, was examined. The effect of delivering LPC conditioning as an aerosol in conjunction with aerosolised LV vector was not examined at this point, because a much larger series of animal experiments would have been needed to establish all of the factors that cause changes in gene expression. The present chapter identified that delivering the vector as an aerosol produced lower levels of gene expression in comparison to bolus delivery, so it is suggested that delivering LPC as an aerosol should only be examined after the protocol for LV aerosol delivery has been optimised.

Although the effects of aerosolising LPC conditioning by itself has not been examined LPC has been formulated with other viral vectors, such as Hd-Ad, and delivered as a coarse spray in other gene therapy studies. Koehler et al. showed that delivering a spray of a Hd-Ad vector formulated with 0.1% LPC to rabbit airways enabled effective gene transduction, which was likely by opening tight junctions in rabbit airways[81]. They also demonstrated that this dose was tolerated well in animals[81]. Further studies conducted by the same group demonstrated that spraying vector formulated with a much lower concentration of LPC (0.01 %) produced effective transduction in porcine airways and was well tolerated by animals in the study[103]. Although a coarse spray of 0.1% LPC formulated with Hd-Ad vector, has been shown to efficiently transduce animal airways its efficacy might vary when delivered as an aerosol using a nebuliser[103]. Delivering 10 µl of 0.1% LPC (same concentration and volume used in the present study) as an aerosol may result in uneven distribution of LPC at the epithelial surface as the volume of LPC used would be too low to cover the entire airway epithelial surface in mice; however, this remains to be investigated in a future study. Future studies would also need to identify the optimal dose volume and LPC concentration for use as an aerosol, bearing in mind that higher concentrations of LPC will likely damage the airway epithelium rather than transiently open tight junctions. They should also determine how uniformly the aerosol delivery method distributes LPC, and whether this enables effective disruption of the tight junctions on the epithelial surface.

3.5 Conclusion

Aerosol delivery of a gene therapy vector formulation is an attractive approach as it has the potential to be non-invasive and could produce a uniform distribution of vector in the conducting airways of animals. This was the first study to investigate the effectiveness of

aerosolising a HIV-1 based LV vector in the airways of mice, using an Aeroneb®Pro nebuliser and flexiVent™ small animal ventilator. *LacZ* gene expression levels produced in the trachea of mice were significantly lower in the aerosol treatment group compared to the bolus treatment group. However, no significant difference in the level of gene expression between the two treatment groups was observed in the left lungs of mice, which was likely due to an outlier in the aerosol treatment group. The effect of vector delivery regimens could not be determined from other regions of the mouse airway, such as the bronchial airways or right lung, as very low levels of gene expression were observed in both treatment groups. Together, these results only identified the lowered efficiency of LV aerosol delivery, using the Aeroneb®Pro-flexiVent™ ventilator circuit, in the trachea of mice. Additionally, the reasons for the lowered efficiency of this nebuliser-ventilator apparatus in delivering LV aerosols was inconclusive. Further investigation is needed to determine the effect of this nebuliser as a viable option in further aerosol delivery studies. A range of factors affecting aerosol delivery of the LV vector through the Aeroneb®Pro nebuliser operated in conjunction with flexiVent™ ventilator are examined further in Chapters 4 and 5.

4 OPTIMISING AEROSOL DELIVERY EFFICIENCY FOR MECHANICAL VENTILATION OF MICE: AN *IN VITRO* STUDY

4.1 Introduction

Chapter 3 demonstrated lowered gene expression in the trachea of mice treated with an aerosol dose of the LV vector compared to those receiving a bolus dose (Section 3.3). In the aerosol delivery group, the LV vector was aerosolised through an Aeroneb®Pro-flexiVent™ ventilator. It was proposed that the lower levels of gene expression observed in this group could be caused by a reduced physical dose volume being delivered to mouse trachea, and/or a reduction in LV viability during the process of aerosolisation. This Chapter investigates the former reason for lowered gene expression. This study quantified the dose volume released at the end of the Aeroneb®Pro-flexiVent™ ventilator circuit when applying the same ventilation parameters that were used to deliver the LV vector to the mouse lungs (Section 3.2.3).

The Aeroneb®Pro nebuliser is increasingly used to aerosolise drug formulations into the lungs of mechanically ventilated animals[146, 181, 182], but there have been consistent difficulties reported in delivering a volume similar to the initial dose through a ventilator circuit. Ferrari et al. aerosolised ceftazidime into the airways of mechanically ventilated piglets and observed deposition that represented 66% of the initial dose volume[183]. Dubus et al. demonstrated deposition of only 13-14% of the initial dose volume on aerosolising 99mTc-diethylene triamine pentacetic acid (DTPA) into the lungs of mechanically ventilated macaques[184]. The dose delivered to lungs of mechanically ventilated mice has been reported to be lower than that delivered to the lungs of larger animal models. Robichaud et al. aerosolised methacholine

to the lungs of mechanically ventilated mice using an Aeroneb®Pro nebuliser with the flexiVent™ ventilator and showed an extremely poor deposition efficiency of 0.1 to 0.2 % of the initial dose at the target site. They reported that a majority of the aerosolised dose was deposited in the ventilator tubing due to impactional deposition, commonly referred to as rainout[146], providing a likely explanation for the low performance of their system. Based on that study, it could be hypothesised that the volume of aerosol produced at the end of the Aeroneb®Pro-flexiVent™ circuit is likely to be significantly lower than a bolus delivery volume. Hence, there is a need to optimise the aerosol delivery efficiency of this system.

The dose volume expelled at the outlet of the ventilator circuit is dependent on nebuliser-related factors, ventilator circuit-related factors, the chosen ventilation parameters, properties of the formulation, and patient (or animal)-related factors[185]. Amongst these, ventilator circuit-related factors and ventilation parameters significantly affect the delivery efficiency of a rodent ventilator system[74]. Ventilator circuit-related factors that affect delivery efficiency are the length and internal diameter of ventilator circuit components[74, 186], the number and type of connectors[186], the presence of tight-radius bends within circuit components[187], and the use of corrugated tubing[188].

As mentioned earlier, deposition of aerosolised formulation within the narrow ventilator tubing was suspected to be the main reason for reduced delivery efficiency in a rodent ventilator system[74, 146]. The width of the ventilator tubing was also shown to influence deposition of the aerosolised dose in clinical studies. Pedersen et al. showed that approximately 4.5% of aerosolised dose was deposited within an 8 mm ET tube, which is the narrowest component of an adult ventilator circuit[187]. However, the dose deposited within a 6 mm ET was 8.4%, under the same delivery conditions[187]. The researchers speculated that

the narrow internal diameter of the 6 mm ET tube could have constricted the aerosol flow path (therefore increasing the gas velocity) and given rise to turbulent airflow within the component[187]. This could have caused impaction and deposition of aerosols against the inner walls of the component, thus explaining increased dose deposition[187]. Hence, it could be hypothesised that using a ventilator circuit component with a larger internal diameter should reduce impactional deposition of aerosols within the component and improve aerosol output.

Ventilation parameters determine the velocity of aerosol flow within the ventilator circuit, and hence influence dose delivered to the lungs[187]. Ventilation parameters, such as RR, V_T, DC, PEEP, and I: E ratio, determine the characteristics of aerosol flow within the ventilator circuit, which in turn affects delivery efficiency[189-191]. Thomas et al. showed that using a low RR and high V_T, while maintaining a constant respiratory minute volume [MV (ml/min) = V_T (ml) × RR (breaths/min)], delivered optimal volume of aerosolised formulation at the tip of the ET tube[192]. Other studies have also confirmed that using a lower RR[192-194], and higher V_T[190, 195] released optimal volume of aerosolised formulation at the tip of the ET tube of an adult ventilator circuit. The researchers speculated that these ventilator settings reduced the velocity of the aerosol and airflow turbulence within the ventilator circuit, which in turn reduced deposition of aerosolised formulation in circuit components, and increased delivery efficiency[192]. Hence, it could be hypothesised that using a lower RR and higher V_T will improve delivery efficiency of a small-animal ventilator circuit, where the largest component is 8 mm in diameter.

Other ventilation parameters, such as the nebuliser DC could also influence dose delivered by the end of the ventilator circuit. Fink et al. reported a significant increase in aerosolised dose delivered to the lungs of mechanically ventilated patients when operating a pMDI at a higher

DC of 0.5 in comparison to a lower DC of 0.25[196]. However, change in DC has not shown to affect aerosol delivery efficiency in an *in vitro* model of a paediatric ventilator circuit[197]. Contrasting results were obtained on testing the effects of DC on delivery efficiency of a small animal ventilator. Robichaud et al. showed that a higher volume of aerosolised methacholine was expelled by the end of the Aeroneb®Pro-flexiVent™ ventilator on operating the nebuliser at a DC of 0.25 compared to a higher DC of 0.5[146]. Thus, it could be hypothesised that using a lower DC produces an increased volume of aerosol at the tip of the Aeroneb®Pro-flexiVent™ circuit.

Researchers have also examined other methods to improve delivery efficiency of nebuliser-ventilator systems. Recent developments include nebulisers that have adaptive aerosol delivery technology, which adapts the nebuliser output to the respiratory pattern of the patient and delivers aerosols in short-pulses during inspiration[198]. This pulsed aerosol delivery has been shown to reduce the velocity of aerosols within the nebuliser system, thus leading to a decreased deposition of aerosols in oropharyngeal regions, and increased lung deposition[198, 199]. The effect of delivering aerosols in short-pulses during mechanical ventilation must still be investigated. It could be speculated that delivering aerosols in short-pulses would reduce aerosol velocity during its transit through narrow tubing of a small animal ventilator, and thus release a higher volume of aerosols at the tip of the nebuliser-ventilator apparatus.

MacLoughlin et al. demonstrated another method to improve delivery efficiency of a rat ventilator operated in conjunction with the Aeroneb®Pro nebuliser. They demonstrated an improvement in aerosol delivery efficiency from ~13% to ~41% of inhaled mass by re-aerosolising the dose deposited in the component adjacent to the Aeroneb®Pro nebuliser;

however, the quality of the re-aerosolised dose material was not known[74]. Similar improvement in aerosol output could be expected on re-aerosolising the dose deposited in the circuit component adjacent to the nebuliser within the ventilator circuit.

4.1.1 Hypothesis and aims

The following hypotheses were developed based on the results from the previous Chapter and the previously published studies outlined above. I hypothesise that the volume of aerosol produced at the tip of the Aeroneb®Pro-flexiVent™ ventilator circuit ET tube will be:

1) Lower than a bolus dose, when using the parameters from the baseline *in vivo* study (Section 3.2.3).
2) Increased by using ventilator circuit containing components with a larger internal diameter, due to minimisation of deposition within the circuit.
3) Higher when using a low RR and high V_T.
4) Improved by reducing the amount of aerosol produced during each breath.
5) Enhanced by re-aerosolising the dose deposited in the Scireq in-line Aeroneb®Pro mount, the circuit component adjacent to the nebuliser.

The aim of this *in vitro* study was to quantify dose volume released from the ET tube at the end of the Aeroneb®Pro-flexiVent™ ventilator circuit when using the same ventilation parameters used in the previous Chapter (Section 3.2.3), by using a dye solution as the surrogate dose fluid. The studies were designed to estimate the amount of fluid that is deposited in each part of the ventilator circuit, and to quantify the effect of making changes, such as replacing the Scireq in-line Aeroneb®Pro mount (the component used to connect to the Aeroneb®Pro nebuliser into the flexiVent™ inspiratory tubing) with one having a larger internal diameter (Figure 4-2), and by altering the ventilation parameters. It was also designed

to assess the effects of delivering aerosol in short time-controlled pulses during each breath, and re-aerosolising the fluid deposited in the Scireq in-line Aeroneb®Pro mount.

4.2 Methods

4.2.1 Aerosol delivery efficiency of the Aeroneb®Pro nebuliser

In this *in vitro* experiment, the Aeroneb®Pro nebuliser (not connected to the ventilator circuit) was mounted to a single well of a 12-well plate containing 500 µl of water. A 20 µl aliquot of dye solution (diluted 50% v/v with water) was aerosolised using the same ventilation parameters as the *in vivo* study (i.e. DC 1), and aerosolised dye solution was captured in the water below (Section 2.2.6).

A standard curve was prepared by plotting known concentration of dye solution against its absorbance, measured using a plate reader (Section 2.2.6). For this experiment, 20 µl of dye solution pipetted into 500 µl of water was considered as 100% of starting volume (i.e. initial dose of dye solution). Other concentrations prepared for the standard curve were 2.5%, 5%, 25%, 50%, and 75% of starting volume, prepared by pipetting 0.5, 1, 5, 10, and 15 µl of dye solution into 500 µl of water, respectively. The volume of aerosolised dye solution released at the outlet of the Aeroneb®Pro nebuliser (expressed as % starting volume) was quantified by interpolating the standard curve.

4.2.2 Aerosol delivery efficiency of the Aeroneb®Pro-flexiVent™ ventilator circuit

The Aeroneb®Pro-flexiVent™ ventilator circuit uses a motor-operated piston to move air through the inlet valve into the inspiratory limb (Figure 4-1 A). Air then flows through the Scireq in-line Aeroneb®Pro mount on which the nebuliser is placed. Aerosols released by the nebuliser are carried into the ventilator tubing, connector tube, Y-piece, and ET tube. In this experiment the aerosol released from the tip of the ET tube was collected under 500 µl of

water in a modified Eppendorf tube (Figure 4-1 B). Aerosol not captured was then released into the expiratory limb which connects to the air outlet valve and a PEEP trap (Figure 4-1 B). When delivering aerosols to airways of mice, the PEEP trap ensures that a positive pressure is maintained in the airways of the animal (Section 3.2.3).

A 20 µl aliquot of dye solution was aerosolised through the circuit (Figure 4-1 A-C) using the same ventilation parameters as per the previous *in vivo* study (Section 3.2.3). The DC, PEEP, and I: E ratio were kept constant for all experiments described in this Chapter, unless noted. Aerosolised dye solution released was collected and assayed later (Section 2.2.6). The circuit was disassembled to gather the fluid from each of the components, and each component was then washed with water, which was collected to be assayed later (Section 2.2.6). The standard curve was used to determine the volume of aerosols released at the tip of the ET tube for all experiments reported in this Chapter.

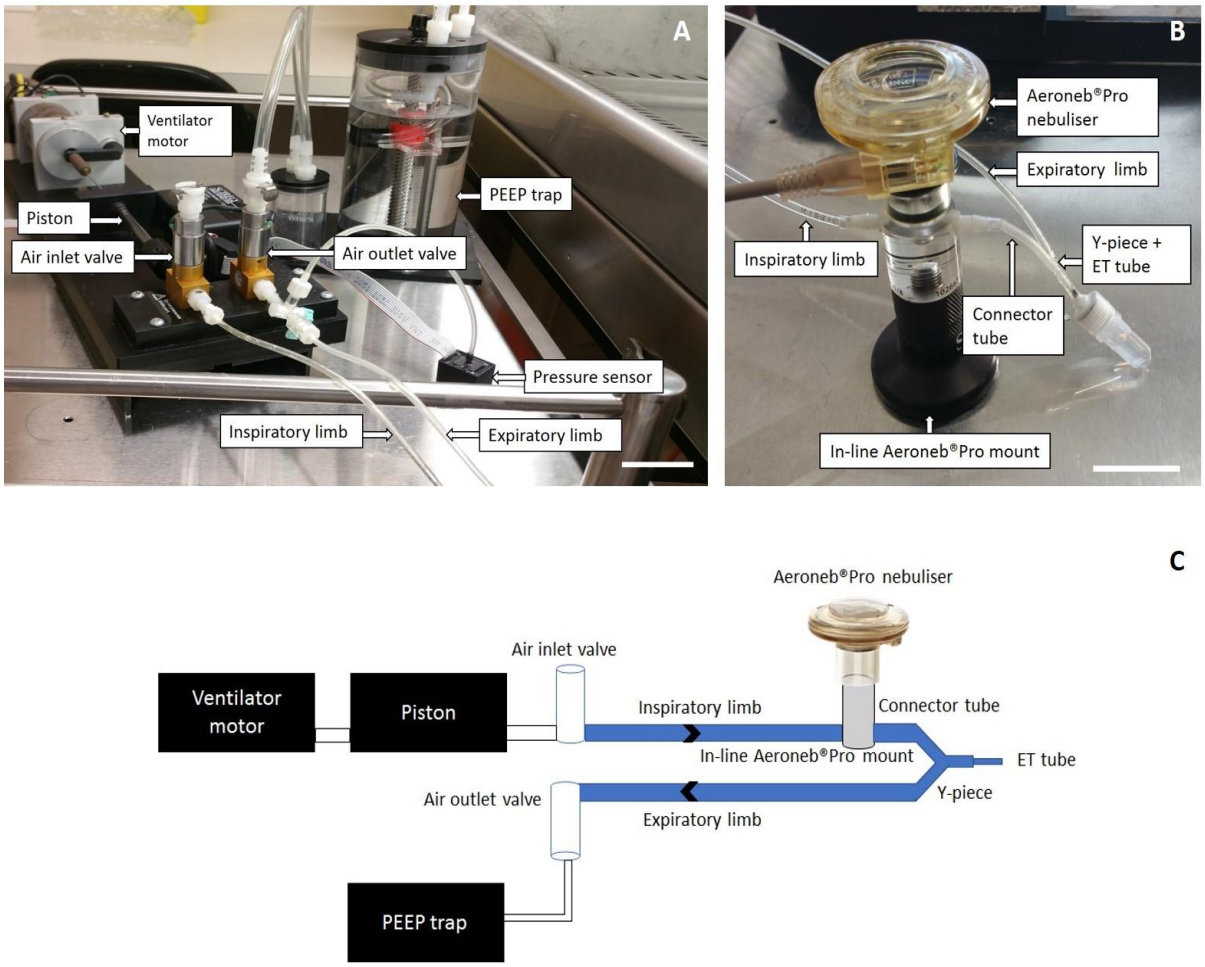

Figure 4-1: Aeroneb®Pro-flexiVent™ ventilator circuit showing the (A) ventilator motor, piston inspiratory limb, expiratory limb, and the PEEP trap (scale bar = 5 cm), (B) Aeroneb®Pro nebuliser connected in-line to the ventilator circuit and ventilator circuit tubing which consists of the connector tube, Y-piece, and the ET tube half-immersed in 500 µl of water contained in an Eppendorf tube with a loosely-screwed cap (Scale bar 2.5 = cm), and (C) diagrammatic representation of the ventilator circuit.

4.2.3 The influence of the internal diameter of the Aeroneb®Pro mount on delivery efficiency

This experiment compared two Scireq in-line Aeroneb®Pro mounts with different internal diameters. The first group used a standard in-line nebuliser mount with an internal diameter of 8 mm (Figure 4-2 A and C, used in Section 3.2.3) as a part of the ventilator circuit. The second group used an in-line nebuliser mount with a larger internal diameter of 12 mm (Figure 4-2 B and D) with an additional connector that has smoother inner walls that should reduce impactional deposition of aerosols. The distance from the centre of in-line nebuliser mount to the adjoining connector tube was 30 mm in the first group (Figure 4-2 C) and 50 mm

in the second group (Figure 4-2 D). Dye solution was aerosolised through the ventilator circuit with the same ventilation parameters used in the earlier *in vivo* study (Section 3.2.3). The volume of dye solution deposited within the in-line nebuliser mount and aerosol output released at the tip of the ET tube were quantified as described earlier (Section 4.2.1). The in-line nebuliser mount that delivered the highest aerosol output was used for the RR experiment described below.

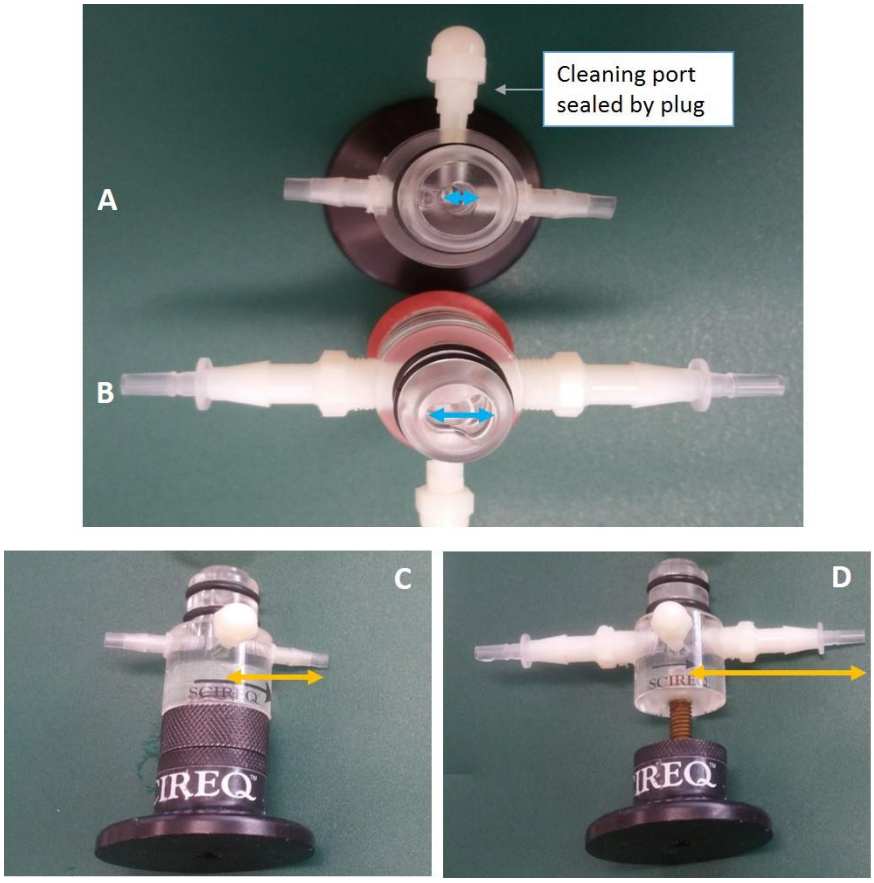

Figure 4-2: Top-view of the (A) standard Scireq in-line Aeroneb®Pro mount with an internal diameter of 8 mm (blue arrow) and (B) another Scireq in-line Aeroneb®Pro mount with the larger diameter of 12 mm (blue arrow). Side view of the (C) standard in-line nebuliser mount, distance from the centre to the tip was 30 mm (yellow arrow) and (D) the in-line nebuliser mount with a larger internal diameter. The distance from the centre to the tip was 50 mm (yellow arrow).

4.2.4 Effect of altering ventilation parameters

4.2.4.1 Respiratory rate

This experiment tested four respiratory rates (RRs); the first group (standard group) used an

RR of 120 breaths/minute, as used in the previous *in vivo* study (Section 3.2.3). This was compared to three other groups with RRs of 60 breaths/minute, 90 breaths/minute, and 150 breaths/minute. The V_T was adjusted so that each group had the same MV of 1.2 ml/min/g, which was the typical MV of C57BL/6 mice[200]. Hence, the V_T used for the standard group was 10 ml/kg and other groups were 20 ml/kg, 13.2 ml/kg, and 8 ml/kg respectively. All other parameters remained the same as the baseline experiment (Section 3.2.3). The RR of the group that produced the highest volume of aerosolised dye solution at the tip of the ET tube along with other ventilation parameters used in the baseline *in vivo* study (Section 3.2.3) was used for the next experiment.

4.2.4.2 Tidal volume

The three V_T tested in this experiment were 10 ml/kg, 13.2 ml/kg (standard group), and 15 ml/kg. The RR (identified from Section 4.2.4.1) was maintained constant for all groups. The dye solution was delivered through the nebuliser-ventilator circuit using appropriate ventilation parameters. The V_T that produced the highest volume of aerosolised dye solution by the end of the ventilator circuit, optimal RR identified from earlier experiment (4.2.4.1), and other ventilation parameters from baseline *in vivo* (study 3.2.3) was used for the subsequent experiment.

4.2.5 Effect of reducing amount of aerosols provided per breath

The amount of aerosols produced per breath could be reduced by either reducing the DC supplied to the nebuliser or delivering the aerosols in short pulses, which were examined in two separate experiments given below.

The duty cycle (DC) of the nebuliser is percentage of time during which the device was active during each respiratory cycle and is altered by changing the on/off periods of the square wave

electrical signal (at a frequency of 25 Hz) applied to the Aeroneb®Pro controller. A DC of 1 (i.e. 100%) means the nebuliser is always on during inspiration, whereas a DC of 0.5 results in the unit producing aerosol for half of the inspiration[146]. The four DC values tested in this experiment were 0.25, 0.50, 0.75, and 1 (standard group, used in earlier *in vivo* study, Section 3.2.3). The dye solution was aerosolised through the ventilator circuit with the appropriate DC. The optimal ventilation parameters RR and V_T identified from previous experiments (Section 4.2.4.1 and 4.2.4.2, respectively) were used in this experiment. The DC that produced the highest volume of aerosolised formulation at the tip of the ET tube was then used in the subsequent experiment.

An alternative strategy for reducing the amount of aerosol produced in each breath is using a single short pulse of aerosol at DC 1. Also, reducing the DC below 0.25 (the lowest DC used in the above study) is not necessarily effective due to the start-up time required to produce aerosol. This *in vitro* experiment had four groups, the standard group consisted of the nebuliser being operated at an optimal DC determined by the previous experiment, as well as groups aerosolising dye solution in pulses of 10 ms, 15 ms, and 30 ms duration per breath. Previously identified ventilation parameters RR (Section 4.2.4.1) and V_T (Section 4.2.4.2) were used to aerosolise dye solution through the ventilator circuit. The pulse parameter that produced the highest aerosol dose at the tip of the ET tube was chosen for the next experiment.

4.2.6 Effect of re-aerosolising dose deposited in the ventilator circuit

Two groups were used in this experiment. In Group 1, dye solution was aerosolised with ventilation parameters previously identified to release optimal volume of aerosols at the tip of the ET tube (Sections 4.2.4.1, 4.2.4.2, and 4.2.5). In Group 2, dye solution was aerosolised

through the ventilator circuit with the same ventilation parameters as Group 1, but the fluid deposited in the Scireq in-line Aeroneb®Pro mount was collected using a pipette, returned to the Aeroneb®Pro, and then re-aerosolised. The volume of the aerosolised dye collected at the tip of the ET tube was then compared to Group 1.

4.3 Results

4.3.1 Aerosol delivery efficiency of the Aeroneb®Pro nebuliser

This *in vitro* experiment measured the volume of aerosol released following delivery through an Aeroneb®Pro nebuliser, without being connected to the ventilator circuit. The volume of aerosols collected after delivery through the Aeroneb®Pro nebuliser was significantly lower than bolus delivery by 13.4% (Figure 4-3).

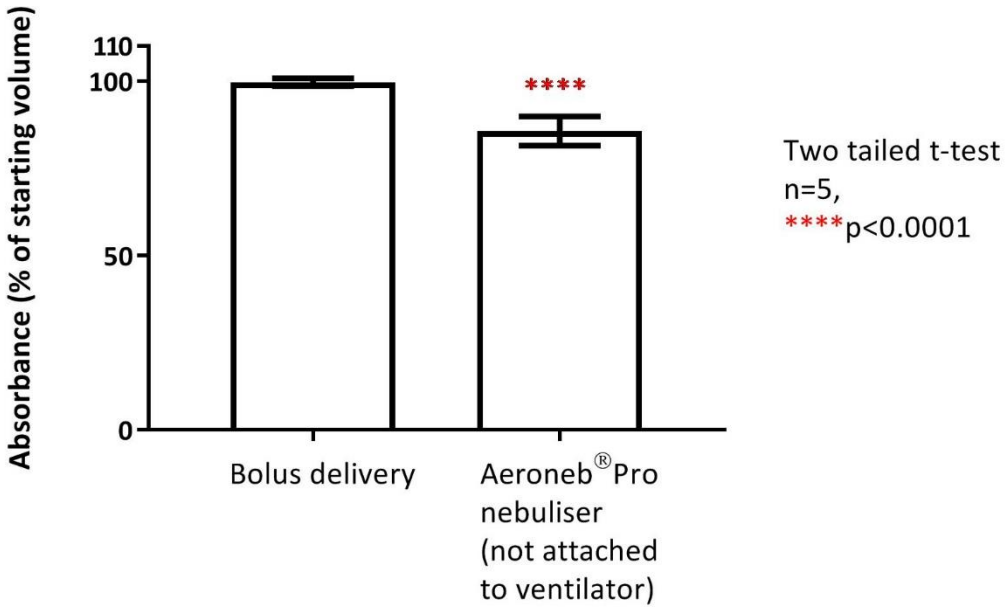

Figure 4-3: Volume of dye delivered as a bolus dose delivery vs an aerosol dose through the Aeroneb®Pro nebuliser.

4.3.2 Aerosol delivery volume efficiency of the Aeroneb®Pro-flexiVent™ ventilator circuit

This experiment quantified the volume of aerosolised fluid deposited in ventilator circuit components and aerosols released at the tip of the ET tube. The majority of aerosolised dye

solution released from the outlet of the nebuliser deposited at the Scireq in-line Aeroneb®Pro mount (83.7% of initial dose volume) (Figure 4-4). Smaller volumes of dye solution were deposited in other components of the ventilator circuit, such as the inner walls of the Aeroneb®Pro nebuliser (10.1% of initial dose volume), connector tube (2.7% initial dose volume), Y-piece (2.1% initial dose volume), and expiratory limb (0.6% initial dose volume) (Figure 4-4). The volume released at the tip of the ET tube was found to be only 2.1% of the initial dose (Figure 4-4).

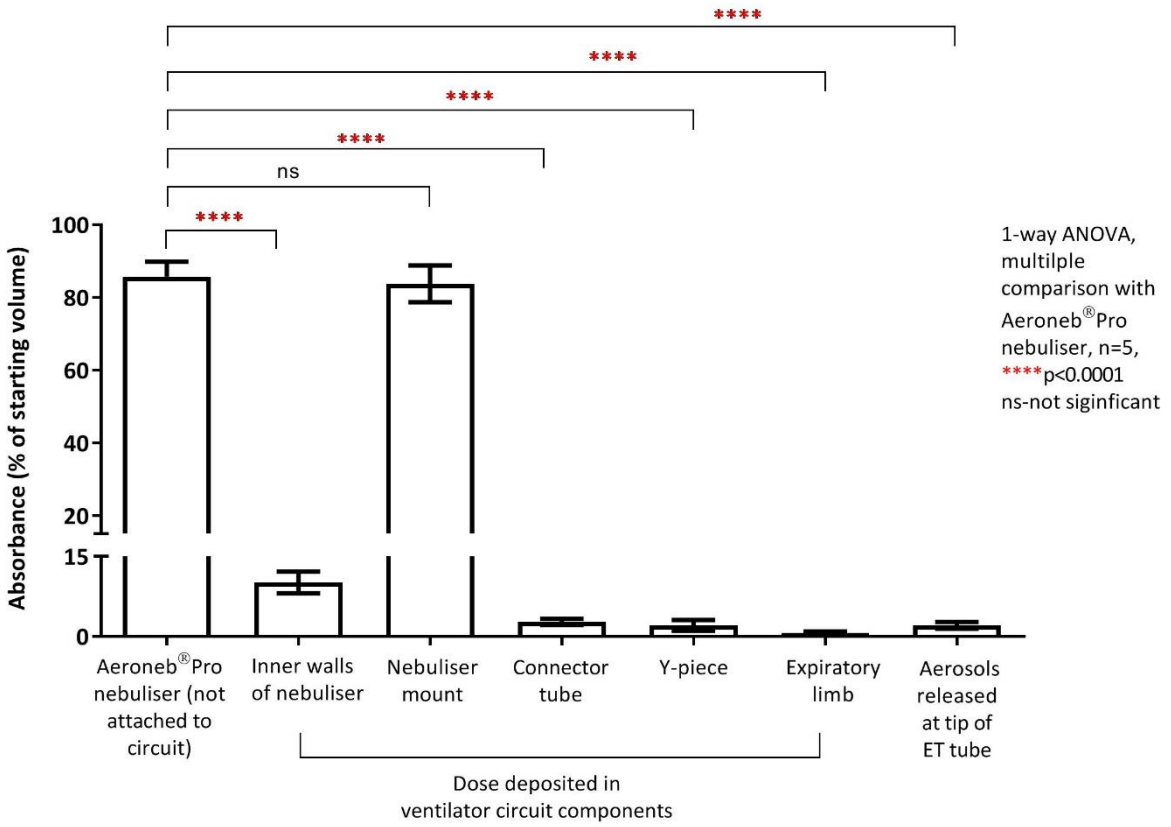

Figure 4-4: Volume of aerosolised dose deposited in different ventilator circuit components and that expelled at the tip of the ET tube.

4.3.3 The influence of the internal diameter of the Aeroneb®Pro mount on delivery efficiency

The effect of replacing the in-line Aeroneb®Pro mount of 8 mm internal diameter with a larger internal diameter of 12 mm, was examined in this experiment. There was no significant

difference in volume of dye solution deposited within the in-line nebuliser mount between the two groups (Figure 4-5 A). However, aerosol output obtained at the tip of the ET tube when using the in-line nebuliser mount with 8 mm internal diameter was 1.7-fold higher than that with 12 mm internal diameter (Figure 4-5 B). As a result, the in-line nebuliser mount with an internal diameter of 8 mm was used for further studies.

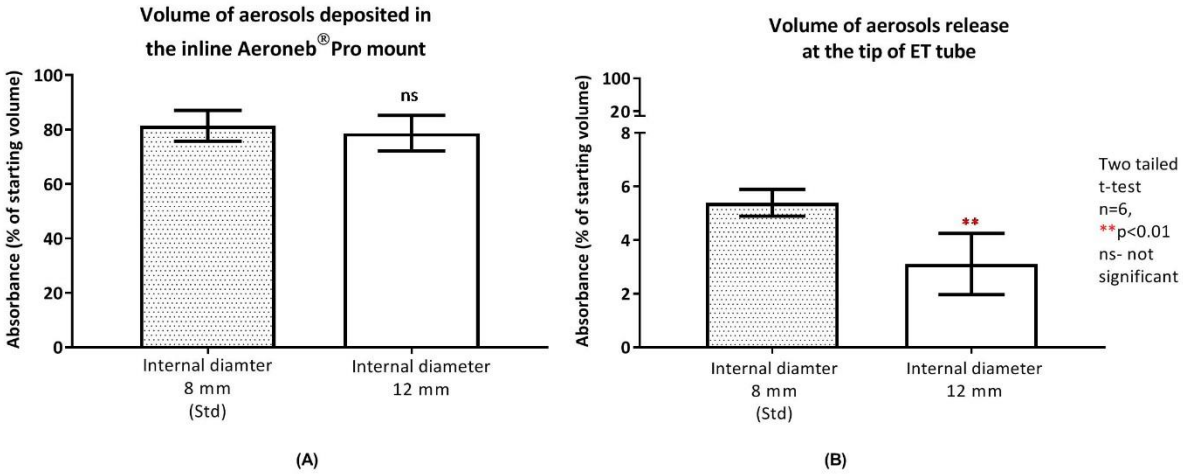

Figure 4-5: Comparison of in-line Aeroneb® Pro mount with 8 mm internal diameter vs. 12 mm internal diameter on the (A) dose deposited at the in-line nebuliser mount and (B) volume of aerosols expelled at the tip of the ET tube.

4.3.4 Effect of altering ventilation parameters

4.3.4.1 Respiratory rate

The impact of changing the RR, while maintaining a constant MV, on aerosol delivery efficiency during mechanical ventilation was investigated. The volume of aerosolised dye solution released at the tip of the ET tube with RR 60 breaths/minute was 1.6-fold higher than RR 120 breaths/minute, the standard group (Figure 4-6). However, no significant difference was observed in the aerosol output between the RR 120 (standard group) and RR 90 breaths/ minute, and RR 150 breaths/minute (Figure 4-6).

Although the optimal aerosol output was obtained using the lowest RR of 60 breaths/ minute,

this RR may not be suitable for *in vivo* studies. Wolthuis et al. demonstrated ventilator induced lung injury in mice using RR as low as 70 breaths/minute[201]. Hence, the second lowest RR 90 breaths/minute was used for further experiments.

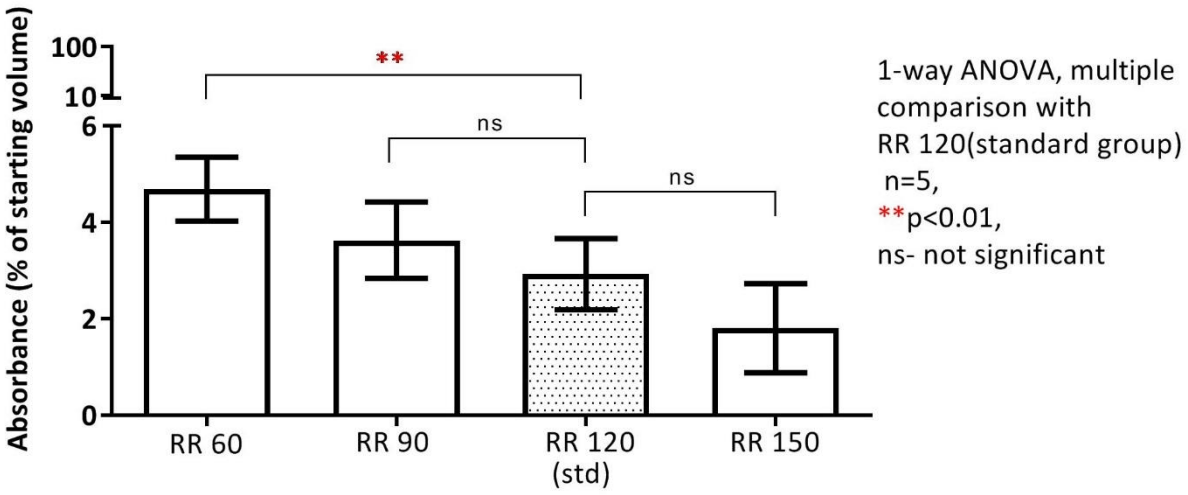

Figure 4-6: The impact of changing RR in breaths/minute, while maintaining constant MV, on the volume of aerosols released at the tip of the ET tube.

4.3.4.2 Tidal volume

This experiment investigated the effect of changing V_T, while maintaining constant RR, on the aerosol delivery efficiency of the Aeroneb®Pro-flexiVent™ ventilator. Results showed no significant change in dose volume expelled at the tip of the ET tube on changing V_T, while maintaining constant RR (Figure 4-7 B). Hence, V_T 13.2 ml/kg was used in the next experiment.

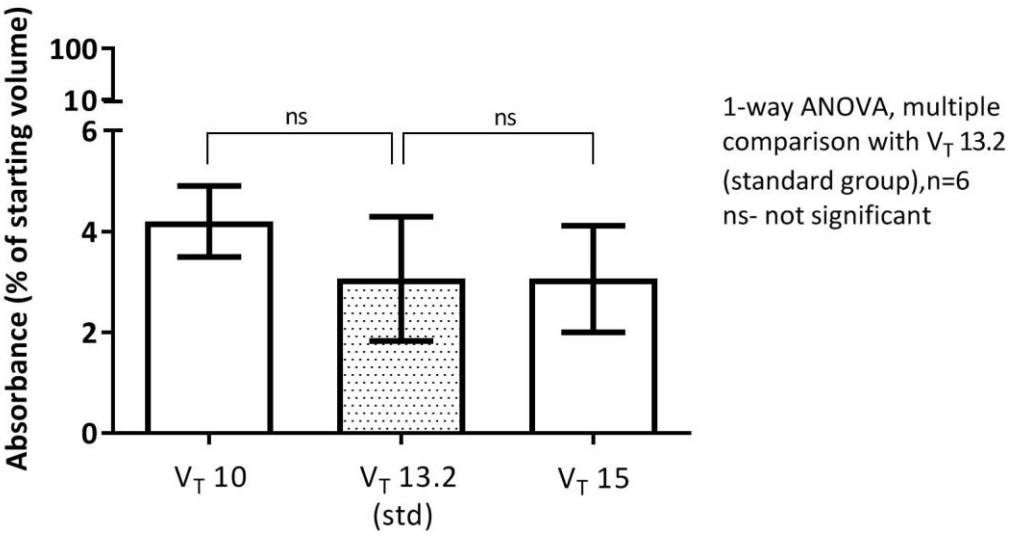

Figure 4-7: Effect of V_T, while maintaining constant RR in ml/kg, on the volume of aerosols released at the tip of the ET tube.

4.3.5 Effect of reducing the amount of aerosols released per breath

Reducing the volume of aerosols produced per breath by supplying a lower DC to the nebuliser resulted in higher aerosol output at the end of the circuit (Figure 4-8). Aerosol output produced was two-fold higher when the nebuliser was supplied with DC 0.25 compared to DC 1 (standard group) (Figure 4-8). However, no significant difference in aerosol output was observed using higher DC settings of 0.5 and 0.75. Hence, DC 0.25 was chosen for further studies to optimise aerosol delivery efficiency.

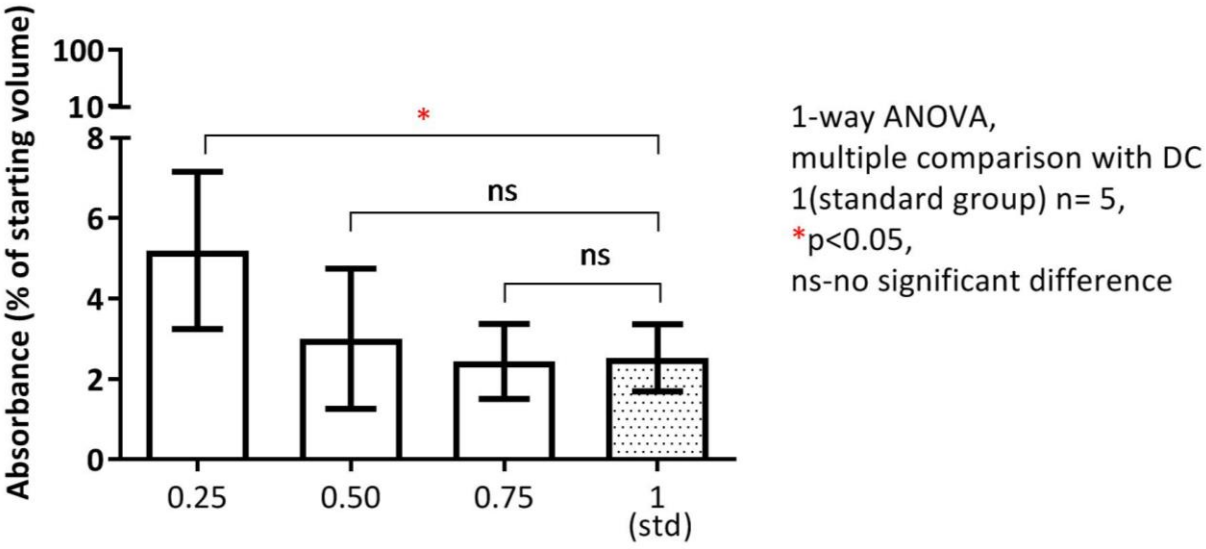

Figure 4-8: The impact of DC on the volume of aerosols released at the tip of the ET tube.

Reducing the amount of aerosols released per breath by delivering aerosols in short pulses also demonstrated an increased aerosol output. The volume of aerosolised dye solution released at the tip of the ET tube was 2.8, 1.9, and 1.9 folds higher on delivering aerosols in short-pulses of 10 ms, 15 ms, and 30 ms, respectively, compared to DC 0.25 (standard group) (Figure 4-9). As the highest aerosol output was obtained on delivering aerosols in pulses of 10 ms, this delivery parameter was used in the next experiments.

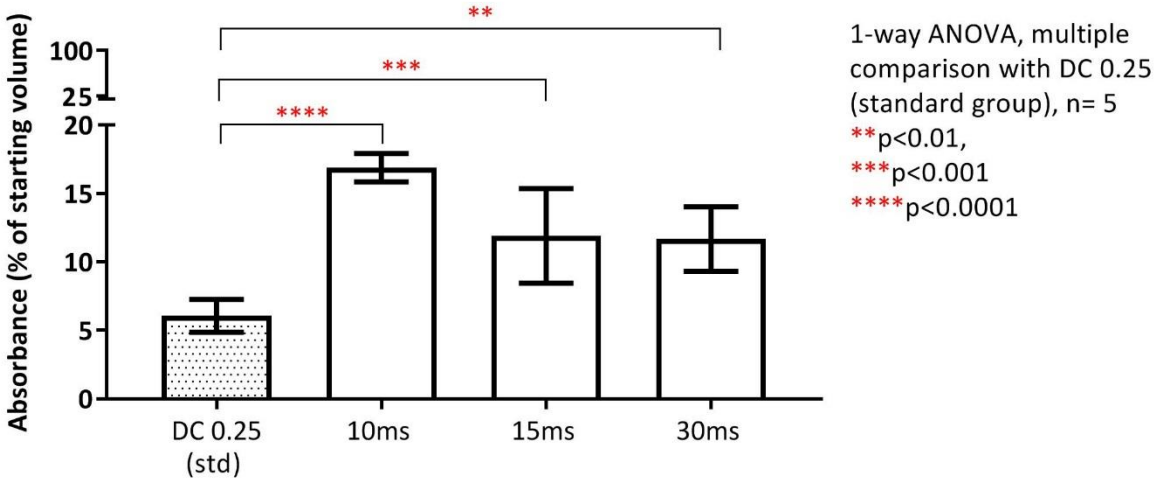

Figure 4-9: The influence of pulsed aerosol delivery on the volume of aerosols expelled at the tip of the ET tube.

4.3.6 Effect of re-aerosolising dose deposited within the Scireq in-line Aeroneb®Pro mount

Re-aerosolising the dose deposited within the Scireq in-line Aeroneb®Pro mount released approximately 21.6% of initial dose volume at the tip of the ET tube, which was 1.3-fold higher compared to delivering aerosols in pulses of 10 ms (Figure 4-10).

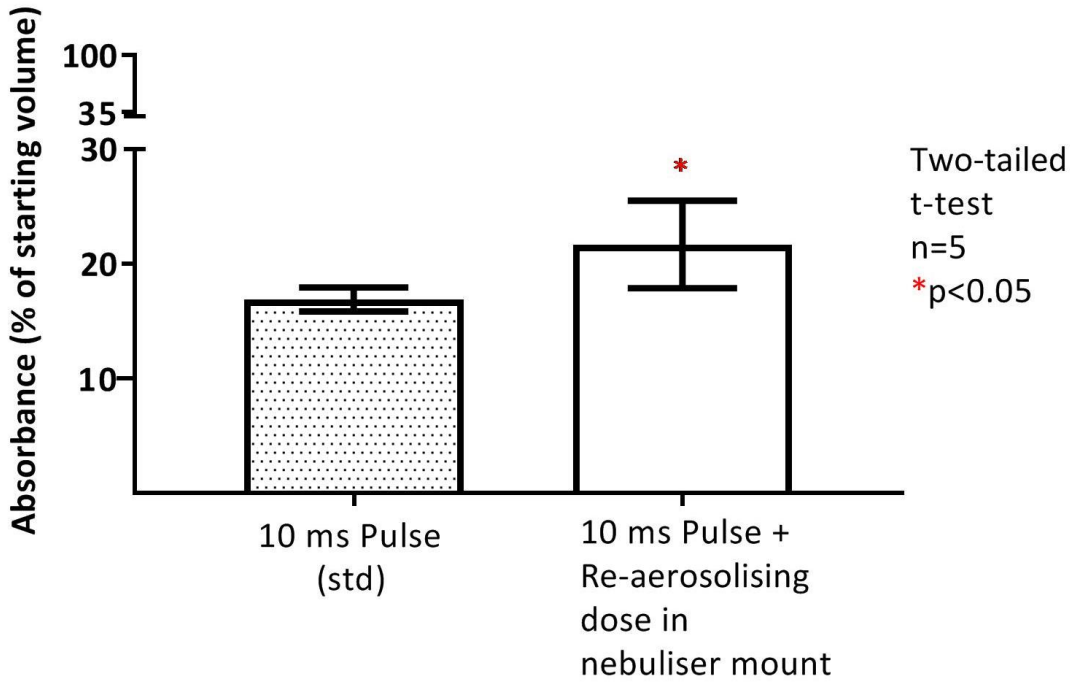

Figure 4-10: Effect of re-aerosolising dose deposited at the Scireq in-line Aeroneb®Pro mount on the physical dose volume of aerosols expelled at the tip of the ET tube.

4.4 Discussion

The experiments in the present Chapter quantified the physical dose volume released at the tip of the ET tube, located at the end of the Aeroneb®Pro-flexiVent™ ventilator circuit, when aerosolising dye solution using the same ventilation parameters as the previous Chapter (Section 3.2.3). Unsurprisingly, the results showed that only 2.1% of initial dose volume was expelled at the tip of the ET tube, validating the first hypothesis. Additionally, a majority of the aerosolised dye solution was deposited in the Scireq in-line Aeroneb®Pro mount (83.7% of initial dose volume). The design of the Scireq Aeroneb®Pro mount was such that air from the ventilator enters the inlet pipe and travels upwards to the base of the nebuliser (Figure

4-11). The gas entering this chamber mixes with the aerosol, which then flows down the outlet pipe where it changes direction by 90° to pass into the adjoining connector tube. The change in the direction of aerosol and gas flow, the high velocity of the aerosol, and the narrow width of the of outlet pipe likely results in impaction and deposition of aerosolised fluid at the bottom of the outlet pipe. A review of literature reported similar deposition of aerosolised formulation in the component placed adjacent to the nebuliser. Dubus et al. aerosolised ^{99m}Tc-DTPA to the lungs of mechanically ventilated macaques and observed similar deposition of the aerosolised dose in the T-piece piece, a nebuliser mount with a larger internal diameter compared to the one used in the present study[184]. MacLoughlin et al. also demonstrated deposition of aerosolised formulation in the T-piece of the ventilator circuit used for rats[74]. They suspected this dose deposition was caused by the narrow internal diameter of the T-piece and high aerosol flow rate[74].

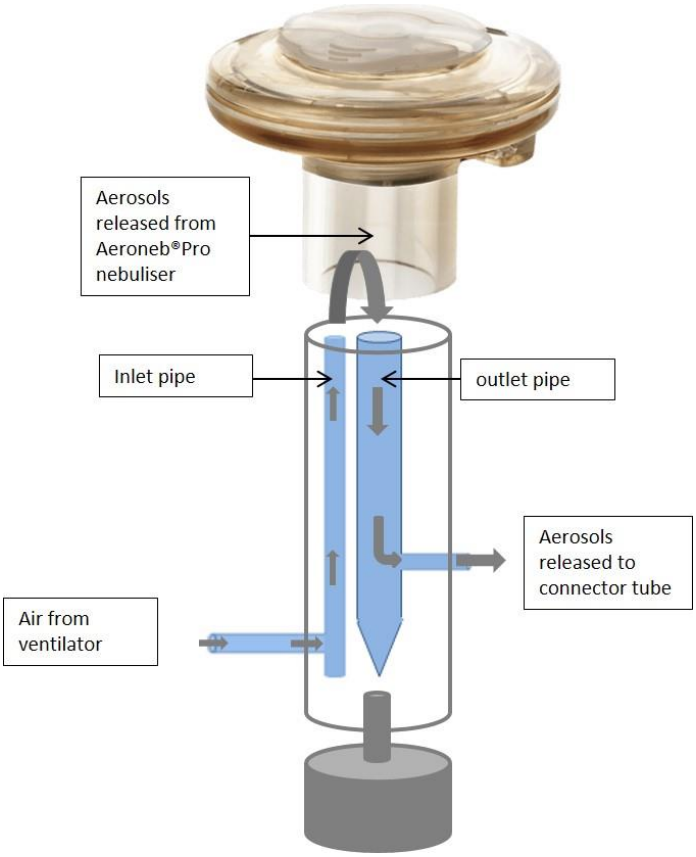

Figure 4-11: A diagrammatic representation of the Scireq in-line Aeroneb®Pro mount showing the direction of aerosol flow.

The next experiment examined the second hypothesis, that using a circuit component with a larger internal diameter would minimise deposition within the component and increase aerosol output. However, the results did not support the second hypothesis as similar levels of aerosolised fluid were deposited within the standard 8 mm internal diameter in-line Aeroneb®Pro mount and that with a larger internal diameter of 12 mm. Deposition in both in-line nebuliser mounts was possibly caused by the 90° bend in the component[187]. Hence, it is possible that a change in internal diameter would not be effective unless the component is re-designed to have a lower angle for smoother transition of airflow. Results also show that aerosol released at the tip of the ET tube, using the in-line nebuliser mount with a larger internal diameter, was lower than the standard nebuliser mount. This was probably because the additional connector attached to the in-line nebuliser mount with a larger internal

diameter was 20 mm longer than of the standard (Figure 4-2), which led to the nebuliser being placed further away from the Y-piece and the tip of the ET tube (aerosol collection point). An *in vitro* study by Ari et al. also showed that position of the nebuliser in the ventilator circuit had a significant impact on aerosol delivery efficiency. They demonstrated higher delivery efficiency when placing the Aeroneb®Pro nebuliser closer to the Y-piece in the inspiratory limb in comparison to placing it closer to the ventilator or between the ET tube and Y-piece[202].

The effect of changing ventilation parameters (RR and V_T) was examined next, as these parameters have been demonstrated to impact aerosol delivery efficiency[74]. The third hypothesis stated that a higher RR and V_T would produce optimal volumes of aerosols at the end of the ventilator circuit. The results partly support the third hypothesis and show that a lower RR of 60 breaths/minute, while maintaining a constant MV, delivered a higher volume of aerosolised dye solution at the tip of the ET tube. A lower RR would have reduced the aerosol velocity within the narrow circuit components thus reducing impaction and deposition of aerosols in circuit components. These results are consistent with previously published studies analysing a human ventilator circuit[192, 193]. The experiments where V_T was changed, while maintaining constant RR, show similar volumes of aerosolised dye solution released at the tip of the ET tube (Section 4.3.4.1). This result was in partial agreement with an *in vitro* study by Berlinsky et al. who showed that the aerosol delivery efficiency of a paediatric ventilator circuit was not affected by changing V_T, when the vibrating mesh nebuliser was placed closer to the ventilator[203]. However, when the vibrating mesh nebuliser was located closer to the Y-piece aerosol delivery efficiency increased with a decrease in V_T [203].

The next experiment examined the fourth hypothesis, which stated that reducing the volume

of aerosols delivered per breath, by supplying a lower DC to the nebuliser and delivering aerosols in short pulses, would improve aerosols released at the end of the ventilator circuit. The results supported this hypothesis as a low DC of 0.25 produced higher volumes of aerosolised dye solution at the tip of the ET tube compared to DC 1. The results obtained in this experiment were in agreement with Robichaud et al. who also showed a DC of 0.25 was optimal for aerosol delivery to the airways of ventilated mice[146]. An explanation for the effect found in the present Chapter is that the lower DC of 0.25 produced lower volumes of aerosol during each respiratory cycle, thus resulting in lower deposition in circuit components and a higher delivered dose volume. The following experiment also supported this hypothesis as delivering aerosols in 10 ms pulses significantly increased aerosol output compared to operating the nebuliser at DC 0.25. Pulsed 10 ms aerosols could have lowered volumes of aerosols produced during the respiratory cycle and reduced the aerosol flow rate at the initial part of the ventilator circuit i.e. within the nebuliser mount. This could have subsequently reduced deposition of aerosolised fluid in nebuliser mount and other circuit components leading to increased aerosol output.

The last experiment examined the fifth hypothesis that stated re-aerosolising the dose deposited in the in-line nebuliser mount would improve aerosol output. Results support this hypothesis and show an increase in aerosol output from 16.8% to 21.7% of initial dose volume. This result was similar to the findings of MacLoughlin et al. who demonstrated an improvement in aerosol delivery efficiency from ~13% to ~41% of inhaled mass after re-aerosolising the dose deposited in the T-piece, using an Aeroneb®Pro- ventilator circuit for use in rats[74].

Other factors that influence aerosol delivery efficiency during mechanical ventilation are

aerosol size and I:E ratio[74, 187]. Aerosol delivery efficiency is dependent on aerosol size as it dictates the impaction and deposition of aerosols through a ventilator circuit[74]. MacLoughlin et al. reported that using an Aeroneb®Pro nebuliser that produced the lowest aerosol size of 3.38 µm (as per manufacturer's specifications) was more efficient than nebulisers producing large aerosol sizes (5.21 µm and 4.29 µm), in a small-bore ventilator circuit used for rats[74]. In another study, Raabe et al. reported that aerosols smaller than 3 µm deposited in the bronchial airways and lungs of mice, while those larger than 3 µm deposited in the naso-pharynx region[204]. As the size of the aerosols generated using the Aeroneb®Pro nebuliser (3.61 µm as per manufacturer's specifications) was close to the optimal size for delivery through a small-bore ventilator circuit and deposition in mice airways, this parameter was not investigated in this Chapter. The I:E ratio also has a significant impact on aerosol delivery efficiency during mechanical ventilation. MacLoughlin et al. compared the effect of different I: E ratios (1:1, 1:2, and 2:1) and reported that an I:E ratio of 2:1 and RR of 45 breaths/minute was optimal for aerosol delivery through a rodent ventilator[74]. As the optimal I:E ratio for aerosol delivery efficiency for a small animal ventilator circuit was demonstrated by MacLoughlin et al.[74] this parameter was not investigated in this Chapter.

Extensive research has been carried out to improve delivery efficiency of adult and paediatric ventilator circuits. Longest et al.[205] showed that using streamlined ventilator circuit components improved the dose produced distal to the ET tube by 1.5-fold in an adult model of a ventilator circuit[205]. Using streamlined components minimises the changes in the direction of the airflow, and use of a constant diameter path minimises disruptions in airflow due to sudden expansion and contraction of the flow path[205]. Together, this likely maintains aerosol velocity and reduces impact deposition. With modern 3D printing techniques

becoming mainstream, it should be possible to design and produce a Scireq in-line Aeroneb®Pro nebuliser mount based on these principles of smooth airflow. In another study, Holbrook et al. demonstrated a 26-fold improvement in delivery efficiency through a paediatric ventilator circuit with streamlined components on aerosolising electrostatically charged aerosols[206]. Hence, future studies could investigate the effect of delivering electrostatically charged aerosols through streamlined circuit components, once they become available (or can be custom manufactured) for a small animal ventilator.

4.5 Conclusion

This *in vitro* study showed that the volume of aerosolised dye solution expelled from the tip of the ET tube with the same ventilation parameters used in the earlier *in vivo* study (Section 3.2.3) was only 2.1% of the initial dose volume. Additionally, most of the aerosolised dose was deposited within the Scireq in-line Aeroneb®Pro mount that lies immediately downstream and adjacent to the nebuliser in the ventilator circuit. Optimising the ventilation parameters, such as V_T, RR, and DC, increased the volume of aerosolised dye solution released at the tip of the ET tube. However, the greatest improvements in aerosol delivery efficiency were observed when delivering the dye solution in pulses of 10 ms, and by re-aerosolising any fluid that deposits within the in-line nebuliser mount. These optimised delivery parameters and pulsed aerosol delivery could be applied in future experiments with larger animals and clinical studies. However, re-aerosolising drug formulation seems less practical for use in larger animal models, as this would require disassembling and re-assembling the ventilator circuit, which may not be feasible.

This study optimised a range of parameters to improve aerosol delivery efficiency of the Aeroneb®Pro-flexiVent™ ventilator circuit. These improvements should be helpful for studies

using the same nebuliser-ventilator apparatus to deliver aerosols to the lungs of mice. However, delivery might also be affected by the physio-chemical characteristics of the aerosolised formulation, and further studies with the particular agent of interest will be needed.

The experiments reported in this Chapter provide significant insight on the methods of improving aerosol delivery efficiency of an Aeroneb®Pro-flexiVent™ ventilator circuit used for small animal models, such as mice. The next Chapter investigates how these optimised delivery parameters can influence aerosolisation of LV gene vector through the Aeroneb®Pro-flexiVent™ ventilator circuit.

5 OPTIMISING AEROSOL DELIVERY OF A LENTIVIRAL VECTOR FOR MECHANICAL VENTILATION OF MICE: AN *IN VITRO* STUDY

5.1 Introduction

Aerosol delivery of LV-LacZ vector produced lower levels of gene expression in the trachea of mice compared to bolus delivery (Chapter 3, Section 3.3). Further examination revealed that only 2.1% of the physical dose volume reached the tip of the ET tube located at the end of Aeroneb®Pro-flexiVent™ ventilator circuit. Additionally, a majority of the aerosolised dose volume (i.e. approximately 97% of the initial dose) was found to deposit within the ventilator circuit components (Chapter 4, Section 4.3.2). The combination of delivering aerosols in pulses of 10 ms, using optimised ventilation parameters, and re-aerosolising the dose deposited within the Aeroneb®Pro nebuliser mount (Chapter 4, Section 4.3.5 and 4.3.6), resulted in an increase in the delivered dose volume from the initial 2.1% to 21.6%. The present Chapter quantifies the biological factors that influence the gene expression of the LV vector fraction that reaches the tip of the ET tube (i.e. would be available for lung delivery).

The effectiveness of any aerosol gene therapy depends on the viability of the vector reaching the respiratory epithelium. While some viral vectors like AAV are stable[110], others such as the Ad[54] and SeV vectors[58] demonstrate reduced viability on aerosol delivery. Viral viability is influenced by the vectors' ability to withstand changes that occur during aerosol delivery [64, 207], such as changes in temperature, pH, concentration, viscosity, and shear stress[58, 208, 209]. For example, AAV vectors are stable at a wide range of temperatures, ranging from 4°C to 55°C, and at a pH of 5.5 to 8.5[210]. However, other viral vectors, such as Ad and LV vectors, are only stable at temperatures lower than 4°C[65] and a narrow pH range [64, 65], which may explain

their reduced viability following aerosol delivery.

Due to these factors, the type of nebuliser used will influence the viability of the aerosolised vector. Griesenbach et al. showed that aerosolising SeV vector through a Pari LC® jet nebuliser reduced viability of the vector to 1% of the initial dose[58]. However, delivering the vector using an AeroProbe™ catheter resulted in vector viability being reduced to 49% of initial dose[58]. Reduced vector viability in both groups was thought to be due to susceptibility of the vector to shear stress produced by the nebulisers during aerosol delivery[58]. Although there are few reports of successful aerosol delivery of LV vectors, this vector type is known to be fragile, as observed during vector production[141, 142, 211]. One approach to combatting this problem has been pseudotyping the LV vector with a VSV-G envelope to improve its stability[212].

The physical and chemical stability of viral vectors has a significant impact on their transduction efficiency[65]. Bolus delivery studies have shown that suspending a viral vector in a protective diluent can improve the physical stability of the vector[65, 213]. SIV- and FIV-based LV vectors have been suspended in diluents containing α-lactose buffer[87, 214] to improve stability[215]. However, recent studies have shown that the presence of protein or serum during vector preparation increases immunogenicity of the LV vector, which further reduces its transduction efficiency[64, 216]. This led to development of serum-free and protein-free cell culture growth media for LV vector production. One such commercially available medium is the FreeStyle™ 293 expression medium (FreeStyle™ medium)[217]. It is a chemically-defined serum-free and protein-free medium developed to culture 293F cells for LV vector production[217]. In a recent study, Alton et al. suspended an SIV based LV vector in FreeStyle™ medium and reported efficient transduction in mice airways following delivery as a bolus dose[218]. Using this medium as a diluent could improve vector viability as this media contains

GlutaMAX™ supplement known to reduce ammonia build up and improve cell viability[217, 219]. The effectiveness of suspending a HIV-derived LV vector in protective agents such as FreeStyle™ medium is yet to be examined and is one focus of this Chapter.

The present Chapter also investigates the efficacy of pulsed delivery of the LV vector through the Aeroneb®Pro-flexiVent™ ventilator circuit. Pulsed aerosol delivery has been used to deliver biological formulations that are susceptible to shear stress, such as viral vectors and pDNA to the airways of animals[81, 220]. Hoggard et al. showed that delivering pDNA as a coarse spray in pulses of 20 ms through an AeroProbe® catheter did not affect pDNA viability[220], so it could be speculated that delivering LV vector in short pulses (here, 10 ms) would also not affect LV viability. Moreover, results obtained from Chapter 4 showed that delivering aerosols in 10 ms pulses, as well as recycling the dose deposited in the in-line Aeroneb®Pro mount, improved volume of aerosols expelled from the tip of the ET tube, compared to the output using baseline delivery parameters of the *in vivo* study (Chapter 3, Section 3.2.3). The present Chapter examines whether these changes also result in improved transduction levels, by quantifying LV gene expression following delivery using pulses of 10 ms and re-aerosolising the dose deposited within the Scireq in-line Aeroneb®Pro mount.

5.1.1 Hypothesis and aims

The following hypotheses are based on results from previous Chapters and the previously published studies detailed above. I hypothesise that the amount of viable LV vector reaching the tip of the ET tube at the end of the Aeroneb®Pro-flexiVent™ ventilator circuit will be;

1) Lower than from a bolus dose, or when using the aerosolisation parameters from the baseline *in vivo* study (Section 3.2.3).

2) Improved when using 10 ms pulsed vector delivery with the optimised ventilation parameters (Chapter 4, Section 4.2.5).
3) Further increased when using the additional step of re-aerosolising the vector that deposits at the Scireq in-line Aeroneb®Pro mount (Chapter 4, Section 4.2.6).
4) Increased when the LV vector is suspended in protective diluents such as α-lactose buffer or FreeStyle™ medium.

The first aim was to quantify the levels of LV gene expression that results when using the baseline aerosol delivery parameters (Section 3.2.3), or bolus delivery. The second aim was to quantify the gene expression when using the optimised delivery parameters (Chapter 4, Section 4.2.5 and 4.2.6). The third aim was to examine how suspending LV vector in FreeStyle™ medium, α-lactose buffer, or standard diluent MS/saline prior to aerosol delivery affects transduction levels. The final aim was to quantify the levels of gene expression produced when delivering the LV vector with both the optimised parameters as well as best diluent (both identified from experiments conducted within this Chapter).

5.2 Methods

5.2.1 Aerosol delivery of LV vector

In this experiment, the Aeroneb®Pro nebuliser was mounted on a single well of a 12-well plate. The well contained 500 µl of cell culture medium supplemented with 10% FCS, pens/strep 1:100 (v/v), 4 µg/ml of polybrene, and 2 µg/ml of gentamycin. A 20 µl aliquot of LV-LacZ vector diluted 1:10 (v/v) in MS/saline was delivered using the baseline *in vivo* study parameters (Section 3.2.3) and aerosols were collected in the cell culture medium present in the well.

The nebuliser was then connected to the ventilator circuit and LV-LacZ vector was aerosolised using the same parameters. Aerosolised vector that reached the tip of the ET tube was

collected under 500 µl of supplemented Hams F12 cell culture medium (Section 2.2.5). The ventilator circuit was then disconnected, each component was separately washed with 500 µl of supplemented cell culture medium (Section 2.2.5), and wash fluid was collected to be assayed later. For a positive bolus-volume control, 2 µl aliquot of the LV-LacZ vector was diluted 1:100 (v/v) in MS/saline and pipetted directly into 500 µl of supplemented cell culture medium which was then stored at 4° C until samples from aerosol treatment group were collected.

To quantify gene expression produced by the LV vector, 200 µl samples of aerosolised vector collected a) at the outlet of the Aeroneb®Pro nebuliser unit; b) at the tip of the ET tube; c) wash of ventilator parts; or d) as a bolus control (Section 2.2.5) were added to CHO-K1 cells on a 24-well plate. Each sample was analysed in duplicate. Three replicates containing untreated CHO-K1 cells were used as a negative control in all experiments. Transfected cells and untreated CHO-K1 cells were incubated at 37°C and 5% CO_2, with a media change after 24 hours using cell culture media. After each experiment all components of the nebuliser-ventilator circuit were disconnected and cleaned with Virkon and then with water (Section 2.2.5).

Two days later, samples were processed with standard X-gal solutions and incubated overnight at 37°C. The cell culture plate was placed on a grid that had four squares located in the centre of each well and away from the edges to avoid spurious results. Each square had an area of 2.5 mm^2, while the total area of each well was 191 mm^2. Images of samples in each square of the well was individually captured by a digital camera attached to a stereo-microscope at 100x magnification (Section 2.2.5). The number of transduced cells per area of field of view (2.5 mm^2) was quantified by image analysis using the MATLAB script (Appendix,

Section 8.2). The average number of transduced cells per field of view was reported as gene expression. In the present Chapter, the functional titre of the LacZ vector was calculated after aerosolisation through the nebuliser by counting the number of transduced cells per field of view. This method was chosen because it was essential to assess how much functional vector was present after aerosolisation, and other titre assessment methods (e.g. PCR-based) would have likely overestimated this by including non-functional vector particles damaged by the aerosolisation process.

5.2.2 Effect of aerosol delivery parameter on LV gene expression

LV-LacZ vector was aerosolised through the ventilator circuit using the three different aerosol delivery parameters listed in Table 5-1. The amount of gene expression obtained in each group was quantified using previously described methods (Section 5.2.1). Ventilation parameters identified (from this study) to produce improved gene expression were used in the next experiment.

Table 5-1: Aerosol delivery parameters used in this experiment.

Groups	Aerosol delivery parameters
1. Baseline *in vivo* (Section 3.2.3)	V_T 10 ml/kg, RR 120 breaths/minute, DC 1.
2. Optimal aerosol volume (Section 4.2.5)	Aerosols delivered in pulses of 10 ms, V_T 13.2 ml/kg, RR at 90 breaths/minute.
3. Optimal aerosol volume combined with re-aerosolisation of Aeroneb®Pro mount captured volume (Section 4.2.6)	Same aerosol delivery parameters as the previous group (group 2) + re-aerosolising fluid deposited in the Scireq in-line Aeroneb®Pro mount.

5.2.3 Effect of diluents on *LacZ* gene expression of aerosolised vector

5.2.3.1 Aerosol delivery via nebuliser alone

Concentrated LV-LacZ vector (suspended in PBS following vector harvest) was diluted 1:10 (v/v) with either MS/saline, FreeStyle™ medium, or α-lactose buffer (40 mg/ml in PBS). The vector was then aerosolised through the nebuliser alone. The nebuliser was operated with the parameters identified as producing the highest levels of gene expression in the previous experiment (Section 5.2.2).

Aerosolised vector was collected under 500 µl of supplemented cell culture medium, which was then used to transduce CHO-K1 cells. The number of transduced cells obtained in each group was quantified using the previously described method (Section 5.2.1). The diluent producing the highest levels of gene expression was then chosen for use in subsequent experiments.

5.2.3.2 *Aerosol delivery through the ventilator*

LV-LacZ vector diluted with either MS/saline, or the optimal diluent identified from the previous experiment (Section 5.2.3.1), was aerosolised through the ventilator circuit with the optimal aerosol delivery parameters (Section 5.2.2). Two modifications were made in the delivery apparatus used in this study; the collection tube was fitted with an O-ring that held the ET tube firmly to prevent aerosol loss, and the tip of the ET tube was immersed in 700 µl of supplemented cell culture medium (Figure 5-1), rather than 500 µl used previously (Section 5.2.1), to better capture of the aerosols released. Consequently, the assay volume used to transduce CHO-K1 cells was also increased from 200 µl (used in earlier experiments) to 300 µl. The level of LacZ expression was revealed using standard X-gal processing (Section 2.3.4) and quantified as above (Section 5.2.1). The diluent that produced the highest gene expression at the end of the ventilator circuit was then used for the next study.

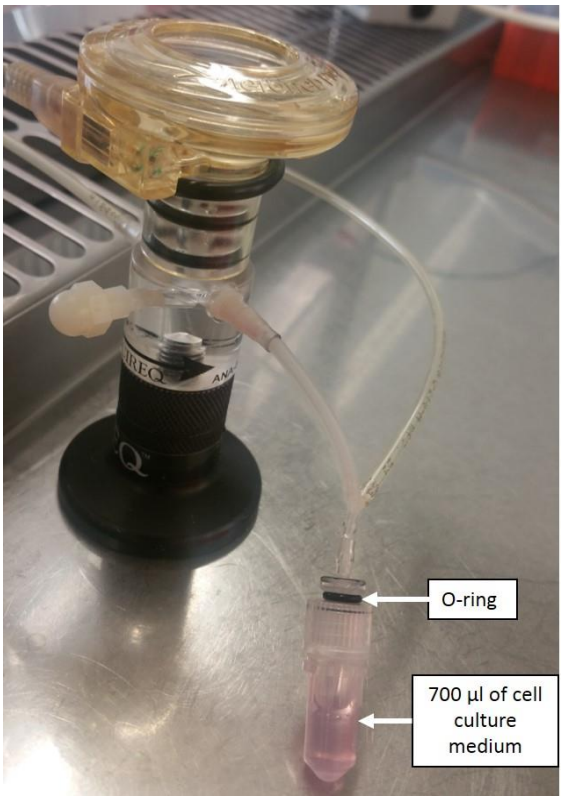

Figure 5-1: The nebuliser-ventilator circuit setup with new modifications.

5.2.4 Effect of optimised parameters on LV aerosol delivery during mechanical ventilation

There were two groups in this experiment; in the first group, the LV-LacZ vector suspended in MS/saline was aerosolised through the ventilator circuit, using the baseline parameters noted in the *in vivo* study (Section 3.2.3). In the second group, the vector was suspended in the optimal diluent (Section 5.2.3.2) and aerosolised using the optimal delivery parameters identified earlier (Section 5.2.2). As a positive bolus control, 2 µl of LV vector diluted 1:100 (v/v) with appropriate diluent was pipetted into 700 µl of supplemented cell culture medium.

In both groups, aerosols were collected under 700 µl of supplemented cell culture medium. CHO-K1 cells were transduced with 300 µl of fluid in the Eppendorf tube from group 1 or group 2 (containing the vector), or from the bolus control solution. The CHO-K1 cells were cultured for 48 hours and stained with X-gal (Section 2.2.5), and levels of gene expression were quantified as described previously (Section 5.2.1).

5.3 Results

5.3.1 Gene expression produced by aerosolised LV vector

Gene expression produced by aerosolised LV vector collected from the outlet of the nebuliser alone was 90.5% lower than bolus delivery (Figure 5-2).

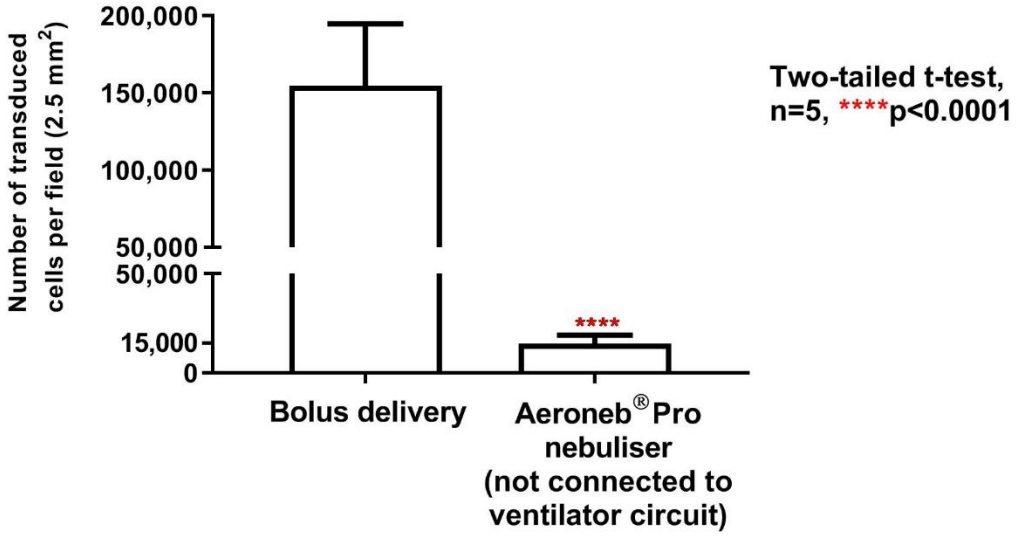

Figure 5-2: LV gene expression produced by bolus delivery vs aerosol delivery through nebuliser alone.

Different volumes of aerosolised LV vector released from the outlet of the Aeroneb®Pro nebuliser deposited in different circuit components, which again contained varying amounts of viable LV vector (Figure 5-3). Gene expression produced by the LV vector present in the wash of the in-line nebuliser mount wash was higher than vector released from the nebuliser outlet (Figure 5-3), which could be a result of evaporation of the vector and impactional deposition of the vector within this component. Also, the wash of the expiratory limb demonstrated negligible gene expression, which suggests that the Eppendorf tube containing cell culture medium was effective in trapping almost all LV vector aerosolised at the end of the ET tube (Figure 5-3).

The gene expression of LV vector obtained from the wash of the in-line nebuliser mount was

only 12.9 % of gene expression produced by bolus delivery. LV gene expression observed from the wash of the circuit components, such as the inner walls of the Aeroneb®Pro nebuliser, connector tube, and Y-piece, was 1.3%, 0.01%, and 0.01%, of bolus gene expression levels, respectively. Finally, the gene expression from the fluid obtained at the tip of the ET tube was only 0.02% of gene expression produced by bolus delivery.

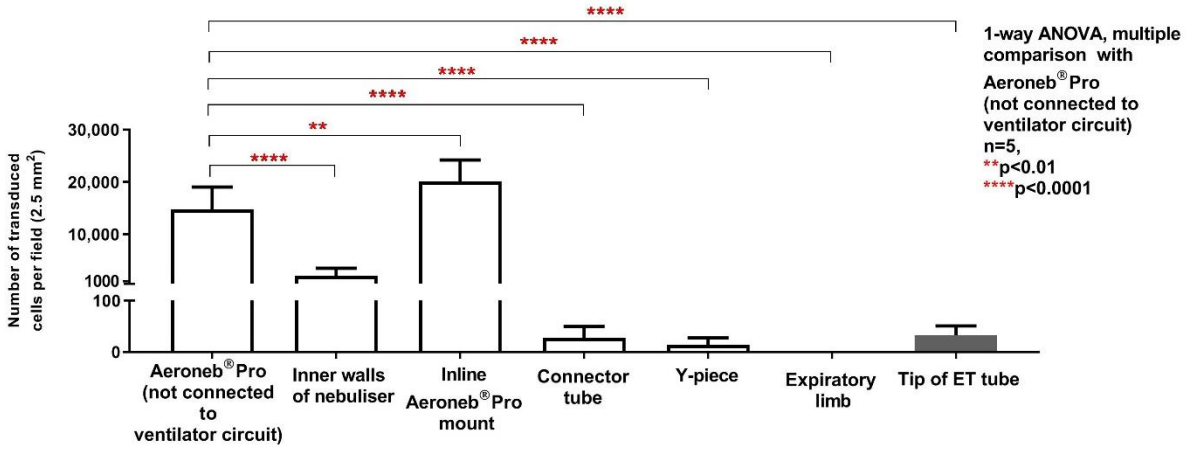

Figure 5-3: Gene expression produced by LV-LacZ aerosol following delivery through the Aeroneb®Pro-flexiVent™ ventilator circuit. The results show the number of LacZ gene expressing cells produced by transducing CHO-K1 cells using the collected fluid from the wash of ventilator circuit components, or the vector collected at the tip of the ET tube. Components of the circuit are shown in Figure 4-1.

5.3.2 Effect of aerosol delivery parameters on LV gene expression

Gene expression obtained from the fluid collected at the tip of the ET tube when delivering the vector in 10 ms pulses was 83.7% higher than obtained using the baseline *in vivo* delivery parameters (Figure 5-4). This result suggested that delivering vector in pulses of 10 ms improved the levels of gene expression of the LV vector released at the tip of the ET tube compared to baseline delivery parameters. Hence this delivery parameter was used in the subsequent study aerosolising LV vector through the ventilator circuit.

In contrast, no significant difference was observed on delivering vector in 10 ms pulses with the additional step of re-aerosolising the dose deposited (approx. 2 µl) within the in-line

nebuliser mount compared to baseline *in vivo* delivery parameters (Figure 5-4). Additionally, high variability in LV gene expression was observed within the 10 ms pulse group and re-aerosolising LV vector group. To reduce variability observed within these aerosol delivery groups a few modifications were made. The ET tube was fitted with an O-ring and volume of cell culture media was increased to 700 µl in the subsequent experiment (Section 5.2.3.2). Studies using dye solution showed that these modifications reduced the variability in the dose volume of food dye solution collected at the tip of the ET tube (15.8% ± 3.2% of initial dose volume) compared to the standard ventilator circuit (15.3% ± 6.2% of initial dose volume).

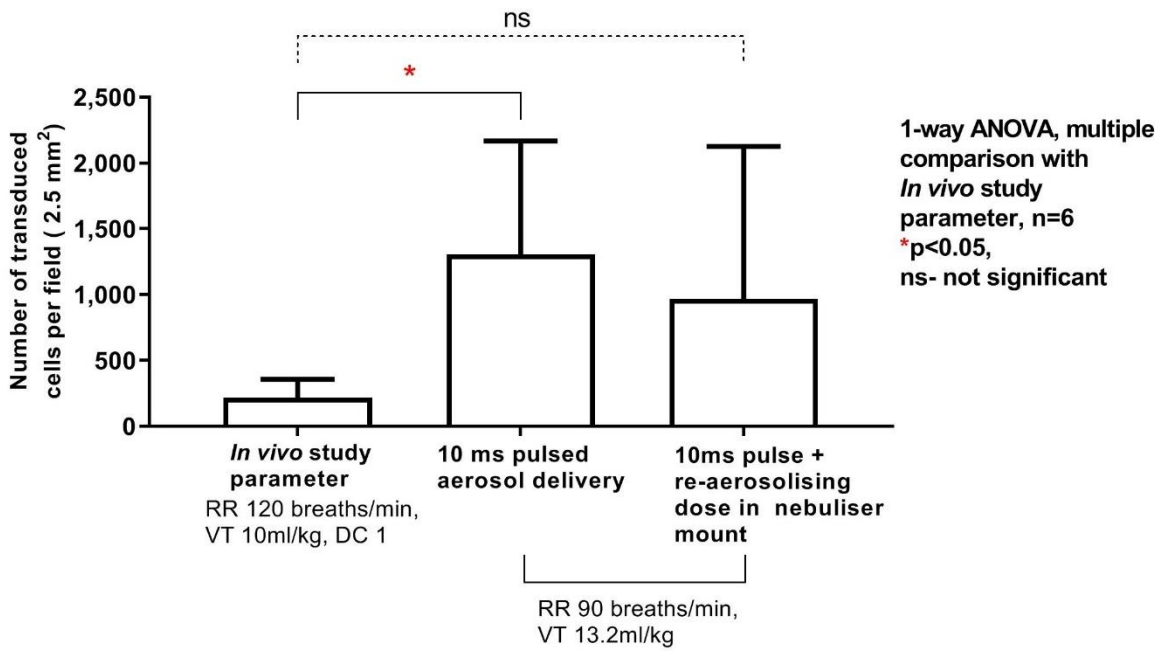

Figure 5-4: Comparison of LacZ gene expression obtained after aerosolising the vector with baseline delivery parameters to 10 ms pulsed delivery or to pulsed delivery with re-aerosolisation of vector deposited within the nebuliser mount.

5.3.3 Effect of diluents on *LacZ* gene expression of aerosolised vector

5.3.3.1 Aerosol delivery through nebuliser alone

This experiment examined the effect of different diluents on LV gene expression, when delivered by the nebuliser alone. The LV vector was delivered in pulses of 10 ms, as this delivery parameter produced the best results in the previous experiment (Section 5.3.2).

Gene expression produced by the vector suspended in FreeStyle™ medium was 49.5% higher than vector suspended in standard diluent MS/saline (Figure 5-5). Additionally, gene expression produced by vector suspended in FreeStyle™ medium was also higher than vector suspended in α-lactose buffer (Figure 5-5). However, no significant difference in gene expression was observed on aerosolising vector suspended in α-lactose buffer compared to vector suspended in MS/saline (Figure 5-5).

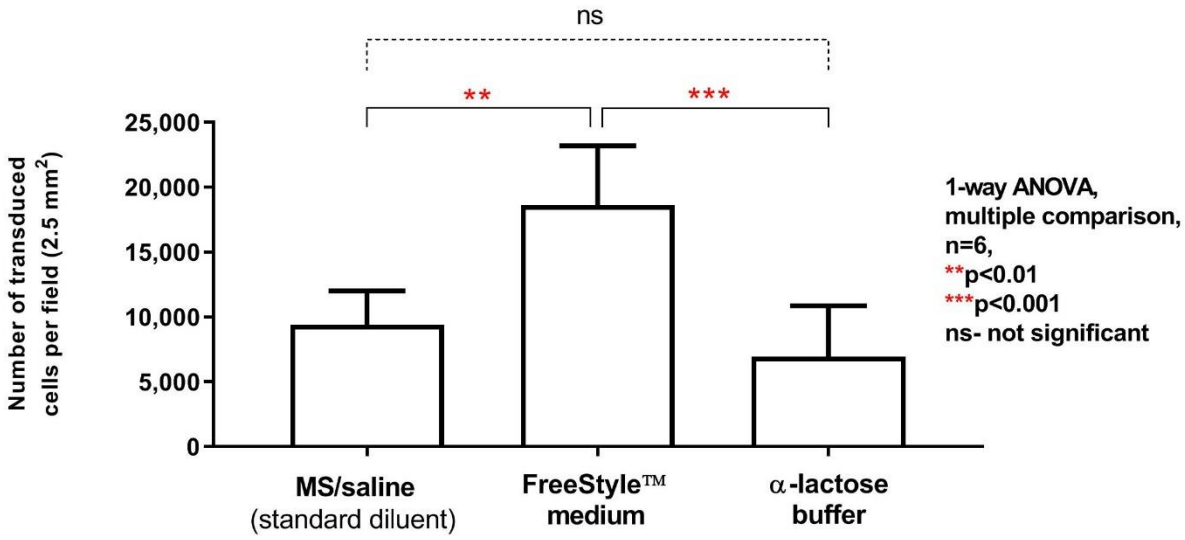

Figure 5-5: Gene expression produced by aerosolised LV vector suspended in standard diluent MS/saline, FreeStyle™ medium, and α-lactose buffer.

5.3.3.2 Aerosol delivery through the ventilator circuit

The effect of delivering LV vector suspended in either FreeStyle™ medium or MS/saline suspension through the ventilator circuit was examined, using the optimal delivery parameters identified in the previous experiment (Section 5.3.2), i.e. 10 ms pulsed delivery. Gene expression produced by the vector suspended in FreeStyle™ medium was 59% higher than vector suspended in MS/saline (Figure 5-6).

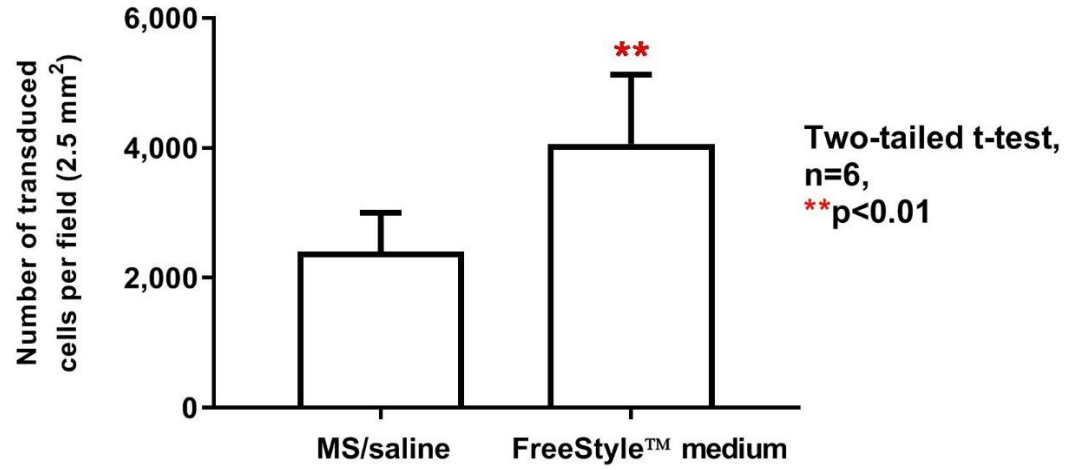

Figure 5-6: Gene expression of LV vector suspended in either FreeStyle™ medium or MS/saline after aerosol delivery through the ventilator circuit.

The combined effect of using these optimal aerosol delivery parameters (i.e. 10 ms pulsed delivery) (Section 5.3.2) and optimal diluent (i.e. FreeStyle™ medium) (Section 5.3.3.2) on LV gene expression obtained at the end of the ventilator circuit was then compared to baseline delivery parameters. Gene expression using optimal delivery parameters was substantially higher than when using the baseline delivery parameters (Figure 5-7).

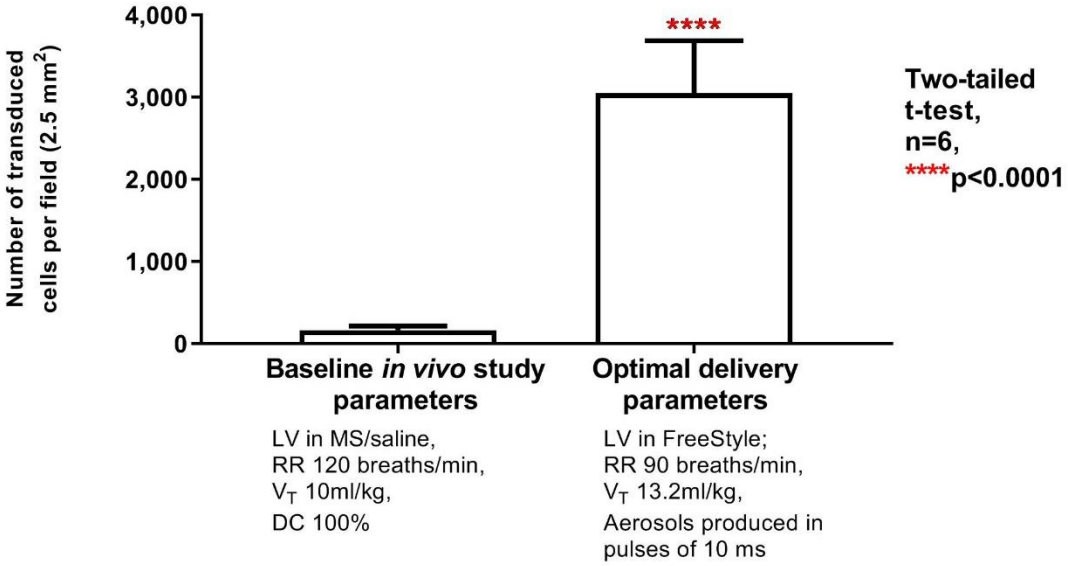

Figure 5-7: Gene expression produced by delivering the LV vector using baseline in the original in-vivo study parameters vs optimal delivery parameters.

Also, the effects of using optimal diluent and aerosol delivery parameters was compared to bolus delivery. The results showed that despite optimising diluent and aerosol delivery parameters, gene expression obtained from LV vector collected at the tip of the ET tube was still significantly lower than bolus delivery (Figure 5-8).

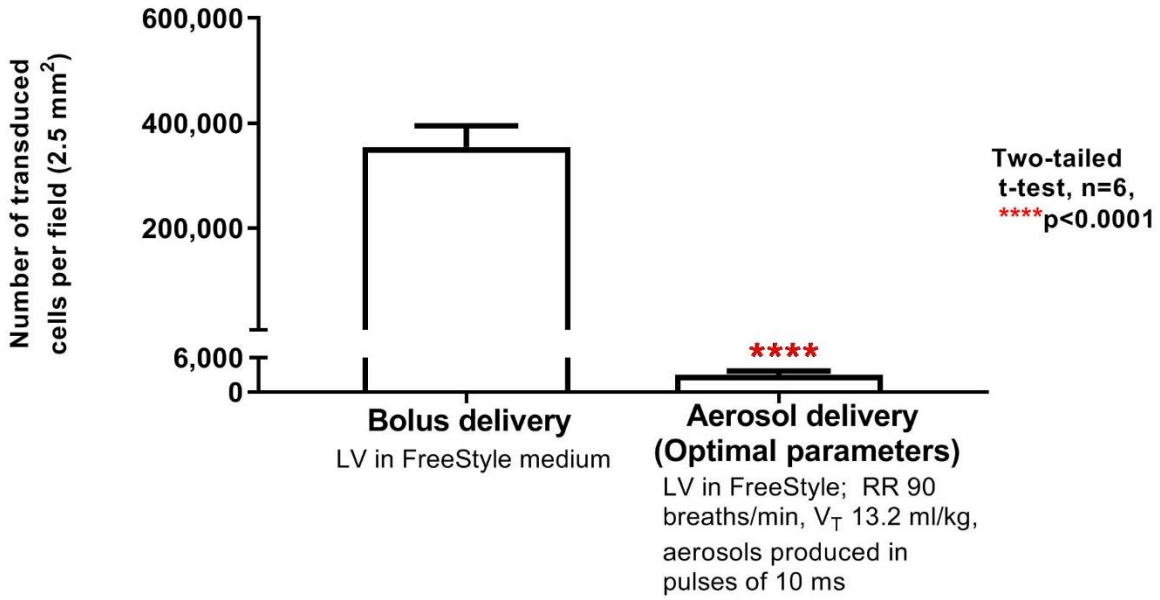

Figure 5-8: Gene expression produced by delivering the LV vector either as a bolus dose or as an aerosol using optimal aerosol delivery parameters.

5.4 Discussion

Chapter 4 demonstrated that the poor transduction in the trachea of mice treated with aerosolised LV vector was a result of the small proportion of the dose volume being delivered to that region (Section 4.3.2). The present Chapter examined the factors affecting aerosolised gene expression through the ventilator circuit to explore how vector viability combined with dose volume is affected as the vector transits through the nebuliser and ventilator circuit.

The first experiment of the present Chapter showed that the levels of gene expression produced by LV vector collected at the tip of the ET tube was 0.02% of bolus delivery

expression levels, thus supporting the first hypothesis. Similar levels of relative reduction in dose volume was observed in Chapter 4, which reported that only 2.1% of the initial volume of aerosolised dye solution reached the tip of the ET tube (Section 4.3.2). Together, these results explain the lowered gene expression levels seen in the trachea of aerosol-treated animals in the baseline *in vivo* study (Chapter 3, Section 3.3).

The first experiment also examined LV gene expression following delivery with the nebuliser alone (not connected to the ventilator circuit). The gene expression produced by aerosolised LV vector collected at the outlet of the nebuliser was 90.5% lower than bolus delivery gene expression levels. In comparison, the results from Chapter 4 showed that the volume of aerosols released after delivery through the nebuliser alone was only 13.4% lower than bolus delivery. The reduced gene expression was likely caused by shear forces acting on the fragile LV vector during transit through the nebuliser.

LV vector production reports highlight the fragile nature of LV vectors compared to other vectors, such as AAV. They suggest that shear forces imposed on the vector as it is passed through an ultrafiltration membrane may result in loss of biological activit[121]. LV vector production methods are typically designed to limit shear stress by pumping fluids at low speeds, minimising bubbles, and utilising low pressures where possible[217]. Other studies have sought to examine the part of the LV vector that makes it shear sensitive, and shown that the envelope of the LV vector influences its viability[221], with the VSV-G envelope being more stable than other envelopes during vector production[222]. Furthermore, Kim et al. demonstrated that subjecting a HIV-1 vector pseudotyped with VSV-G envelope to ultracentrifugation resulted in survival of 70% of the vector particles[223]They also found that after ultracentrifugation the number of functional particles of a HIV-1 vector with a VSV-G

envelope was five-fold higher than a murine leukemia virus (MLV) vector pseudotyped with the same VSV-G envelope, thus demonstrating that the core of the viral particle also determines its stability[223]. Regardless of the cause, this shear sensitivity during production suggests that care must also be taken during delivery to not render the LV vector biologically inactive.

As discussed in Section 1.6.4, vibrating mesh nebulisers such as the Aeroneb®Pro contain a mesh that is vibrated up and down by a few micrometres during operation, with the force of displacement generating aerosols. This rapid displacement likely generates shear stresses on the LV vector. Challenges associated with aerosolisation of shear-sensitive substances are not limited to LV vectors. A previously published study showed that when a shear and temperature sensitive protein solution, L-Lactic dehydrogenase, was passed through a vibrating mesh nozzle, (similar to the Aeroneb®Pro nebuliser design) used to spray dry the protein formulation, a loss of 78% of enzymatic activity was recorded[224]. The results from that study showed that the reduction in enzymatic activity was due to heat and shear stress produced by the vibrating nozzle and due to circulation of fluid within the pores of the nozzle[224]. Hence, it could be speculated that similar shearing of the LV vector occurs as it passes through the vibrating mesh plate of the Aeroneb®Pro nebuliser. Future studies should concentrate on developing novel LV shear reduction methods for preventing this degradation during aerosolisation. Therefore, the results presented in this Chapter demonstrate significant reduction in vector viability along with small reduction in dose volume following aerosolisation through the nebuliser. These findings agree with previously published studies that showed aerosolising viral vectors, such as Ad and SeV, resulted in lower vector viability following delivery through a nebuliser[54, 58].

To identify any sources of reduction of LV gene expression within the ventilator circuit, samples of deposited LV aerosol were drawn from each circuit component for analysis. Gene expression produced by the fluid collected at the in-line nebuliser mount was only 12.9 % of gene expression produced by bolus delivery, which could be due to impactional deposition of LV aerosols within this component. However, results from Chapter 4 reported deposition of approximately 83.7% of initial dose volume within the in-line nebuliser mount (Section 4.3.2). Together, these results suggest that although deposition of the majority of the dose volume occurs within the nebuliser mount, reduction in LV gene expression due to deposition within this component was minimal.

Vector recovered from other circuit components also demonstrated reduced LV gene expression as a result of impactional deposition of aerosolised vector within the circuit components. LV gene expression collected from the wash of inner walls of the nebuliser, connector tube, and Y-piece, was 1.3%, 0.01%, and 0.01%, of bolus gene expression levels, respectively. However, results from chapter 4 demonstrated deposition of dose volume within the inner walls of the nebuliser, connector tube, and Y-piece to be 10.1%, 2.7%, and 2.1% of initial dose volume, respectively (Section 4.3.2). Together these results suggest that the lowered delivery efficiency of the Aeroneb®Pro-flexiVent™ apparatus when delivering LV vector was caused primarily by reduction in the vector viability following aerosol delivery through the nebuliser and secondly by deposition of viable vector within circuit components.

To address the problem mentioned above, the second experiment examined the effect of aerosolising the vector with the optimal delivery parameters that were shown to reduce deposition of aerosolised fluid within circuit components (identified in Chapter 4). Delivering LV vector in pulses of 10 ms and with optimised ventilation parameters (Section 4.2.5)

produced higher levels of gene expression from the fluid collected at the tip of the ET tube compared to the baseline *in vivo* study parameters (Section 3.2.3), thus supporting the second hypothesis. Delivering the vector in short pulses lowers the dose volume released per respiratory cycle, which in turn may reduce impaction against the inner walls of the circuit component and result in higher amount of viable vector delivered to the tip of the ET tube compared to baseline *in vivo* study parameters, hence explaining the above result.

Many CF gene therapy studies have used this method of pulsed vector delivery to the airways of animal models and CF patients. Kohler et al. delivered a Hd-Ad vector formulated in LPC and PBS in pulses of 1 s followed by 0.1 s of air-only pulse using an AeroProbe™ catheter to the lungs of rabbits and reported transduction of 66% in tracheal epithelial cells[81]. However, the authors did not compare the effect of pulsed delivery to non-pulsed delivery on vector viability. In another study, Harvey et al. delivered a first-generation Ad-CFTR vector in pulses of 2 s using a bronchoscope and demonstrated 5% of CFTR mRNA expression in the airways of CF patients, which was transient, lasting between 4 to 30 days[98]. The researchers also did not compare the effect of pulsed vector delivery vs non-pulsed delivery on vector viability. The pulse used in the above-mentioned studies were 100 to 200 times longer than the 10 ms pulse used in the present Chapter. This leads to the speculation that the LV vector would still be viable when pulsed for a longer period of time (i.e. >10 ms); however, this might reduce the dose volume delivered at the tip of the Aeroneb®Pro-flexiVent™ ventilator apparatus (Section 4.3.5) and hence remains to be investigated.

Chapter 4 reported that re-aerosolising the dose deposited within the Aeroneb®Pro mount significantly improved dose volume expelled at the end of the ventilator circuit (Section 4.3.6). However, the present study showed that the levels of gene expression produced by re-

aerosolising the vector deposited within the in-line nebuliser mount was similar to the baseline *in vivo* study, thus not supporting the third hypothesis. This result suggests that re-aerosolising the vector could have reduced LV viability as the reintroduced vector was subject to nebuliser shear forces and impactional forces against the inner wall of the circuit components for a second time, thus reducing the amount of viable vector produced at the tip of the ET tube. As re-aerosolising the vector did not produce any significant difference in gene expression studies compared to baseline *in vivo* study it was not used further.

In an attempt to overcome reduced vector viability associated with aerosolisation through the nebuliser, the vector was suspended in different diluents to assess whether they would protect vector particles during aerosolisation. In support of the fourth hypothesis, vector suspended in FreeStyle™ medium produced increased levels of gene expression when delivered through the nebuliser alone (Section 5.3.3.1) as well as through the ventilator circuit (Section 5.2.3.2), compared to vector suspended in α-lactose buffer or the standard diluent MS/saline. The increased gene expression with FreeStyle™ medium may be a result of its GlutaMAX™ and Pluronic F-68 components (see Section 5.1 for an explanation why GlutaMAX™ improved viability of the HIV vector). Pluronic F-68 is a non-ionic surfactant commonly used to reduce shear stress produced in culturing cells in bio-reactors for vector production[217], and could also have contributed to improved LV gene expression.

Aerosolising the LV vector when suspended in an optimal diluent and using optimal aerosol delivery parameters produced higher levels of gene expression than when parameters of the baseline *in vivo* study were used. However, gene expression produced by the vector collected at the end of the ventilator circuit using these optimal parameters remained significantly lower than bolus vector delivery. Although not conducted within the same experiment, the

gene expression produced by the LV vector suspended in optimal diluent (FreeStyle™ medium) following delivery through the nebuliser alone (Figure 5-5) was still significantly lower than bolus delivery (Figure 5-8). These results suggest that an aerosolised vector would be unlikely to match gene expression efficiency produced using a bolus dose in the airways of mice. Further speculations could be drawn on what might happen if the initial aerosol dose volume was increased to match the bolus dose. With improvement in the optimal delivery parameters, the starting volume of the aerosol sample delivered would need to be increased 117 times (i.e. 20 µl to 2.4 ml) to deliver the same number of functional particles as a bolus dose. However, this is not feasible because delivering a large volume of fluid through the Aeroneb®Pro flexiVent™ ventilator circuit would result in substantial fluid deposition within the narrow tubes of the delivery circuit, resulting in blockage and delivery failure. Simply increasing the starting volume loaded into the Aeroneb®Pro to match the LV bolus dose is clearly not a practical solution. This is far too much vector to use per animal, and it would still be unlikely to result in comparable transduction levels. Furthermore, production of the large volumes of expensive vector formulations required would not be feasible or justified.

Finally, results of the experiments conducted in this Chapter, along with those of Chapter 3 and 4, suggest that aerosolising a HIV-based LV vector through the Aeroneb®Pro nebuliser was not effective in producing similar levels of gene expression as bolus delivery. Although a review of literature had proposed that this nebuliser would be ideal to deliver expensive vector formulation compared to other nebulisers (Section 1.6 and 3.1) and was effective in aerosolising other shear sensitive biological formulation[144, 161] results reported in this study highlight significant drawbacks that could limit its effectiveness, such as reduction in vector viability. The low gene transduction efficiency of this nebuliser alone might not be suitable to

deliver LV vector to the airways of larger animal models; however, this would need to be further investigated. As this nebuliser was shown to not be effective in delivering enough viable LV vector particles as bolus delivery, there is a need to investigate alternative delivery systems that could successfully aerosolise viral vector formulation or other shear-sensitive biological formulations.

5.5 Conclusion

The work described in this Chapter covered a range of experiments designed to quantify the gene expression of LV vector aerosolised through a ventilator circuit designed for use in mice, using the delivery parameters derived from the baseline *in vivo* study. Aerosolisation of LV vector resulted in lower gene expression than bolus delivery. The literature indicates that this lowered gene expression is primarily due to shear stress imposed on the vector during aerosol delivery and secondly, impaction of the vector against the inner walls of the ventilator circuit. Short pulses of vector increased gene expression compared to that achieved using baseline ventilation parameters. This suggests that pulsed vector delivery was effective and could be used for future studies aerosolising LV vector or other viral vector through a delivery circuit. This Chapter also identified FreeStyle™ medium – a serum-free, and protein-free formulation – as an optimal diluent that protected viral vector particles during aerosol delivery and could possibly overcome immune response problems caused by using serum containing medium[217]. For these reasons, the effectiveness of this serum-free medium for use in CF gene therapy studies needs to be investigated further. Despite efforts to improve gene expression of the aerosolised LV vector through the Aeroneb®Pro-flexiVent™ ventilator circuit, results showed that it was still lower than bolus delivery. Although this nebuliser had been shown to effectively aerosolise other shear-sensitive biological formulations[144, 161], the results from this

thesis demonstrated that the Aeroneb®Pro vibrating mesh nebuliser was not effective in producing enough viable HIV-based LV vector to produce similar levels of gene expression as bolus delivery. The low gene transduction efficiency of the Aeroneb®Pro nebuliser further suggests that it might not be effective in delivering sufficient viable LV vector to the airways of larger animal models.

6 DISCUSSION

One of the current challenges that affects therapeutic outcomes of gene therapy is the vector delivery regimen. Although the lungs are readily accessible, delivering vector formulation to them has been challenging[48]. In past clinical trials, vector formulation was delivered either as a liquid/bolus dose delivered via a bronchoscope[49] or as an aerosol using a nebuliser[93]. Alternatively, a vector formulation has also been delivered as a coarse spray to the lungs of CF patients, using an intra-tracheal sprayer[98]. The relative ease of delivering vector formulation and uniform distribution of the vector when delivered as an aerosol makes it an attractive delivery option[54, 60]. However, only six of 27 gene therapy clinical trials have incorporated aerosol delivery regimens to transfer viral vector to the airways of CF patients[92, 93, 98, 99, 106, 107, 109]. These clinical trials, along with other pre-clinical studies, reported that the primary difficulty in delivering a viral vector as an aerosol was it produced inadequate levels of gene expression[54, 58]. Hence, developing an optimal delivery regimen is dependent on using appropriate nebulisers to aerosolise sensitive vector formulations[66]. Advances in nebuliser technology have led to production of delivery systems that effectively deliver shear-sensitive and temperature-sensitive formulations. A recently developed ultrasonic nebuliser, the SAW nebuliser, has been shown to aerosolise sensitive biological formulations like stem cells and proteins[80, 225]. However, aerosolising the LV vector using the SAW nebuliser produced lower levels of gene expression than bolus delivery (Appendix, Section 8.1.4.1), suggesting that this nebuliser was also not efficient in delivering viable aerosols of LV vector. As a result, further work with this nebuliser in this thesis was abandoned. Further studies examined the effect of delivering LV as a coarse spray (rather than an aerosol) using the MADgic™ atomisation device. Results demonstrated similar levels of gene expression on spraying the vector compared to

bolus delivery (Appendix, Section 8.1.4.2), which suggests that this coarse sprayer is effective in delivering the LV vector and warrants further investigation. However, there are certain drawbacks in using this device as its use in patients would be invasive and a minimal volume of 1 ml is required to deliver a spray (30 to 100 µm) of the vector formulation. In spite of this result, an optimal nebuliser to deliver fine aerosols of the LV vector is yet to be identified and was the main goal of this thesis.

Another type of nebuliser that has been shown to retain stability of shear-sensitive biological formulations following aerosol delivery is the vibrating mesh nebuliser[159]. The Aeroneb®Pro is one such commercially available vibrating mesh nebuliser that has been approved for clinical use and is currently utilised to deliver drug formulations to the airways of mechanically ventilated patients[166, 167]. Additionally, this nebuliser has also been used to deliver aerosols to the airways of mice mechanically ventilated with a small animal-ventilator (flexiVent™)[146, 226]. This allows the delivery efficiency of expensive and shear-sensitive vector formulations through a clinical grade nebuliser to be tested in a smaller animal model. It also permits development of scalable-aerosol delivery protocol at a reduced cost, which could be further used in larger animal models.

HIV-based LV vectors are promising as they transduce both dividing and non-dividing cells and have shown to produce long-lasting gene expression[132]. The CFARG group in Adelaide have developed a LV vector delivery protocol that has been demonstrated to correct the CF gene defect for up to 12 months in CF mice[132]. Bolus delivery studies using this vector demonstrated effective gene transduction in the airways of animal models[57, 132, 140]. There is a necessity to translate this bolus vector delivery regimen to an aerosol form for clinical realisation, because aerosol delivery regimen is more likely to be non-invasive and enable

uniform distribution of the vector in patient airways. HIV-based LV vectors have been successfully aerosolised in lung cancer studies[122]; however, the efficiency of aerosolising this vector has not been quantified for CF airway delivery.

Hence, the aims of this thesis were to: (1) determine the efficiency of the Aeroneb®Pro nebuliser for aerosolising a HIV vector pseudotyped with the VSV-G envelope into the lungs of mice, during mechanical ventilation with a flexiVent™ ventilator and compare distribution of the LV vector delivered as an aerosol or bolus dose in lungs of mice; (2) quantify the viability of the aerosolised LV vector at the point at which it enters the mouse trachea, i.e. the viability of the LV vector released at the end of the Aeroneb®Pro-flexiVent™ ventilator circuit; (3) quantify the volume of aerosols reaching the mouse trachea, i.e. how much aerosol is released at the end of the Aeroneb®Pro-flexiVent™ ventilator circuit compared to bolus delivery; and (4) optimise the delivery parameters to develop an effective LV aerosol delivery protocol that could be used in further pre-clinical studies.

Chapter 3 investigated the first aim, which was to determine the efficiency of delivering the LV vector as an aerosol in the lungs of mechanically ventilated mice. In this study, two groups of mice were pre-treated with a fluid dose of LPC conditioning solution (i.e. a bolus dose) followed by an aerosol dose of LV vector to one group of mice and bolus dose of the vector to the other group. The results demonstrated significantly lower levels of gene expression in the trachea of aerosol-treated mice compared to those receiving a bolus dose. However, the comparative effect of the two delivery regimens in other regions of mice airways were not conclusive due to one outlier. It was hypothesised that the lowered gene expression in the trachea was caused by the reduced dose volume reaching the animal, and/or lowered viability of aerosolised vector delivered. These possibilities were investigated in Chapters 4 and 5,

respectively. Additionally, delivering LPC as a fluid dose prior to LV aerosol delivery could be another cause of lowered gene expression, as there could be a mismatch in the areas treated with LPC fluid and LV vector aerosol doses. To overcome this problem, LPC could be delivered as an aerosol, which would distribute it uniformly in the airways and would ideally overlap with regions treated with aerosolised LV vector. However, delivering higher concentrations of LPC can damage the respiratory epithelium and cause oedema[169, 170], and examining the effect of LPC aerosol was not within the scope of this thesis.

Another drawback was that the distribution of LV aerosols in the airways of mice could not be examined due to the low gene expression produced by this delivery regimen compared to bolus delivery. The airways of bolus-treated mice demonstrated patchy gene expression similar to other studies reported in the literature[57, 140]. Together, results from Chapter 3 identified lowered transduction efficiency of delivering LV aerosol using the Aeroneb®Pro-flexiVent™ apparatus compared to a bolus delivery group in the trachea of mice; however, the effectiveness of the LV aerosol delivery in other regions of the lung was not conclusive. As a result, additional studies were carried out to examine aerosol delivery efficiency of this nebuliser-ventilator circuit.

Chapter 4 investigated the second and fourth aims, which were to quantify the volume of aerosols released from the ventilator circuit ET tube (using *in vivo* baseline ventilation parameters) and to optimise the delivery parameters to develop an efficient LV aerosol delivery protocol. This study showed that only 2.1% of the starting dose volume reached the tip of the ET tube and a majority of the aerosolised dose was deposited within the Scireq in-line Aeroneb®Pro mount. This result likely explained the low levels of gene expression observed in the trachea of aerosol-treated mice in Chapter 3. This study demonstrated the

problems associated with impactional deposition of aerosolised fluid within a small-bore ventilator circuit and the challenges in adapting a human clinical-grade Aeroneb®Pro nebuliser for use with a small laboratory animal. These findings supported results obtained by other researchers who also demonstrated lowered delivery efficiency of the Aeroneb®Pro nebuliser used in conjunction with a rodent ventilator[74, 146]. Hence, to improve the delivery efficiency of the nebuliser-ventilator circuit the parameters that control aerosol production and airflow were examined. Significant improvement in aerosol output was observed on changing some ventilation parameters, delivering aerosols in short time-controlled pulses, and re-aerosolising the dose deposited at the ventilator circuit component. These results suggested that optimising delivery parameters could control aerosol flow and deposition within the ventilator circuit that could in turn impact the delivery efficiency of the system. The only limitation was the process of re-aerosolising, which required disassembling and re-assembling the ventilator circuit, and might not be practical for use in a larger animal model or clinical studies. Thus, the experiments conducted in Chapter 4 contributed to the understanding of aerosol delivery efficiency through a small-bore ventilator circuit used for mice. The optimised aerosol delivery parameters identified in Chapter 4 could also be applicable for other studies using the same ventilator circuit. However, further testing may be required as results may vary depending on the physio-chemical nature (e.g. viscosity, etc) of the drug formulation.

Chapter 5 examined aims three and four, which were to investigate LV gene expression following delivery through the nebuliser and the ventilator circuit, and to optimise delivery parameters to develop an efficient delivery protocol. Results demonstrated that the levels of *LacZ* gene expression from the vector collected at the nebuliser outlet was lower than bolus

delivery by 90.5%. The low levels of gene expression could be due to lowered viability of the LV vector as a result of destructive shear forces produced by the nebuliser and the lowered volume of the LV vector fluid collected at the nebuliser outlet compared to bolus delivery. Although this nebuliser was thought to produce reduced levels of shear stress on the aerosolised formulation, it could have been sufficient to damage the fragile LV vector. Additionally, wash of the circuit components also revealed deposition of small quantities of viable LV vector (< 15% of viable vector) within the circuit, although Chapter 4 had shown that majority of the dose volume (i.e. approximately 97% of the initial dose) deposits within the circuit components due to impactional deposition. Furthermore, the gene expression produced by the fluid collected at the end of the delivery circuit was only 0.02% of bolus delivery, which could explain the low levels of gene expression observed in the trachea of live mice exposed to LV-LacZ aerosols (Chapter 3). Together these results from Chapter 5 demonstrate the challenges in aerosolising the LV vector through the vibrating mesh nebuliser and a small-bore ventilator circuit.

Chapter 3 reported elevated levels of gene expression observed in the lungs of the outlier animal. The reasons for the drastically high levels of gene expression in the outlier animal may have been due to a dosing error in the volume of LPC delivered to the lung of the animal, improper cleaning of the ventilator circuit tubing, or an inherent and unexplained variation in respiratory characteristics of that mouse, rather than delivering twice the dose of the LV vector. As described in Chapter 5 (Section 5.4), it is likely that increasing vector volume would still result in very low levels of viable LV vector produced at the outlet of the nebuliser and produces an additional set of challenges.

To improve efficiency of the nebuliser-ventilator system, used in mice, in delivering the LV

vector it was aerosolised using the optimal delivery parameters identified in Chapter 4. The levels of gene expression improved when delivering the LV vector in pulses; however, re-aerosolising the LV vector trapped in the in-line nebuliser mount did not increase gene expression compared to baseline parameters. Re-aerosolising the vector could subject it to additional shear-forces that damage the vector, and with a second impaction against the inner walls of the circuit components these effects together could have caused this finding.

Other experiments in Chapter 5 examined methods to preserve vector viability during aerosol delivery, such as suspending LV vector in protective diluents. Suspending LV vector in Freestyle™ medium produced higher levels of gene expression than the standard diluent MS/saline following delivery through the nebuliser alone and delivery circuit. These efforts to preserve the viability of the LV vector during mechanical ventilation and increase the amount of aerosol reaching the ET tube tip improved the total transduction level, but it was still significantly lower than bolus delivery.

The results from Chapter 5 suggest that the Aeroneb®Pro-flexiVent™ ventilator circuit was not effective in delivering sufficient quantities of viable LV vector to produce similar levels of gene expression as a bolus dose. Although these studies were conducted in a small-animal ventilator circuit, it highlighted the practical challenges of aerosol delivery regimen that could be expected in large animal models and clinical studies. Chapter 5 also showed that delivering the LV vector in short pulses improved vector viability. However, to deliver a larger volume of LV vector to the airways of humans and larger animal models the pulse length would likely need to be increased for practical reasons (so that delivery time is not overly long). Such an increase in the pulse length would likely reduce LV viability. However, if further improvements in nebuliser design, to reduce shear stress, were to occur these could drastically improve the

LV viability released from the nebuliser. To overcome problems associated with LV viability, future research could also investigate pseudotyping the LV vector with other viral vector envelopes resistant to shear stress and/or encapsulating the vector with a protective chemical agent, such as polyethylene glycol.

Combined with improvements to the delivery circuit to reduce LV deposition an aerosol delivery system could still potentially be used to deliver larger volume of vector formulation to the airways of large animals and humans. The Aeroneb®Pro nebuliser was initially designed for use in humans and has been used in clinics to deliver antibiotic formulations inline, such as colistin, to the airways of CF patients[227]. It has also been shown to be effective in delivering drug formulation to the airways of larger animals, such as non-human primates[228]Thus, delivery of larger volumes of vector formulation is possible, provided the nebuliser design and delivery route are optimised.

Another factor that needs to be examined in the future is delivery an LV vector carrying the larger therapeutic *CFTR* gene (~4.4 kb)[108] in place of the *LacZ* gene (~3.1 kb) used in this thesis (Section 8.3). A previous study has shown that titre of the LV vector decreased with increasing size of the transgene[229] as this affects the packaging efficiency of the virions. However, aerosolising either a LV-CFTR vector or a LV-LacZ vector of the same titre through a nebuliser could be speculated to produce similar number of viable LV particles, because the physical properties (particle size and envelope) are not altered by the transgene that is packaged. Nonetheless, this remains untested. In conclusion, regardless of the results from Chapter 5, the question of "which is the optimal aerosol delivery system or nebuliser to aerosolise the HIV-based LV vector?" remain unanswered. Hence, there is a need to seek new delivery systems that have shown to aerosolise shear-sensitive biological formulation so as to test

their effectiveness in delivering the LV vector.

While the search for an optimal fine particle nebuliser remains ongoing, there is a need to optimise other methods to deliver LV vector for the first clinical trial in CF patients. This thesis identified that the LV vector could be delivered as a coarse aerosol using the MADgic™atomisation device (Appendix, Section 8.1.4.2). The MADgic™atomisation device was more effective in delivering the LV vector than either the Aeroneb®Pro nebuliser or SAW nebuliser, which may be due to the aerosol generation mechanism. As explained in Section 8.1.1, the MADgic™atomisation device generates coarse aerosols through the atomiser, located at the tip of the device, by applying manual pressure to the bottom of the syringe that pushes the liquid formulation through the tubing. In the Aeroneb®Pro nebuliser, electric current is supplied to the mesh plate with finely tapered holes on which the formulation was placed. This results in vibration of the mesh plate and generation of aerosols, as described in Section 1.6.4. The mechanism of SAW nebuliser involves an electric signal supplied to the interdigital transducer (IDT), which generates travelling SAW waves that aerosolises the liquid delivered to the substrate via a capillary tube, as explained in Section 8.1.1.

The frequency of the vibrating mesh of the Aeroneb®Pro nebuliser was 128 kHz[230] and the frequency of the SAW waves employed in the SAW device was 10 to 100 MHz[80], while there are no vibrations produced by the MADgic™atomisation device. Using these high frequency vibrations (in the MHz range) could be the source of the shear stress that likely destroys some of the LV particles during the aerosolisation process. Additionally, the heat generated at the point of aerosolisation within the SAW nebuliser (Section 8.1.5) could have also further denatured the LV vector, resulting in its poorer performance compared to the other two devices.

Future studies should optimise the design of the Aeroneb®Pro nebuliser-flexiVent™ ventilator circuit to reduce the deposition of the aerosolised dose within the circuit. Published studies have shown that using streamlined ventilator circuit components downstream of the nebuliser reduces dose deposition within the circuit and thus enhances vector delivery[205]. Streamlined circuit components reduce sudden changes in the diameter of tubing, turbulence, and have adequate flow separation resulting in uniform flow of aerosol within the component[205]. Longest et al. showed that using a streamlined T-piece component, which is placed adjacent to the nebuliser in the delivery circuit, reduced deposition within this component by a factor of four[205].

The design of the Aeroneb®Pro nebuliser and SAW nebuliser could also be improved to enable effective delivery of shear-sensitive biological formulation, such as gene therapy vectors. The mesh plate of the Aeroneb®Pro nebuliser could also be re-designed using different metallic alloys that are compatible to deliver LV vectors. In a study by Choi et al., a vibrating mesh plate nebuliser was fabricated using a bio-compatible palladium–nickel alloys and showed effective delivery of mouse fibroblast and human basal epithelial cells[231]. The effectiveness of these types of modifications on LV vector delivery remains unknown.

Heat produced by the SAW nebuliser was suspected to be an obstacle in its efficient delivery of LV vectors. To overcome this, experiments could examine the effectiveness of different heat-sinks, such as copper or aluminium alloys, in absorbing the heat generated. Also, the SAW nebuliser could be operated in a cooled chamber (at 4°C) to further reduce the heat generated. Another method to capture the heat generated by the SAW nebuliser is by using Peltier cooling plates. A study conducted by Hertel et al. showed that attaching a micro Peltier cooling plate attached to the nebuliser reservoir reduced the average temperature of the

reservoir by 8°C[232]. This reduction in temperature enabled the effective aerosol delivery of a thermolabile protein through the nebuliser. Until such improvements in nebuliser design and ventilator circuits occur research efforts should focus on the optimisation of the MADgic®atomisation device, combined with alterations to the vector diluents to improve stability and reduce sensitivity, as an optimal delivery device for further pre-clinical LV gene therapy studies.

Further cell culture studies could examine the effectiveness of the MADgic™atomisation device for delivering LV vector suspended in optimal diluents, delivered in short-pulses, and priming the device with protective agents prior to vector delivery. Additionally, future studies would also need to examine the distribution of the vector in animal airways and effectiveness of this vector delivery regimen in CF animal models. A major drawback of using the MADgic™atomisation device is that the patient needs to be sedated and undergo endo-tracheal intubation. In spite of this drawback, it could be used in the first clinical trial to uniformly distribute the HIV-based LV vector to the airways of CF patients, provided it produces promising results in the aforementioned studies. Other research groups, like the UK Cystic Fibrosis Gene Therapy Consortium have also examined similar intra-tracheal sprayers to deliver SIV vector[218]. They demonstrated successful delivery of the SIV vector through a Trudell AeroProbe® catheter and a metered nasal spray device that could be used in the first CF gene therapy trial using this vector[218].

In the event that LV aerosol/spray experiments produce unfavourable results, the LV vector could be delivered as a bolus dose via a bronchoscope. Although this method might not result in uniform spatial distribution of the vector in the lung, and requires sedation of the patient, it has been effective in delivering other viral vectors, such as AAV, to the airways of CF

patients[55]. FIV based LV vector has been effectively delivered to the airways of pigs using a bronchoscope[233]. A pilot study conducted by Liu et al. demonstrated low gene expression levels after delivering a HIV-based LV vector as a bolus dose to the lungs of sheep using a bronchoscope[140]. The researchers speculated that low gene expression could have resulted from the low volume of LV vector delivered to the lungs of sheep and small size of the study[140]. However, a recent study by the CFARG confirmed that the HIV-based LV vector could be effectively delivered through a miniature bronchoscope, where it produced effective and lobe-specific gene transduction in the lungs of rats[234].

As the realisation of using an LV vector for CF gene therapy draws closer, there is an urgent need to identify optimal methods to deliver expensive vector formulations to the airways of patients. Besides vector stability, there are other challenges that affect delivery of vector formulations to appropriate sites in airways. These challenges include bacterial colonisation and mucus obstruction[235] that could limit vector deposition at appropriate sites in airways[236]. Immune response produced against the viral vector or transgene products could also result in reduced efficacy on vector re-administration[236] as well as other clinical complications such as pulmonary inflammation and pneumonia[105]. Therefore, the effect of aerosolised antibacterial and mucolytic agents alongside LV vector treatment need to be investigated in the airways of CF animal models. The effect of drugs for suppressing the immune response and LV vector delivery should also be examined, because the safety of using these agents combined with a gene therapy treatment is still relatively unknown. Targeting vector treatment to certain regions of the lung also needs to be investigated as this could reduce the burden of producing expensive vector formulations. Finally, there is a need for newer, more non-invasive nebuliser designs that could efficiently aerosolise shear-sensitive LV vector

formulations to the airways of CF patients. Hence, there is still much work that needs to be done to successfully deliver a HIV-based LV vector to correct or prevent disease in the airways of CF patients.

7 CONCLUSION

This thesis aimed to identify an ideal nebuliser capable of effectively delivering a HIV-based LV vector pseudotyped with a VSV-G envelope developed by the CFARG in Adelaide. The effectiveness of a recently developed SAW nebuliser in delivering aerosols of this vector was examined in an *in vitro* study. Results demonstrated significantly lowered gene expression compared to bolus delivery, which could be due to the fragile nature of the virus. In contrast, the next study showed that the MADgic™atomisation device was effective in delivering the HIV-based LV vector as a coarse spray because levels of gene expression obtained were similar to bolus delivery. However, using this device could be invasive and requires the sedation of patients. Therefore, there is a need to examine other nebulisers that could be non-invasive and effectively deliver fine aerosols of the LV vector to patient airways.

Further experiments examined a commercially available vibrating mesh nebuliser, the Aeroneb®Pro, which has been shown to effectively aerosolise shear-sensitive formulations. To my knowledge, this was the first study to examine the effect of aerosolising this LV vector using the Aeroneb®Pro nebuliser into mouse airways. This study highlighted the challenges in delivering aerosol formulation to the narrow airways of mice and the fragile nature of delivering the LV vector as an aerosol. Further bench studies documented the effect of ventilation parameters, pulsed aerosol delivery, and re-aerosolising dose deposited within circuit components on delivery efficiency of the Aeroneb®Pro-flexiVent™ ventilator used for smaller animal models. This knowledge could also be useful in other aerosol delivery studies using the same ventilator apparatus. Subsequent cell culture studies elucidated the effect of aerosol delivery parameters of LV vector through the ventilator circuit and identified the use of serum-free FreeStyle™ medium as a suitable diluent that improves vector LV gene

expression following aerosol delivery. Further investigation is warranted for using this medium as a diluent for clinical studies. Despite optimising delivery parameters, the Aeroneb®Pro-flexiVent™ ventilator apparatus was found to be ineffective in delivering enough viable LV vector to produce similar gene expression levels as bolus delivery.

Research efforts to identify an optimal nebuliser to effectively aerosolise the HIV-based LV vector remain ongoing. Future research effort needs to focus on delivering this vector as a spray or bolus dose. The effectiveness of the MADgic™ atomisation device in delivering the HIV-based LV vector as a spray has been reported in this thesis. This result provides initial data for future studies that could examine the effectiveness of this device in delivering LV vector formulations to the lungs of animal models. Such research effort could be comparable to other research groups, like the UK Cystic Fibrosis Gene Therapy Consortium, who have also examined the effectiveness of a similar intra-tracheal sprayer (Trudell AeroProbe ® catheter) to deliver the SIV vector in preparation for the first CF gene therapy trial [218].

Besides the above-mentioned studies, research is also needed to examine the effect of aerosolising the LPC airway conditioning treatment prior to aerosolising the LV vector. Other issues that might severely limit the outcomes of aerosol gene therapy treatment, such as mucus obstruction in the airways of CF patients and immune responses to the vector, also need to be addressed. The experiments reported in this thesis highlight the advantages and disadvantages associated with LV aerosol delivery. Much work still needs to be done to develop a non-invasive, and effective vector delivery method for use in the first CF gene therapy trial using the HIV-based LV vector.

8 APPENDIX

8.1 Aerosol delivery of the LV vector using other types of nebulisers

8.1.1 Introduction

As discussed in previous Chapters, aerosol delivery of a HIV-based LV vector is challenging, due to the fragile nature of the vector[156]. The Aeroneb®Pro nebuliser was chosen in these studies to aerosolise the LV vector due to its record of successful aerosolisation of shear-sensitive formulations, such as proteins and liposomes[160, 161, 237] (Introduction, Section 1.6.4). However, prior to examining the Aeroneb®Pro nebuliser other new delivery devices were briefly assessed. This Appendix investigates the effectiveness of two devices; a prototype Surface Acoustic Wave (SAW) nebuliser, and the commercially available MADgic™ atomisation device.

8.1.1.1 SAW nebuliser

Advances in engineering technology have led to development of the SAW nebuliser[238]. This nebuliser has been successfully used to aerosolise drug formulations such as β_2 agonist salbutamol sulphate and shear sensitive biological formulations, such as proteins[225], pDNA[79], and stem cells[80], suggesting that it might also be suitable for LV vector delivery. The SAW nebuliser (Figure 8-1) makes use of transverse-axial polarised electroacoustic waves, also known as SAW waves, which have a displacement amplitude of a few nanometres. SAW waves are generated by applying a sinusoidal electric field to the interlaced fingers of an interdigital transducer (IDT). These waves then travel along the adjacent piezoelectric lithium niobate (LN) substrate that helps contain most of the energy generated. The travelling SAW waves give rise to capillary waves along the surface of the liquid placed on the substrate, these waves then lead to liquid breakup and formation of aerosols[239]. The size of aerosols

produced by this device range from 1 to 10 µm, depending on characteristics of the liquid formulation[240]. On inhalation, larger aerosol particles (5 to 10 µm) deposit in the central airways of patients[241] (the primary region targeted by CF gene therapy), which might make this a suitable nebuliser for use in future clinical studies[42].

The frequency used in the SAW nebuliser ranges from 10 to 100 MHz, which sets it apart from the 10 kHz-1 MHz frequency range of traditional ultrasonic nebulisers[225]. Other nebulisers like the Aeroneb®Pro, use a much lower frequency range of 128 kHz to aerosolise a liquid formulation[242]. The frequency used in the SAW nebuliser induces vibrations with a shorter time period than the molecular relaxation time scale of large molecules in liquid, thereby reducing the possibility of denaturing molecules of the drug formulation[225]. As this could translate to less shear forces, the present Chapter reports on pilot experiments to determine whether the SAW nebuliser could be an effective method for delivering the LV gene vector.

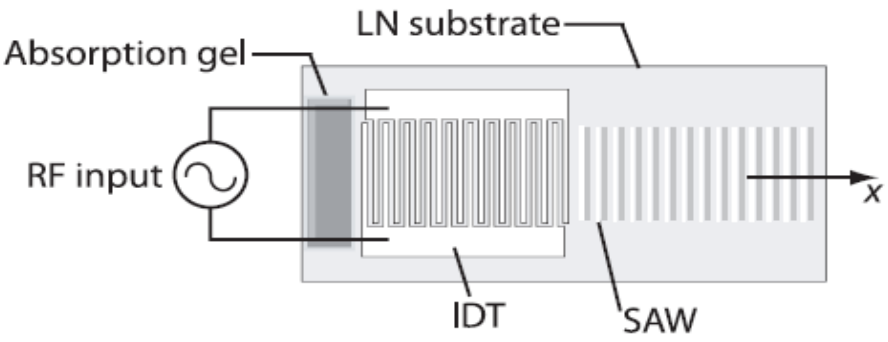

Figure 8-1: SAW waves generated by applying a sinusoidal electric field to the finger of an interdigital transducer (IDT) placed on the lithium niobate (LN) substrate[1].

8.1.1.2 Intra-tracheal sprayers

Intra-tracheal sprayers or nebulisation catheters deliver a coarse spray of drug formulation to the airways of patients[98] and animal models[58, 81, 103]. To deliver a drug formulation, the nebulising catheter needs to be guided to the trachea using a bronchoscope[58]. Although this procedure is invasive and requires sedation of the subject[81, 103], it has significant advantages,

as majority of the drug formulation reaches the bronchial airways and lungs, reducing the dose deposited at other regions like the pharynx and vocal cords[98], and maximising the potential effectiveness of expensive drug formulation[243]. The Microsprayer® (PennCentury, Philadelphia, USA) and the AeroProbe™ (Trudell Corporation, USA) catheter are two intra-trachea devices commonly used to deliver viral vectors in many CF gene therapy studies[81, 103, 111, 114, 116]. As mentioned in the introduction, the production of both the Microsprayer® and AeroProbe™ catheter have been halted (PennCentury™ closed for business in 2015 and Trudell discontinued the AeroProbe™ in its product line), so new sprayers like the MADgic™ atomisation device (Teleflex, Ireland), have been developed (Figure 8-2 A).

8.1.1.3 MADgic™ atomisation device

The MADgic™ atomisation device has been used in clinics to deliver topical anaesthesia to the airways of patients[244, 245] (Figure 8-2 A). The device comprises of an atomiser with tiny holes, located at the tip of 28 cm of tubing, to allow its use in intubated patients (Figure 8-2 A and B). It also has separate tubing to allow oxygen delivery to the lungs of patients. One drawback of this device is that it can only spray a large volume of liquid formulation (1 ml to 5 ml) and cannot to be used to deliver smaller volumes (µl) of formulation, like that used for mice. Hence, in this *in vitro* study the starting volume of vector formulation was increased to 1 ml by diluting the vector with standard diluent and delivered it as a coarse spray. To deliver a spray of the liquid, the formulation is loaded into a 5 ml syringe that is then attached to the tubing of the atomiser. Manual pressure is then applied to the bottom of the syringe to push the liquid formulation through the tubing to reach the atomiser situated at the tip of the device (Figure 8-2 C). The bolus formulation goes through the atomiser, which contains microscopic holes (Figure 8-2 D) to generate aerosol droplets that range from 30 to 100 µm[246]. A study using a similar trachea-bronchial sprayer (Microsprayer®), which delivers aerosol

droplets of the same size, has demonstrated deposition of the AAV vector in rhesus macaque major conducting airways[111], which is also the target region for human CF gene therapy[42].

The MADgic™ atomisation device was recently used to deliver vector formulation in a CF gene therapy study. Cooney et al. were the first to use this device to effectively deliver an FIV based LV vector carrying the therapeutic *CFTR* gene to the airways of CF pigs[87]. They demonstrated partial correction of the CF gene defect two weeks later in the excised bronchial and tracheal airways of the treated animals[87]. Hence, this pilot study was designed to examine the effectiveness of the MADgic™ atomisation device in delivering coarse aerosols of the LV vector used in this thesis.

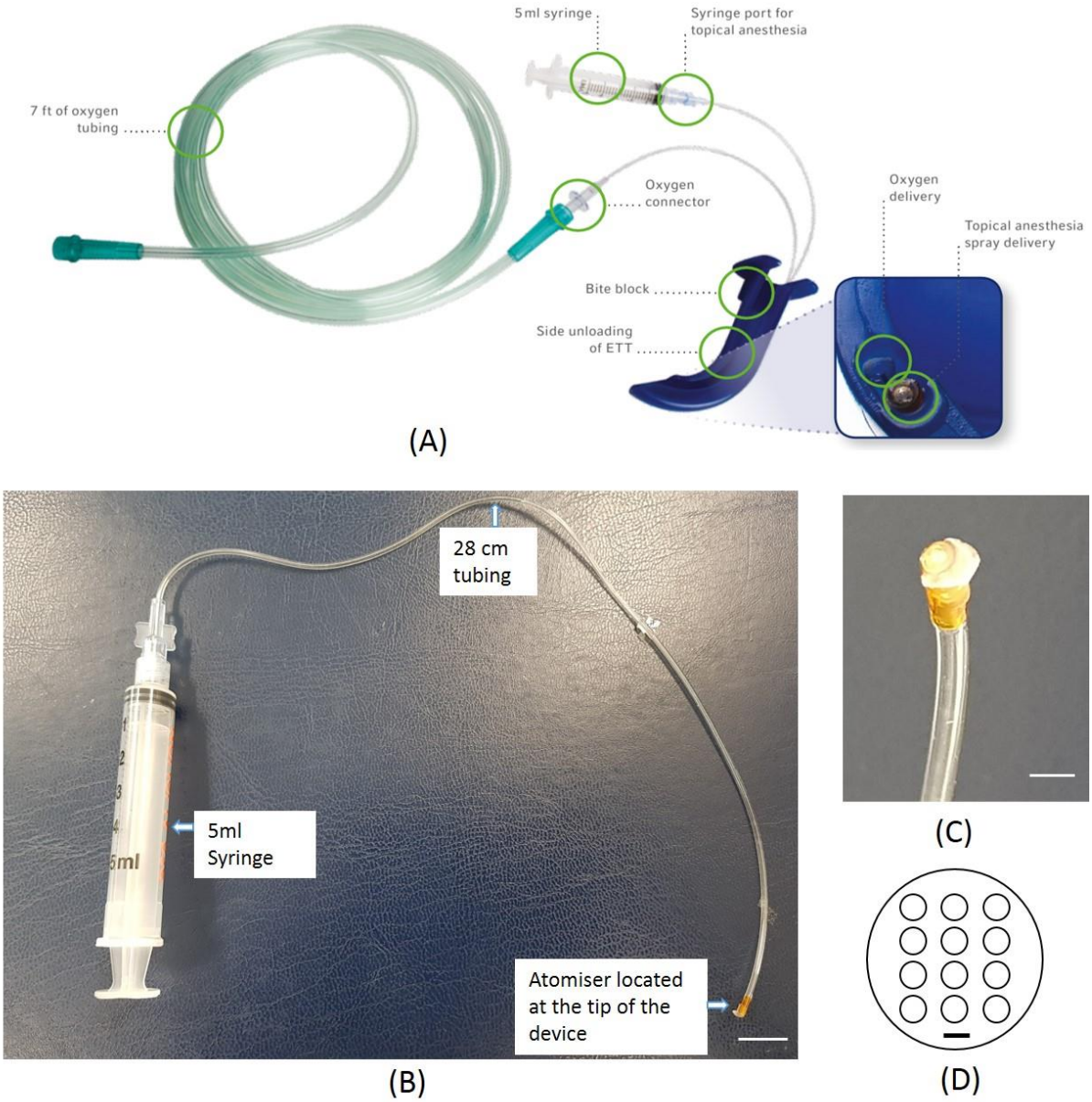

Figure 8-2: (A) MADgic™ atomisation device consisting of a 5 ml syringe with the atomiser at its tip and separate tubing to enable oxygen delivery to the lungs of intubated patients[247], (B) magnified view of 5 ml syringe attached to tubing with the atomiser at the tip (scale bar =1 cm), *(C) magnified view of the tip (scale bar = 0.5 cm), and (D) diagrammatic representation of the atomiser tip showing microscopic holes through which the liquid formulation is pushed to generate coarse aerosols (scale bar = 30 µm).*

8.1.2 Hypothesis and aims

The hypothesis of the present study was based on the results of previously published studies described in Section 8.1.1. I hypothesised that delivering a HIV-based LV vector as an aerosol/spray through the:

1) SAW nebuliser would produce similar levels of gene expression as bolus delivery.

2) MADgic™ atomisation device would produce gene expression levels comparable to bolus delivery.

The aim of this pilot study was to quantify the gene expression produced by the LV vector following aerosol delivery through the SAW nebuliser or the MADgic™ atomisation device and compare that to the gene expression obtained by delivering the vector as a bolus dose.

8.1.3 Methods

8.1.3.1 Aerosol delivery using the SAW nebuliser

The SAW nebuliser was mounted onto a metal base to provide support. A hole was made in the cap of a 50 ml Falcon tube and was fitted with an O-ring. This cap was then mounted on top of the SAW nebuliser to direct the plume of aerosols downward towards the cells (Figure 8-3 A and B).

Two aspects of the SAW nebuliser were examined in this study. The first experiment quantified how much of the original dose was retained by the nebuliser during aerosol delivery, using coloured dye solution. The second experiment examined the effectiveness of the nebuliser for delivering a LV vector and compared it to bolus delivery. Results of the first experiment were used to adjust the starting volume of the LV vector dose delivered by the SAW nebuliser in the second experiment.

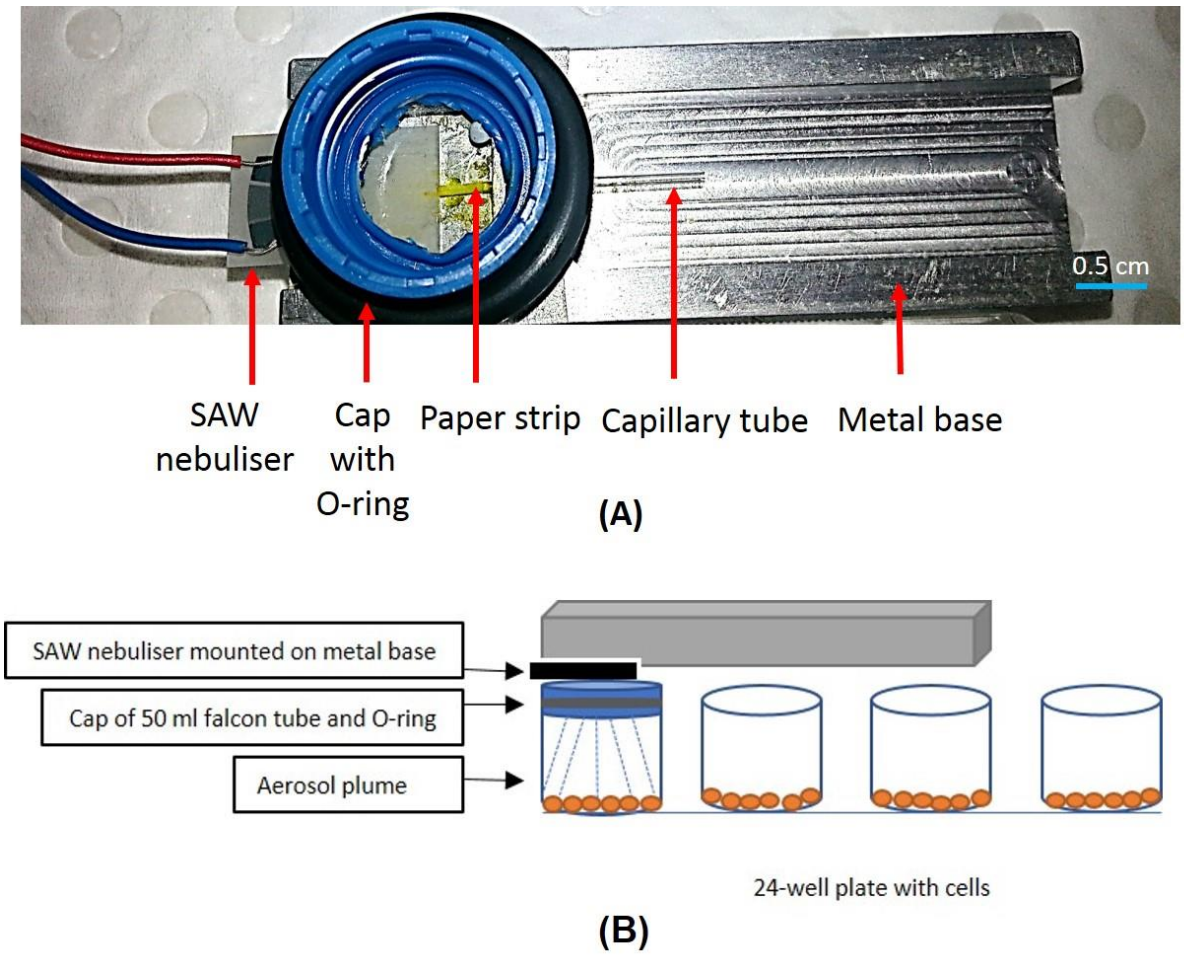

Figure 8-3: (A) SAW nebuliser apparatus used in the present study (B) diagrammatic representation of the SAW nebuliser delivering aerosols onto CHO-K1 cells in a 24-well plate.

8.1.3.2 Quantifying the residual dose volume retained by SAW nebuliser on aerosol delivery

A 10 µl aliquot of dye solution (diluted 50% v/v with water) was loaded onto the capillary tube and aerosolised by supplying the SAW nebuliser with an electric potential of 100 mV. The combined weight of the paper strip and capillary tube was measured before and after aerosol delivery was complete (n=5). The difference in weight of the paper strip and capillary tube represented the dose volume retained by the SAW nebuliser.

8.1.3.3 Quantifying the gene expression of LV vector aerosolised using the SAW nebuliser

A 10 µl aliquot of diluted LV-LacZ vector (diluted 1:250 in PBS, v/v), along with additional dose volume of formulation retained by the SAW nebuliser identified from the previous experiment (Section 8.1.3.2), was loaded into the capillary tube (unlike previous experiment

that used a starting vector dose of 20 µl, the present study used 10 µl of LV vector because the maximum volume held by the capillary tube was 15 µl). A thin film of the vector formulation was then transferred to the edge of the SAW nebuliser via the paper strip due to capillary force within the tube. A plume of LV aerosols released by the SAW nebuliser was then directed towards the 0.25 $x10^6$ CHO-K1 cells placed in the well. A fresh paper strip was used for each sample. Between aerosolisation samples the entire SAW nebuliser setup (Figure 8-3) was rinsed once with 5 ml of Virkon solution and three times with 5 ml of water and was then dried. As a control, 10 µl of diluted LV-LacZ vector was delivered as a bolus dose onto CHO-K1 cells using a pipette (n=3/group). Three wells containing untreated CHO-K1 cells were used as negative controls. The cells were cultured for 48 hours and then stained with X-gal overnight (Section 2.2.5). Three random images of each well were captured using a digital camera attached to a stereo-microscope at 40x magnification. The number of transduced cells per area of field of view (5 mm^2) was quantified using the MATLAB image-analysis script (Section 8.2). Gene expression was reported as the number of transduced cells per field of view.

8.1.3.4 Quantifying gene expression of LV vector sprayed using the MADgic™ atomisation device

The MADgic™ atomisation device needed a minimum of 1 ml starting dose volume to generate coarse aerosols. Therefore, the LV vector formulation was diluted 1:10,000 with MS/saline (v/v) and 1 ml of this diluted vector formulation was used in this study (rather than 1:250 dilution used in the previous experiment, Section 8.1.3.3). Also, unlike earlier studies, the starting volume of vector formulation of the present study was high (i.e. 1 ml compared to 20 µl used in previous studies) and delivering 1 ml of vector formulation could flood the cells; hence, the vector formulation was not directly sprayed onto the cells in the well.

The diluted LV vector was loaded into the barrel of the device and a coarse spray was generated by depressing the syringe plunger for a duration of three to four seconds. The coarse aerosols of the LV vector were collected in a 15 ml tube. The vector formulation retained in the tubing of the device was collected separately and weighed. A 20 µl aliquot of the sprayed vector was then used to transfect CHO-K1 cells cultured on a 24-well plate. As a replicate, the same volume of the sprayed vector was used to transduce cells in another well on the 24-well plate. After each sample, the apparatus was rinsed once with 2 ml of Virkon solution and three times with 5 ml of water. As a bolus control, 20 µl of the diluted LV-LacZ vector was delivered onto CHO-K1 cells (n=5/ group) using a pipette. Cells were cultured for 48 hours, X-gal stained overnight, and four random images of each well were captured at 100x magnification (Section 2.2.5). The number of transduced cells per area of field of view (2.5 mm^2) was quantified using the MATLAB image-analysis script (Section 8.2). Gene expression was reported as number of cells present per field of view.

8.1.4 Results

8.1.4.1 SAW nebuliser

This pilot study (n=3) quantified how much of the 10 µl original dye dose (i.e. 10 mg) was retained in the SAW nebuliser during the delivery process. The average combined weight of the paper strip and capillary tube before and after delivering dye solution was 17.15 ± 1.6 mg (mean ± S.D) and 18.51 ± 1.6 mg, respectively. The maximum weight of the dye solution retained in the capillary tube and paper strip following aerosol delivery was 1.5 ± 0.1 mg, which was approximately 15% of the initial dose volume. As the maximum dose retained by the device was 1.5 mg (i.e. equivalent to 1.5 µl) the volume of the LV vector aerosolised using the SAW nebuliser in the subsequent cell culture experiment (Section 8.1.3.3) was set higher at 11.5 µl (10.0 µl + 1.5 µl) to account for the dose expected to be retained in the nebuliser.

Results showed that gene expression obtained on delivering the LV vector through the SAW nebuliser was only 0.16% of bolus delivery (Figure 8-4). Additionally, the number of transduced cells in the bolus group of the present study cannot be compared to the bolus studies performed later in other experiments (Section 5.3.1 and 5.3.3.2), as the titre of the LV vector used in the present study was different to the vector used in the Chapter 5.

Figure 8-4: LacZ gene expression obtained on delivering the LV vector as a bolus dose or as an aerosol using the SAW nebuliser.

8.1.4.2 MADgic™ atomisation device

The efficiency of delivering the LV vector as a coarse spray using the MADgic™ atomisation device was then examined. After aerosolisation of 1 ml of vector formulation, 0.09 ± 0.02 g (i.e. equivalent to 90 µl) of the dose was retained in the MADgic™ atomisation device, i.e. approximately 9% of the dose volume. Unlike the previous study using the SAW nebuliser, no correction for the retained dose was made because delivery volume was far larger (1 ml) than the SAW nebuliser and Aeroneb®Pro nebuliser experiments (10 to 20 µl). Similar levels of gene expression were observed on delivering the LV-LacZ vector through the MADgic™ atomisation device compared to the bolus delivery group (Figure 8-5). This result shows that the MADgic™ atomisation device could be an effective way to deliver a coarse spray of the LV

vector. Similar to the previous study, the number of transduced cells per field of view in the bolus delivery group of the present study was also higher than the bolus delivery group in Chapter 5 (Section 5.3.1and 5.3.3.2).

Unlike the previous study of the SAW nebuliser (section 8.1.4.1), the titre of the LV vector used in Chapter 5 and the present study were the same, and so it was possible to make further comparisons. The difference in gene expression level was due to differences in the time taken to deliver the LV vector onto CHO-K1 cells between experiments reported in Chapter 5 and the present study. The time taken to spray the LV vector through the MADgic™ atomisation was 3 to 4 seconds; as a result, samples from both aerosol and bolus treatment groups were delivered to CHO-K1 cells under 30 seconds. However, time taken to deliver the LV vector through the Aeroneb®Pro-flexiVent™ ventilator circuit was 6 to 7 minutes; therefore, samples from both treatment groups were delivered to CHO-K1 cells under 8 minutes. During aerosolisation experiments, samples from the bolus treatment group were stored at 4° C until used. Higashikawa et al. had shown that the half-life of HIV-based LV vector decreases when stored at 4° C (and above)[64], which could help explain the difference in the number of transduced cells between the bolus treatment group of present study and Chapter 5.

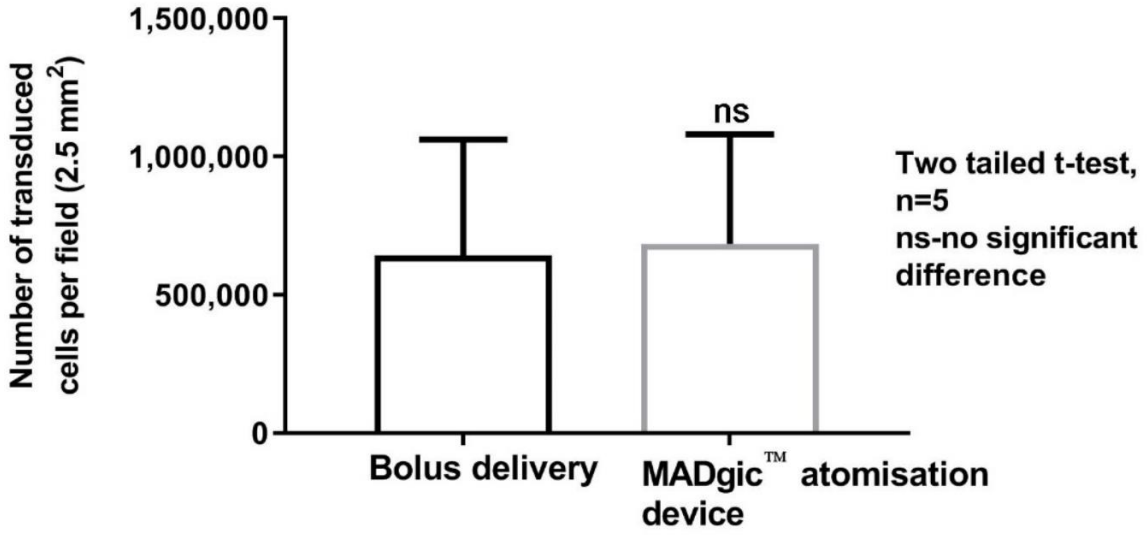

Figure 8-5: LacZ gene expression obtained on delivering the vector as a bolus dose or as a coarse spray using the MADgic™ atomisation device.

8.1.5 Discussion

The characteristics of two additional aerosol delivery devices with potential for delivering a LV vector were examined in this Appendix. The first experiment with the SAW nebuliser identified the volume of initial dose retained by the nebuliser; however, one limitation of this study was that it did not examine whether increasing the initial dose volume compensated for the retained dose losses. Nonetheless, this likely had little impact because the results from the subsequent experiment demonstrated negligible levels of gene expression are achieved after aerosolising the LV vector through the SAW nebuliser compared to bolus delivery (Figure 6-4). Thus, the first hypothesis was not supported. One flaw in the design of this experiment was that there were no replicates for samples reported in the above experiment (Section 8.1.3.3). These preliminary studies were observational and while they cannot be used to uncover factors controlling the level of gene expression observed, the work of others can provide clues to explain the negligible gene expression associated with LV aerosol delivery through the SAW nebuliser. One factor may be increased temperature in the LV vector formulation during SAW operation and aerosol delivery. Jugo et al. showed that aerosolising

a liquid formulation through the SAW nebuliser increased temperature of the formulation to 50˚C[248]; and temperatures well below this level known to inactivate HIV-1 based LV vectors like that used in the present study[64]. Shear stress produced during the transfer of vector formulation from the loaded capillary tube into the adjoining SAW nebuliser section may also be a factor, although shear stresses in the SAW nebuliser are thought to be minimal[79, 238]. A third factor could be that LV vector particles were retained in the paper strip during aerosol delivery. Analysis and quantification of vector retention in the paper strip was not performed in this study but will be an important facet of future SAW nebuliser development for LV vector aerosolisation. Thus, this pilot study showed that the SAW nebuliser in its current form was not effective in delivering viable aerosols of the LV vector for use in mouse studies.

In contrast, the coarse spray produced by the MADgic™ atomisation device demonstrated similar levels of gene expression to bolus delivery. This result supports the second hypothesis and was consistent with what is known about the MADgic™ atomisation device. The device probably imposes minimal shear stress, so the sprayed LV vector would remain largely viable. The size of the aerosols produced by this device (30–100 µm) would also have played a role. Delivering aerosols of a larger size may have reduced the number of vector particles on the surface of the aerosol that were exposed to shear stresses or other factors affecting viability. However, the effect of aerosol size on biological viability of LV vector, even through other aerosol delivery devices, remains unknown. As the MADgic™ atomisation device was recently introduced for use in gene vector delivery studies, researchers are yet to fully assess the efficiency of this device in delivering viral vectors.

Chapter 5 showed that suspending the LV vector in protective diluent improved levels of LV gene expression following delivery through the Aeroneb®Pro nebuliser (Section 5.3.3). Thus,

similar experiments could be conducted to further increase efficiency of the MADgic™ atomisation device in delivering the LV vector. Cmielewski et al. demonstrated that the viability of a HIV-based LV vector was improved during transit through a 15 mm tube by priming the tube with bovine serum albumin[56]. Thus, future studies could also examine the effectiveness of priming the MADgic™ atomisation device (or any other device identified to effectively aerosolise LV vector) with protective agents, such as FreeStyle™ medium.

A major limitation of the MADgic™ atomisation device for gene therapy research development is that it requires a minimum of 1 ml of fluid formulation to deliver a coarse spray of aerosols, which restricts the use of this device in small animal models such as mice. The large size of aerosol spray delivered by this device (30–100 µm) would not be suitable for aerosol delivery to the airways of mice. This is because the size of aerosols in studies using mice should lie between 0.5 to 5 µm to achieve effective deposition in mice airways and lungs[249]. However, this device could be used to deliver vector to the airways of larger animal models, such as pigs and sheep, where larger volumes are essential to reach sufficient portions of the airway or lung; the literature suggests volumes from (0.45 to 5 ml) would be needed[103, 140, 233]. In a recent gene therapy study, Cooney et al. instilled 2 ml of FIV vector to the airways of newborn pigs using the MADgic™ atomisation device[87]. The volume of aerosolised vector formulations used in the airways of patients in past CF gene therapy trials varied between 1 to 5 ml, depending on the type of vector, nebuliser, concentration of the vector formulation, patient lung capacity, and so on[92, 93, 250]. As the MADgic™ atomisation device can deliver up to 5 ml of formulation[247] it has the capacity to be used in coarse spray delivery of LV vector for future clinical trials, and hence warrants further investigation.

8.1.6 Conclusion

The ability of two novel aerosol delivery devices to deliver viable LV vector was examined in this Appendix. Results from this preliminary study show that in its current form the SAW nebuliser does not effectively aerosolise the LV vector formulation in an *in vitro* setting, despite previously being successfully used for aerosolising other biological formulations, such as pDNA[79] and stem cells[80]. While these findings resulted in this nebuliser not being used in further *in vivo* LV vector delivery studies in this thesis, it is a novel aerosolisation method with potential still to be fully realised. The MADgic™atomisation device, in contrast, provided levels of gene expression very similar to that of bolus LV delivery. To the knowledge of the author, this is the first *in vitro* study to demonstrate effective delivery of a HIV-based LV vector using the MADgic™ atomisation device and the success suggests further *in vitro* and *in vivo* studies are warranted to better understand the benefits and limitations, and to optimise LV aerosol delivery in animals.

8.2 Image analysis – MATLAB script

The levels of gene transduction in a 12-well or 24-well plate were quantitated using images taken at 40x or 100x magnification via stereo-microscope. The characteristic blue colour observed in each well of the cell culture plate is indicative of successful gene expression by the LV-LacZ vector. The intensity of blue colour and number of cells present on the cell culture plate producing that colour was quantitated using the MATLAB script given below.

```
start_path = 'L:\Equipment Data\Microscope\Harsha\';
input_format = 'jpg';
output_format = 'jpg';
Hmin = 0.4;      % Colour (ie blue)
Hmax = 0.6;
Smin = 0.15;     % How saturated the colour is
Vmax = 1;       % How bright it is
numpixels = 5;    % Number of pixels in cluster required for detection
markerSize = 5;    % Size of the marker used to show identified cells
```

```
% Load the blank file
[blank_name,blank_path] =
uigetfile([start_path,'\*.',output_format],'Select the blank image');
blank = imread([blank_path,'\',blank_name]);

% Get the folder to process
folder_name = uigetdir(start_path,'Select the folder containing the files
to process');
files = dir([folder_name,'\*.',input_format]);

% Process each image
for i = 1:length(files),

  % Load the input file
  input_name = files(i).name
  input = imread([folder_name,'\',input_name]);

  % Perform the normalisation with the blank image
  normalised = double(input)./double(blank);

  % Convert from RGB to HSV color space
  hsv_image = rgb2hsv(normalised);

  % Select regions of the image that meet the Hue and Saturation max/min
criteria
  BW = (hsv_image(:,:,2) > Smin) & (hsv_image(:,:,1) > Hmin) &
(hsv_image(:,:,1) < Hmax & (hsv_image(:,:,3) < Vmax));

  % Clean up the results
  BW = bwareaopen(BW, numpixels);

  % Mark the detected cells
  STATS = regionprops(BW, 'Centroid');

  if ~isempty(STATS),
  im =
insertMarker(normalised,cat(1,STATS.Centroid),'x','Size',markerSize,'Color'
,'red');
  end

  % Count the number of cells
  cells = num2str(length(STATS));
  proportion = num2str(sum(sum(BW)) / (size(BW,1) * size(BW,2)) * 100);

  % Display images and save the output image
  figure(1), imshow(input); title('Input');
%   figure(2),imshow(hsv_image(:,:,1)); title('Hue'); impixelinfo
%   figure(3),imshow(hsv_image(:,:,2)); title('Saturation'); impixelinfo
%   figure(4),imshow(hsv_image(:,:,3)); title('Value'); impixelinfo
  figure(5),imshow(BW); title('Mask');
%   figure(6),imshow(BW); title('BW')
  figure(7),imshow(im);
  titletext = ['Detected ', cells, ' cells (', proportion, '% area)'];
  title(titletext);

  % Save the output image
  imwrite(im,[folder_name,'\',input_name(1:length(input_name)-4),' -
',titletext,'.',output_format]);
```

```
end
```

8.3 Plasmid map of pHIV-MPSV-nlsLacZ

The plasmid map of pHIV-MPSV-nlsLacZ used in this thesis is shown in Figure 8-6.

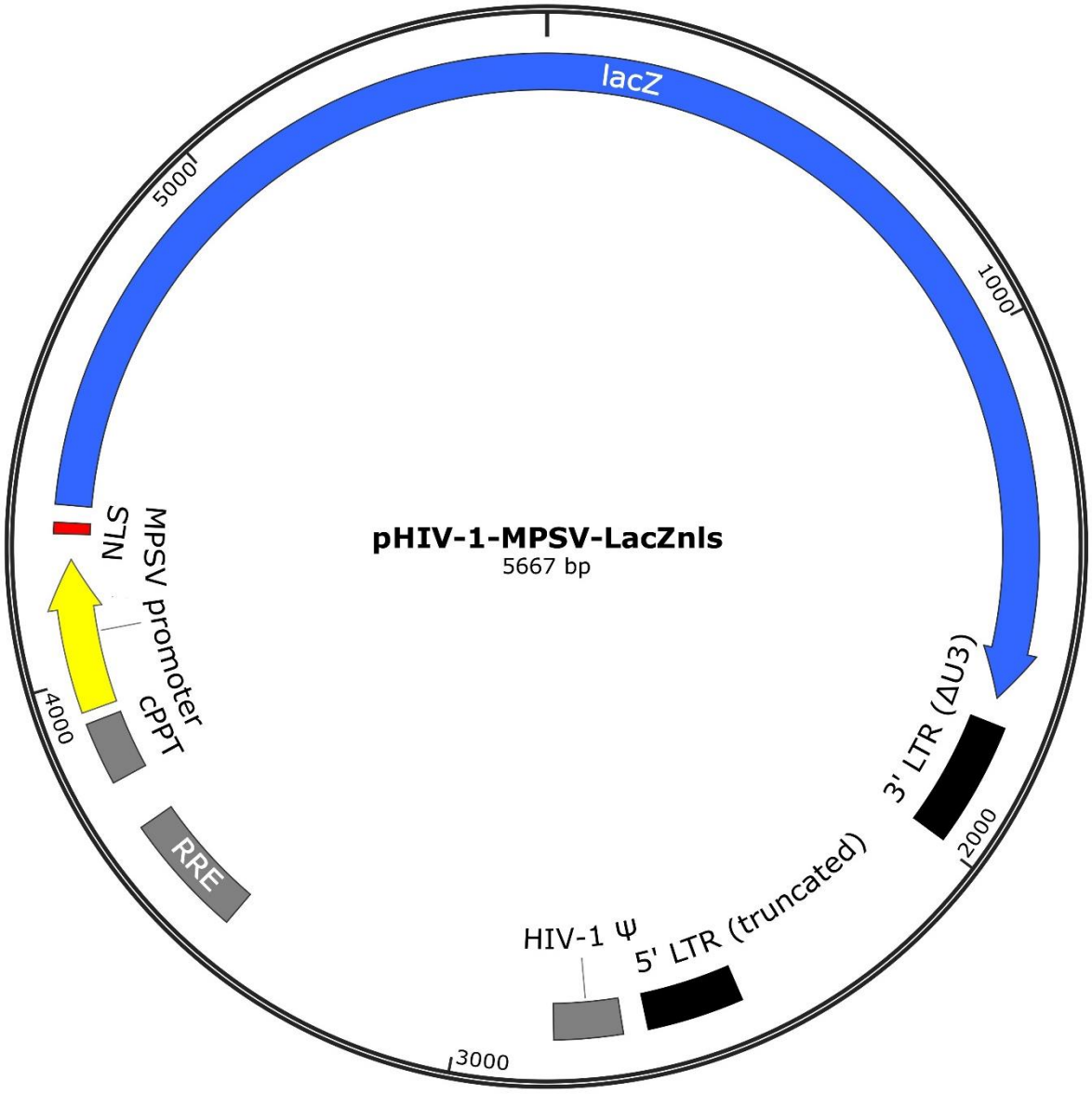

Figure 8-6: Plasmid map of pHIV-MPSV-nls LacZ.

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