An *in vitro* hybrid method used to model realistic airway deposition and post-deposition behaviour for orally inhaled products

A thesis submitted to fulfil requirements for the degree of Master of Philosophy

by

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This is to certify that to the best of my knowledge, the content of this thesis is my own work. This thesis has not been submitted for any degree or other purposes.

I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

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THESIS AUTHORSHIP ATTRIBUTION STATEMENT

Chapter 2 of this thesis has been accepted as a conference paper in the Respiratory Drug Delivery Europe 2017, Book 2 as:

Validating a Realistic Physical Airway Model to Investigate *In Vitro* Drug Deposition, Dissolution, and Epithelia Transport of Orally Inhaled Products

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Dale R. Farkas, Worth Longest and Michael Hindle contributed in this conference paper by supplying us the CAD airway models for us to 3D print and use in the study. I designed the study methodology and was responsible for the experimental work, analysis of the data and preparing the manuscript.

Original copy of conference paper is attached in Appendix (Attachment 1).

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The Development and Validation of an *In Vitro* Airway Model to Assess Realistic Airway Deposition and Drug Permeation Behaviour of Orally Inhaled Products

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Original copy of journal article is attached in Appendix (Attachment 2).

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ABSTRACT

This research focuses on the development and validation of a new *in vitro* test method capable of characterising realistic airway deposition of inhaled pharmaceutical aerosols, and the events following deposition at the airway epithelia. An existing physical model of a medium-sized Virginia Commonwealth University (VCU) mouth throat (MT) and tracheal bronchial (TB) upper airway was modified and validated to integrate Calu-3 monolayers cells cultured onto Snapwell® inserts for the collection of deposited drug. The model was housed in a chamber and validation was carried out using two nebulised ciprofloxacin formulations: a controlled-released encapsulated liposomal solution termed "Ciprofloxacin for Inhalation" (CFI), and an un-encapsulated "free" solution termed "Free Ciprofloxacin for Inhalation" (FCI).

Validation was carried out to ensure modifications to the airway model resulted in sufficient collection of drug for analysis, whilst ensuing minor changes to *in vitro* airway deposition behaviour. Studies were carried out to assess how experimental variables influenced *in vitro* drug permeation behaviour, i.e., choice of membrane material, formulation type and aerosol collection mechanism. Most importantly, the integrity of the barrier formation and tight junction functionality of the monolayers were assessed post airway deposition to evaluate if dynamic stresses caused from the airflow could affect *in vivo* drug transport behaviour.

In conclusion, a novel hybrid *in vitro* test method has been extensively validated to successfully characterise realistic airway deposition of two different nebulised solutions of ciprofloxacin drug. It is hoped that this model can be used by others as a potential screening tool for head-to-head comparative studies without resorting to expensive clinical trials, as well as a foundation for the design of new inhalation platforms with improved performance efficiency and reproducibility.

SYMBOLS AND ABBREVIATIONS

µ - Micro

°C – degrees Celsius

ABS – Acrylonitrile butadiene styrene

ACI – Anderson cascade impactor

AIT – Alberta idealised throat

ALI – Air-liquid interface

ANOVA – Analysis of variance

API – Active pharmaceutical ingredient

ATCC – American Type Culture Collection

CFD – Computational fluid dynamics

CFI – Ciprofloxacin for Inhalation

COPD – Chronic obstructive pulmonary disease

CT – Computed tomography

DMEM – Dulbecco's Modified Eagle's medium

DPI – Dry powder inhaler

e.g. – For example

et al – and others

EMA – European Medicines Agency

EVOM – Epithelial volt-ohmmeter

FCI – Free Ciprofloxacin for Inhalation

FDA – Food and Drug Administration

Flu-Na – Fluorescein-Sodium

FPF – Fine particle fraction

GSD – Geometric standard deviation

HBSS – Hank's Balanced Salt Solution

HFA – Hydrofluoroalkane

HPLC – High performance liquid chromatography

i.e. – That is

IP – Induction port

IVIVC – In vitro – in vivo correlations

kPa – kilopascal

L/min – Litres per minute

LCC – Liquid covered culture

MMAD – Mass median aerodynamic diameter

MOC – Micro orifice collector

MSLI – Multi-stage liquid impinger

MT – Mouth-throat

NGI – Next generation impactor

OIP – Orally inhaled product

PA – Polyamide

P_{app} – Apparent permeability coefficient

PBS – Phosphate buffered saline

PE – Polyester

PET – Position emission topography

pMDI – Pressurised metered dose inhaler

Ph. Eur – European Pharmacopoeia

RH – Relative humidity

SD – Standard deviation

SMI – Soft Mist Inhaler

SPECT – Single photon emission computed topography

TB – Tracheal-bronchial

TEER – Trans epithelial electrical resistance

UFLC – Ultra-fast liquid chromatography

USP – United States Pharmacopeia

VCU – Virginia Commonwealth University

VMD – Volumetric median diameter

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CHAPTER 1

Current and Emerging *In Vitro* Test Methods for Orally Inhaled Products

To date, the development of *in vitro* test models used to assess the drug delivery characteristics of orally inhaled products (OIPs) has become a growing research trend in pulmonary drug delivery. With the development of novel drug formulations, coupled with advances in inhaler technology design, there are a range of compendial *in vitro* test apparatuses currently listed in the Pharmacopoeias intended for quality control testing of pharmaceutical inhalation products. Recently, such methods have been widely adapted to resemble *in vivo* circumstances, in attempt to predict airway deposition behaviour and estimate the events following airway deposition *in vivo*, i.e., drug permeation, metabolism and potential drug interferences at the airway epithelia. Developing robust, discriminative *in vitro* test methods capable of predictive outcomes is highly desirable; however, it has been made apparent that extensive validation is required to make predictive statements.

This chapter fundamentally describes the development and advances of *in vitro* test methods and airway models used to enrich pulmonary drug delivery research. Principle decisions to find a suitable *in vitro* tool for the question being asked, i.e., from a quality control or predictive perspective are discussed, as well as their associated advantages and limitations.

1.1 Introduction

The delivery of pharmaceutical medication via the pulmonary route offers significant advantages over systemic delivery, such as oral or intravenous administration. These advantages include, though are not limited to, the following key aspects: the ability to administer smaller doses directly to the lung, consequently mitigating adverse side effects [1, 2]. The anatomical and histological features of the airways, i.e., large surface area for drug deposition in the lung, good vascularisation and thin airway epithelial lining renders rapid drug absorption, hence making pulmonary drug delivery a promising alternative to treat both local and systemic disease [3, 4]. The non-invasive nature of drug administration directly to the lung makes it a safer and more convenient alternative compared to intravenous administration of medication.

The clinical response of inhaled drugs is strongly dependent on the total lung deposition and the associated distribution of the deposited dose throughout the region of the lung [5]. Deposition of inhaled aerosols throughout the lung could be understood by considering both the physical airway geometry and deposition mechanisms. Fundamentally, there are three main deposition modes which govern lung deposition: inertial impaction, gravitational sedimentation, and to a lesser extent, diffusion [6]. A schematic representation of these deposition mechanisms is illustrated in Figure 1. The air velocity in the oropharynx and large upper airway (including the trachea and bronchi) is relatively high compared to the lower airways, and so larger aerosols, typically greater than 5 μm in aerodynamic diameter, are likely to deposit at these regions, as well as the airway bifurcations due to their inability to follow the changes in air flow direction of the inspired air stream. Consequently, this may lead to suboptimal treatment outcomes and/or adverse effects [7, 8]. Smaller particles within the 1 to

5 µm aerodynamic size range have less inertia than larger particles immitted from a inhaler and are likely to follow the air flow direction and pass further downstream from the oropharynx and upper regions of the lung, possibly depositing in the bronchioles. At these regions, the air velocity is relatively low and deposition is likely to occur via gravitational sedimentation [6]. Transitioning downstream are the terminal bronchioles and alveolar region, where air velocity is negligible and deposition of very fine aerosols (less than 1 µm in aerodynamic diameter) typically occurs in a randomised fashion via Brownian diffusion.

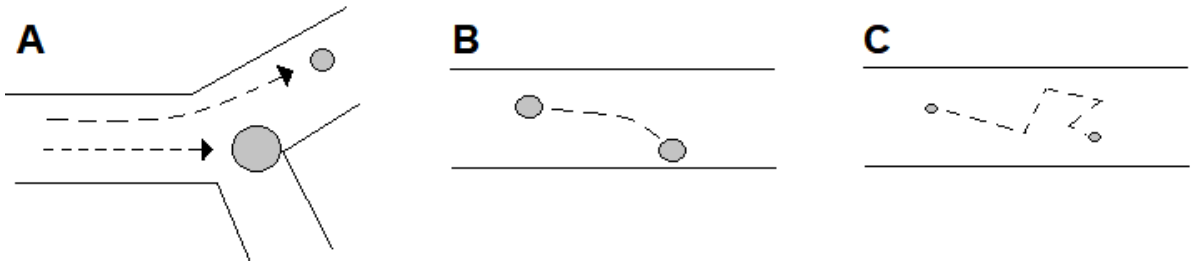

Figure 1. Schematic representation illustrating the main deposition mechanisms occurring at different regions within the airway: A) inertial impaction of larger aerosols at the upper airways and branched airway bifurcations, B) gravitational sedimentation of aerosols at the bronchiole regions, and C) random Brownian diffusion at lower airway regions.

Optimal drug delivery to the lung for the treatment of local diseases such as asthma or chronic obstructive pulmonary disorder requires successful and consistent generation of aerosols within the respiratory size range: 1 to 5 µm aerodynamic diameter [7, 8]. Aerosols are generated from orally inhaled products (OIPs), which are a class of medical device product constituting of a drug formulation with one or more active pharmaceutical ingredients (API), with or without added excipients, delivered in either one of four different inhaler device platforms: the pressurised metered dose inhaler (pMDI), the dry powder inhaler (DPI), the nebuliser, and the more recently developed soft mist inhaler (SMI) [9-11]. With differing device designs,

formulation types and aerosol generation mechanisms, all inhaler platforms strive to generate aerosols within the respiratory size range. Table 1 shows a list of commercial inhaler platforms illustrating structural differences. Most nebuliser, pMDI and SMI platforms are formulated as solutions or suspensions that reside within the inhaler platform, and upon device actuation, generate fine aerosol droplets from the inhalers' mouthpiece. On the other hand, DPIs consist of a dry powder either formulated in pure-drug (in micronised or as a spray dried form), or with a lactose carrier excipient to improve powder flowability upon device actuation. The lactose excipients are added to increase the dosing accuracy and minimise dose variability seen with most DPI formulations [12]. The breath-actuated DPIs rely on the patients' respiratory effort to entrain powder from the device and break up the bulk formulation into finer particles suitable for inhalation while pMDI, DMI and nebulisers are active devices that impart aerosolisation energy to achieve a fine aerosol.

Table 1. List of some commercial inhaler platform devices intended to administer pharmaceutical aerosols to the lung.

Pressurised metered dose inhaler (pMDI)	- Ventolin® (suspension formulation, GlaxoSmithKline) - Flovent® (suspension formulation, GlaxoSmithKline) - QVAR® (solution formulation, Teva)
Dry powder inhaler (DPI)	- Pulmicort Turbuhaler® (carrier-free, AstraZeneca) - HandiHaler® (capsule, Boehringer Ingelheim)
Nebuliser	- Microair® NE-U22 (Mesh, Omron) - PARI LC® Sprint Plus, (Mesh, PARI)

Soft mist inhaler (SMI)	- Respimat® Soft Mist (Boehringer Ingelheim)

With current advances in aerosol formulation and inhaler device technology, the clinical performance and drug delivery efficiency of aerosols suitable for inhalation typically relies on the multifaceted interactions between the formulation characteristics and dispersion mechanisms. Therefore, assessing the extent of total lung deposition *in vivo*, and the distribution patterns of inhaled aerosols throughout the airway following device actuation, more so for locally-acting asthmatic drugs such as bronchodilators and anti-inflammatory agents, should theoretically predict their clinical effects, both beneficial and adverse [13, 14]. For instance, the distal small airways are the major site of air flow obstruction for patients suffering from chronic obstructive pulmonary disease; therefore, a lack of drug administered to this region could potentially contribute to poor disease control [15, 16].

Overall, this chapter provides an integrated overview of the past, current and emerging *in vivo* and *in vitro* approaches used to evaluate the delivery performance of inhaled aerosols generated from OIPs, and its rationale in the perspective of:

- Traditional radionuclide imaging techniques;
- Stringent regulatory requirements for quality control practices;
- Fundamental principles to improve and evaluate aerosol drug delivery systems;
- Means to establish robust *in vitro – in vivo* correlations (IVIVCs); and,
- Predicting the pulmonary fate following lung deposition.

1.2 Non-invasive radionuclide imaging techniques

Traditional means to quantify *in vivo* lung deposition behaviour of inhaled aerosols have typically relied on non-invasive radionuclide imaging techniques, which can range from conventional two-dimensional gamma scintigraphy techniques, to more advanced three-dimensional approaches such as single photon emission computed topography (SPECT) and positron emission topography (PET) [17]. Broadly speaking, such imaging techniques have proved to be a valuable screening platform for clinicians and researchers, providing information on the extent of lung deposition and regional distribution patterns in the oropharynx, whole lung (central, intermediate and peripheral zones) and the residual amount residing in the inhaler following dosing [18-21]. Such information has been used to make predictive statements regarding the pulmonary fate of the deposited aerosols, i.e., whether the deposited drug is absorbed from, cleared, or retained in the lung tissue, thereby giving information on the therapeutic response on the treatment regime.

However, making predictive statements regarding the clinical response of inhaled aerosols based purely on *in vivo* lung deposition data is not straight forward. Firstly, radiolabelling of the drug formulation is technically challenging, and extensive *in vitro* validation is required to ensure no significant changes in formulation composition during the radiolabelling processes would affect drug deposition behaviour in the lung [22-24]. Furthermore, the radiolabelled marker must act as an adequate marker across the full range of generated aerosol sizes. Secondly, the amount of radioactive material must be low to minimise any risks associated with high radiation doses and consequently, images may suffer from poor spatial resolution [25]. *In vivo* studies are also expensive, time consuming and suffers from poor discriminatory power due to inter-subject variabilities, i.e., inhaler technique/usage and anatomical differences, make it difficult for quick and discriminative head-to-head comparisons between

different inhaler platforms and formulation types. For these aforementioned reasons, it is desirable to establish simpler and quicker test alternatives that are cost and time efficient, robust and sufficiently discriminative that can be used for comparative purposes, and/or make predictive statements on the clinical performance on the selected treatment regimes.

Compared to imaging techniques, *in vitro* tests offer greater flexibility and control over experimental conditions in assessing how critical parameters such as formulation composition, or flow rate can affect overall delivery performance during the early product development stages. To provide such context, several different *in vitro* methods have been proposed, with strengths and weaknesses used to investigate lung deposition behaviour [26-28], and to an extent been developed to estimate the fate of inhaled aerosols following lung deposition i.e., drug dissolution, permeation and transport across the airway epithelia [29-31].

1.3 Regulatory requirements for orally inhaled products

The United States Food and Drug Administration (FDA) and the European Medicines Agency (EMA) have drafted several guidance documents intended to evaluate the overall product quality and delivery performance of new OIPs prior to market approval [32]. Overall, it is essential for manufacturers and/or developmental scientists to show that the inhaler platform is capable of delivering drug efficiently and reproducibility to the patient, with two essential parameters assessed: the emitted dose and the aerosol size distribution of the emitted dose [33-36]; the former is an indicated of patient delivered dose and the later, regional lung deposition. The United States Pharmacopeia (USP) and the European Pharmacopoeia (Ph. Eur) monographs have explicitly outlined a series of mandatory *in vitro* test procedures using standardised protocols, all which needs to meet specified acceptable limits before approval into the market [33-36]. Furthermore, such *in vitro* methods can be used to make head-to-head

comparisons between existing products, and/or used to demonstrate therapeutic equivalence between generic and reference products, provided satisfactory and comparative *in vitro* results [37].

1.3.1 Delivered dose uniformity content

For OIPs, the delivered dose uniformity content of the API and/or excipients present in a single delivered dose is to be evaluated across multiple batches to ensure consistent and reproducible dosing [38, 39]. Furthermore, testing of multiple or consecutive doses may be required from regulatory agents if stated otherwise. Satisfactory criterial is met from the USP monograph if: *" the dosage uniformity are met if not less than 9 of the 10 doses are between 75 % and 125 % of the label claim, and none is outside the range of 65% to 135% of the label claim"* [39]. Failure to comply to such specifications will result in failure to register and launch the product to market.

It is important to note that testing conditions and specified general requirements listed in the monographs do differ between different inhaler platforms. For instance, it is required to test pMDIs from the mouthpiece at a fixed air flow rate of 28.3 L/min, whereas for DPIs, depending on the intrinsic resistance of the inhaler [40, 41], the sampled air flow rate should be selected such that it generates a differential pressure drop of 4.0 kPa across the inhaler, tested at a duration to withdraw 4.0 L of air [36]. Therefore, it is up to the manufacturer and developmental scientist to become acquaint with the regulatory guidelines to ensure that the selected test method is suitable for their final product. Additionally, dose uniformity testing is to be conducted throughout its' container life, and simulated under different patient-usage conditions, i.e., a range of inhalation flow rates, and for (example for pMDIs) such testing should be conducted under simulated conditions, i.e., shaking of the canister, or sampled with

add-on devices (spacers and holding chambers). Potential issues pertaining to inconsistent dosing, such as lower-than-expected dosing may be associated with a decrease in therapeutic benefit, or higher-than-expected dosing may lead to an increase in unwanted side-effects, both being problematic from a safety and efficacy view point.

1.3.2 Aerosol size and lung deposition behaviour

Not only is dosing consistency a necessity from a regulatory stand point, the ability to demonstrate information pertaining the extent of drug deposition and distribution in the lung is one key characteristic that should be assessed thoroughly for OIPs. The aerodynamic size distribution of aerosols generated from the inhaler mouthpiece is an important parameter which is required from regulatory agents, and according to the Pharmacopeia guidelines *"may be expected to correlate with the drug dose or that fraction of the drug dose that penetrated the lung during inhalation"* [36].

A number of Pharmacopoeia monographs have specified a list of *in vitro* apparatuses used to characterise the emitted dose in terms of aerodynamic diameter in order to obtain relevant sizing profiles [39, 42]. Similarly, with delivered dose uniformity, such sizing determinations needs to be conducted from a single dose, or multiple doses (if stated otherwise), sampled across multiple batches, tested throughout its container life, and simulated under different patient-usage conditions [39, 42]. Inconsistent sizing profiles may result in poor treatment outcomes particularly for locally-acting drugs, since size dictate where the aerosol deposit in the lung.

1.3.2.1 Integrating impingers and cascade impactors into the monographs

A range of *in vitro* sizing apparatuses currently exist in numerous monographs. Shown in Figure 2, these devices range from the simple two-stage glass twin impinger (in the earlier British Pharmacopoeia), to the more advanced, higher resolution apparatuses, present in both USP and Ph. Eur. Monographs: the multi-stage liquid impinger (MSLI), the eight-stage Andersen cascade impactor (ACI), and the most recently developed seven-stage next generation impactor (NGI). All differing in designs, test specifications and tolerances, each apparatus consists of a series of stages, with stage consisting of a nozzle plate with one or more jet orifices directly positioned above an impaction plate for drug collection [43, 44]. Figure 3 illustrates a schematic representation of the ACI demonstrating its operating principle. Upon testing, the emitted dose is sampled across the sizing apparatus at a constant air flow rate, where the dose is separated into different size fractions, depending on the inertia of the generated aerosols, i.e. aerodynamic diameter and aerosol velocity. Larger aerosols carrying greater inertia will break away from the air stream and deposit onto the impaction plate, while smaller aerosols will remain entrained in the air stream and pass through to the next consecutive stage, potentially impacting on the following surface plate. Each succeeding stage will remove a fraction of the emitted dose, with each stage arranged in an order of decreasing cut-off size. Principally, this is thought to resemble the deposition behaviour of aerosols in the lung, with larger aerosols depositing at the upper stages, i.e., oropharynx and large upper airways, and smaller aerosols depositing at the lower stages, i.e., bronchioles and alveolar regions.

Figure 2. List of compendial apparatuses used for routine sizing characterisation, showing the: A) Two-Stage Liquid Impinger, B) Multi-Stage Liquid Impinger, C) Andersen Cascade Impactor, and D) Next Generation Impactor [45].

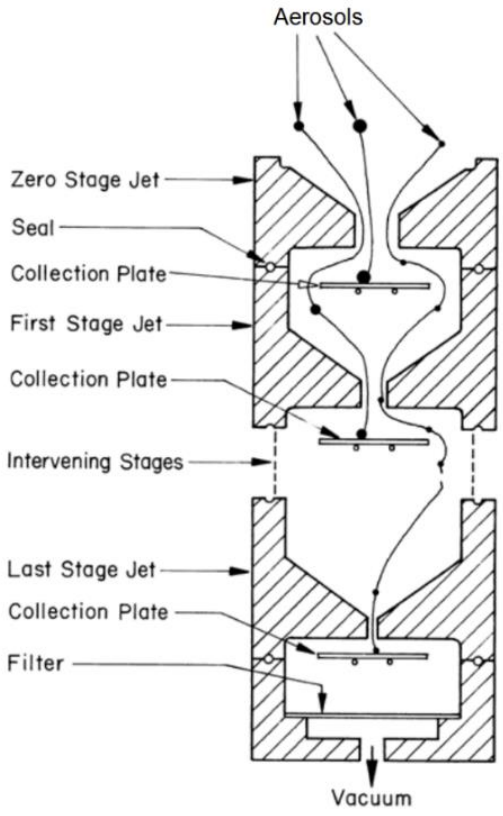

Figure 3. A schematic illustration showing the operating principle and separation of aerosols via inertial impaction within the Anderson Cascade Impactor. Image from source: [36].

The two-stage glass twin impinger operates at a calibrated, constant air flow rate of 60 L/min, which separates the aerosols at a pre-defined cut-off aerodynamic diameter of 6.4 µm. Given the reduced resolution in the size distribution within the "respirable size" range, the twin stage impinger may not provide sufficient information or discretionary power for adequate head-to-head comparison. Regulatory insistence on improved separation apparatuses to obtain additional resolution within this size range have been advocated [33-36].

The five-stage MSLI, calibrated at a constant air flow rate of 60 L/min separates the delivered dose into the following aerodynamic cut-off size diameters: stage 1 – 13.0 µm, stage 2 – 6.8 µm, stage 3 – 3.1 µm, stage 4 – 1.7 µm, and the filth stage being the filter stage captures finer aerosols less than 1.7 µm [46]. The ACI is calibrated at a constant air flow rate of 28.3 L/min, separating the emitted dose into different size fractions ranging from 0.4 to 10.0 µm [36]. Since monographs specifically state pMDIs to be tested at 28.3 L/min, the ACI is an ideal test device for pMDI size evaluations; however, this is rather restrictive for DPIs testing since the selected flow rate depends purely on the intrinsic resistance of the DPI, which may not necessarily be targeted at 28.3 L/min [47, 48]. To resolve this dissatisfaction, the monographs have suggested to "*omit Stage 6 and Stage 7*" from the ACI impactor when sampling at air flow rates greater than 60 L/min [36, 42]; however, Kamiya *et al* have reported significant wall losses in the modified ACI, resulting in drastic shifts in size profiles [49]. Discussions amongst the pharmaceutical industry and scientists about the inadequacies pertaining current impingers/impactors at the time had led to the development of the NGI. The NGI was designed to sample aerosols at a wider range of calibrated air flow rates, i.e., from 15 to 100 L/min, making it ideal for nebuliser testing at very low air flow rates, to DPI sampling at higher flow rates [50-52].

1.3.2.2 The development of the USP/Ph. Eur Induction Port

With the array of different impingers and impactors listed in the monographs, the sizing profiles generated are fundamentally dependent on the overall design of the test apparatus, and the selected air flow rate. To ensure consistent sampling of the emitted dose into the test apparatus, the development of the USP/Ph. Eur induction port (IP), which is a simple, well-defined right-angle bend serves as a standardised interface between the inhaler mouthpiece and the entrance of the test apparatus during routine testing of OIPs [36, 42]. A schematic illustration of the compendial USP/Eur IP used in conjunction with the NGI is shown in Figure 4. Additionally, this inlet is thought to resemble the oropharyngeal region, hence mass deposition on the USP/Eur IP is thought to resemble deposition in the oropharynx, *in vivo*.

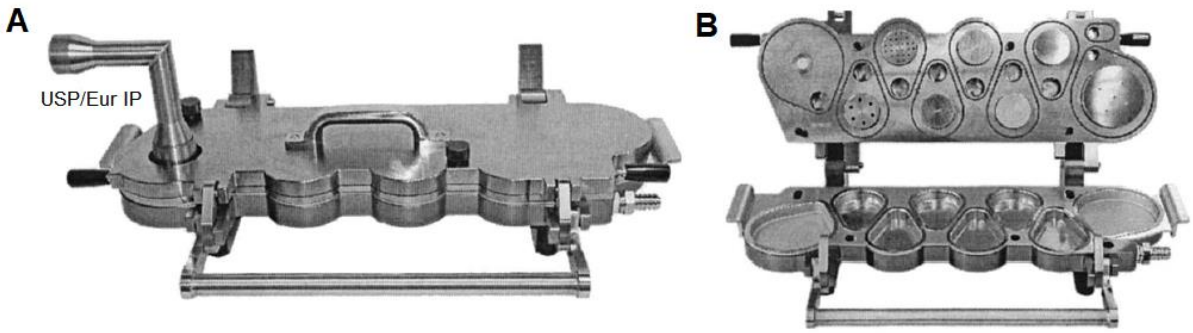

Figure 4. An illustration of the Next Generation Impactor used in conjunction with the USP/Eur Inlet Port, operated in A) closed form, and B) open form. Images from source: [50].

1.3.2.3 Can derived-sizing parameters be used for predictive aerosol performance?

The primary advantage of these sizing apparatuses is that direct mass measurements of the API can be quantified on each consecutive stage, using chemical assaying techniques such as high-performance liquid chromatography (HPLC). Following mass quantification, critical sizing parameters can be derived from the generated aerodynamic size distribution profile. The most critical parameters commonly used for comparison purposes are the mass median aerodynamic diameter (MMAD), which is defined as the central tendency of the generated size distribution,

and the fine particle dose (FPF) and fraction (FPF), typically defined as the mass or fraction of the size distribution that is less than 5 μm, the fraction that resides within the optimal "respiratory size range" for inhalation products. Accurate determination of the aerodynamic size distribution profiles in impactors are paramount since it is thought to be the metric most indicative of lung deposition and thus *in vivo* outcomes. However, it is important to note that such impactors are limited to fractioning aerosols by aerodynamic diameter and do not incorporate realistic anatomy. Thus, questions such as "can induction port deposition be translated to make statements pertaining oropharyngeal deposition?" or "can the FPF be used to estimate the portion of the emitted dose that is likely to reach the smaller airways? " need to be asked. Overall, these derived parameters have been used fruitfully within the pulmonary research field to investigate and improve the device design and formulation properties to increase overall delivery performance for better treatment outcomes, however direct relationships to predicted *in vivo* should always be considered with care.

1.4 Establishing *in vivo- in vitro* methods for predictive estimates

Intended for routine testing and quality control assessment, it is important to realise that impactor-based sizing apparatuses are not surrogates of the human airway, and that the operating conditions are overly-simplified, i.e., sampling the delivered dose at a constant flow rate as opposed to a variable inhalation profile seen with patients inhaling through their inhalers. Until recently, these Pharmacopoeial methods have been adopted and further refined as *in vitro* predictive models by mimicking test conditions that closely resemble *in vivo* circumstances [26, 31, 53, 54]. Ideally, if an *in vitro* test method could be established and standardised to predict *in vivo* behaviour for inhaled aerosols, such test methods could be utilised as a vital screening tool during the early product development stages.

1.4.1 Developing physical realistic airway models

With the USP/Ph. Eur IP developed primarily to ensure consistent sampling of the emitted dose into the size apparatus during routine testing, it is apparent that its simplistic design cannot act as a physical surrogate used to mimic oropharyngeal deposition behaviour seen *in vivo* [55-58]. To address this issue, researchers from the University of Alberta constructed the anatomically-relevant Alberta Idealised Throat (AIT) airway model cast, derived from an extensive database of computed tomography (CT) scans. The AIT was developed as a representative interface between the inhaler mouth-piece and the sizing apparatus for routine testing, as well to provide better air flow realisation in the oropharynx region [28]. Figure 5 shows the structural differences between the AIT and USP/Eur IP models. The AIT is commercially available and is manufactured in aluminium.

Figure 5. The Alberta Idealised Throat (left, in open form) and the USP/Ph. Eur induction port (right) [28].

1.4.1.1 The USP/Eur IP underestimates oropharyngeal deposition behaviour seen *in vivo*

Comparative *in vitro* studies reported higher deposition behaviour in the AIT model to the compendial USP/Ph. Eur IP for a variety of commercial pMDI and DPI products [28]. The complex geometry of the AIT is thought to capture the coarser aerosols from the emitted dose, resulting in better correlation with mean *in vivo* lung deposition data obtained from gamma scintigraphy studies compared to the compendial USP/Ph. Eur IP [59, 60]. By integrating patient-related factors, the development of the AIT has highlighted the potential applicability in establishing more representative *in vitro* test methods used not only for routine testing for OIPs, but also for predictive measures on product delivery performance.

1.4.1.2 Capturing the *in vivo* inter-subject variability

For inhaled aerosols, large *in vivo* inter-subject variability has been reported from gamma scintigraphy lung deposition studies, with variations ranging from 20.0 – 60.0 % of the total metered dose deposited in the lung for a single test inhaler [61]. As highlighted earlier, while predicting and correlating mean *in vitro* lung deposition data to mean *in vivo* gamma scintigraphy outcomes could be established, given appropriate *in vitro* tools, of equal importance is the ability to predict the inter-subject variations that are due to apparent differences in airway geometries and inhalation manoeuvres [53].

To explore this concept further, researchers from the Virginia Commonwealth University (VCU) developed a "medium-sized" physical realistic upper airway model of the mouth-throat (MT) and tracheal-bronchial (TB) region to study realistic airway deposition *in vitro* [26]. The medium-sized MT model was constructed from CT scans based on a healthy individual adult [62], while the upper TB model was developed based on anatomical cast dimensions reported by Yeh *et al* [63], and for practical purposes, only extends from the trachea down to the third

airway bifurcation. To capture the morphological variations seen *in vivo*, the medium MT-TB airway model was volumetrically scaled to generate "small" and "large" versions of the upper airway in order to capture the mean and "extreme" anatomical ranges within a healthy adult population (Figure 6) [26]. By coupling these realistic physical airway models with representative inhalation profiles acquired from healthy trained patients using a breath simulator, the researchers were able to successfully capture this *in vivo* inter-subject variability in terms of lung deposition for a range of commercially available inhalers [26, 27].

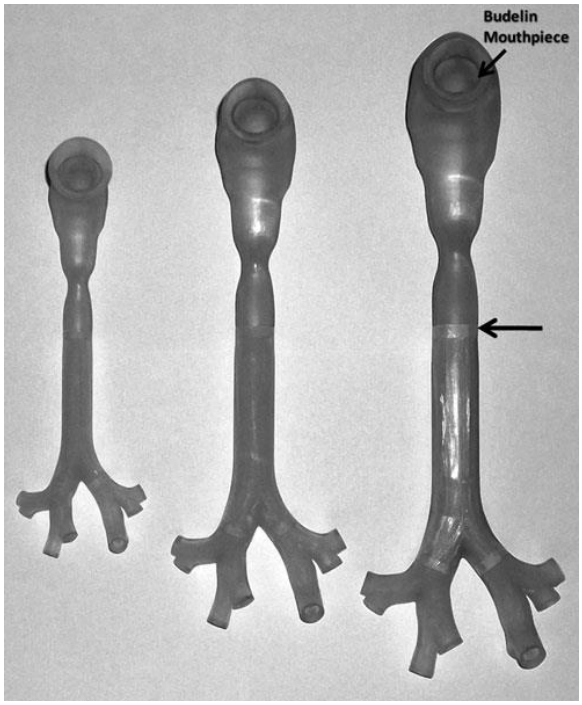

Figure 6. The small (left), medium (middle), and large (right) Virginia Commonwealth University mouth-throat and tracheal-bronchial upper airway models. The mouth-throat and tracheal-bronchial airway model were designed to snap-fit together to form one part [26].

1.5 Is Lung Dose a Good Predictor for Clinical Response?

Significant advances with representative *in vitro* test methods used to study and predict lung deposition behaviour *in vivo* have been sufficiently promising to make estimates on therapeutic outcomes for OIPs. Up to this point, it is inviting to assume that the total lung dose can be directly correlated with clinical outcomes; unfortunately, this assumption may be overly simplified. A higher total lung dose alone does not necessarily correlate to better treatment outcomes since the extent of local and regional deposition are of equal importance, especially for locally-acting drugs. For instance, if the inhaled drug is delivered at an inadequate amount, or to region(s) of the lung lacking target receptors, the effectiveness of the inhaled drug may be compromised.

Pertaining this perspective, Longest *et al* proposed an *in vitro – in silico* approach to track regional lung deposition behaviour of inhaled aerosols [64, 65]. Typically, their approach involves measuring either the aerosol size distribution of the initial emitted dose exiting the inhalers' mouthpiece, or the size distribution of the dose exiting the representative VCU MT model using existing compendial impactors. These measurements are then used as inputs for *silico* computational fluid dynamics (CFD) simulations used to track and quantify both local and regional deposition patterns of aerosol, providing an in-depth examination in the complex aerosol transport behaviour in the respiratory tract. Figure 7 shows a CFD simulation illustrating the regional deposition behaviour of aerosolised Budesonide powder emitted from the commercial Novolizer® DPI within the medium size VCU MT-TB airway model [65]. Good correlations between *in silico* and *in vitro* deposition was observed in their study.

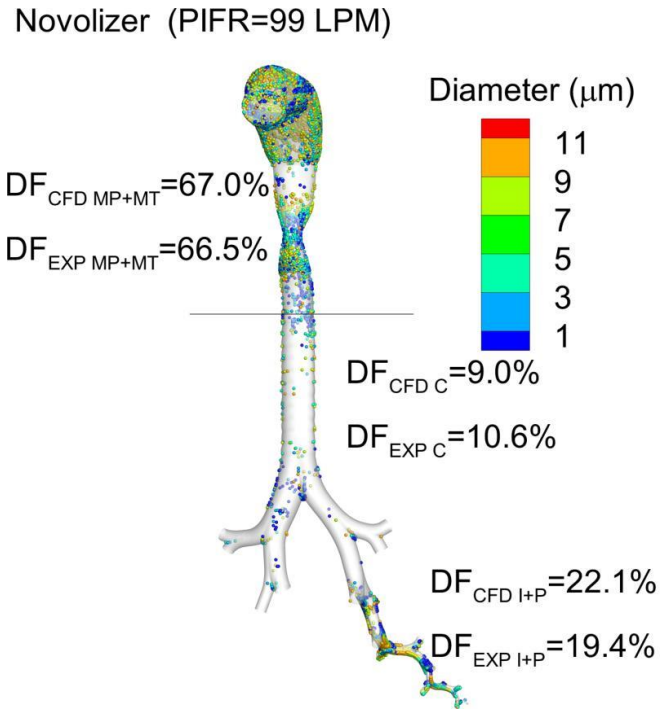

Figure 7. Comparison of *in vitro* and *in silico* predictions of deposition fraction in different regions of the airways for the Novolizer DPI with a powdered budesonide formulation [65].

Of equal importance is the need to characterise the event following lung deposition, i.e., the dissolution and cellular transport behaviour of deposited drug occurring at the airway epithelia. The airway epithelium, together with the mucus layer lies at the interface between the host and the external environment and forms a physicochemical protective barrier against inhaled foreign particulate matter [66, 67]. As for many locally-acting drugs, depending on their solubility with the airway epithelium lining fluid, the deposited aerosols may readily be absorbed across the epithelium and to some extent may be redistributed in the lungs via clearance mechanisms (cough or via mucociliary clearance), or simply taken up by macrophages [1, 68]. Overall, the local bioavailability and systematic drug absorption depends on the fraction of deposited dose that dissolves in the lung. Hence, it is important to recognise the practical limitations in using *in vitro* lung deposition data to make predictive estimates on therapeutic response.

There has been a tremendous interest to better understand the pulmonary fate of inhaled aerosols following lung deposition [69-71]; that is, *how do inhaled aerosols interact with the lung cells once deposited on the airway epithelia? How do deposited aerosols transport across the epithelial barrier and penetrate deep into the lung tissue?* and *How does aerosol size affect cellular uptake across the airway lining?* A lot of developmental work has been forwarded to establish a range of *in vitro* test methods used to answer these questions.

1.6 Current *in vitro* drug dissolution and release studies for inhaled aerosols

Currently, there are a range of established *in vitro* test apparatuses and guidance documents used to study drug dissolution and release profiles for solid oral dosage forms and transdermal systems [72, 73]. There are however, no standardised Pharmacopoeial methods used to assess such profiles for OIPs; instead, existing methods for solid oral dosage forms have been widely adapted to characterise the dissolution and solubility behaviour of aerosols generated from inhaler devices [72, 73]. Assessing the dissolution behaviour of generated aerosols is vital to evaluate the efficacy of the treatment regime, since drug dissolution is a strong determinant of drug bioavailability and may correlate with the rate and extent of drug action at the targeted site.

1.6.1 Current dissolution and drug release test apparatuses

1.6.1.1 Rotating basket dissolution apparatus

The rotating basket dissolution test method (USP apparatus 1) was the first official method used to study *in vitro* drug dissolution profiles for solid dosage forms. It consists of a metallic

drive shaft connected to a cylindrical basket where the dosage form resides, positioned within an enclosed hemi-spherical cylindrical vessel (Figure 8 A). Pre-warmed dissolution media is kept inside the vessel at a controlled temperature of 37 ± 0.5 ºC. Upon operation, the analyst must ensure accurate positioning of the shaft and basket in the vessel to specified test limits and the drive shaft rotates freely to ensure reproducible and consistent dissolution results. Samples are repeatedly taken from the solution vessel over time to measure drug dissolution into the surrounding media.

1.6.1.2 Rotating paddle dissolution apparatus

The rotating paddle dissolution test method (USP apparatus 2), similar to the USP apparatus 1 except that the basket is substituted for a coated two-blade paddle, and the dosage form is positioned at the base of the vessel (Figure 8 B). By placing the dosage form freely at the base, several limitations are apparent with this set-up. The major limitations are adherence of drug to the paddle shaft and/or potential floating of the insoluble drug in the media, which can be collected during the sampling, significantly interfering with data reproducibility and consistency [74]. In an attempt to make up for some of these shortfalls, caging the dosage form with small, loose, non-reacting wire baskets (Figure 8 C), or placing a mesh above the dosage form (Figure 8 D) have been modified into this test set-up [75].

Designated to evaluate the dissolution and drug release profiles for transdermal systems, the paddle over disk (USP apparatus 5) is identical to the USP apparatus 2, with the addition of a disk assembly located at the base of the vessel (Figure 8 E).

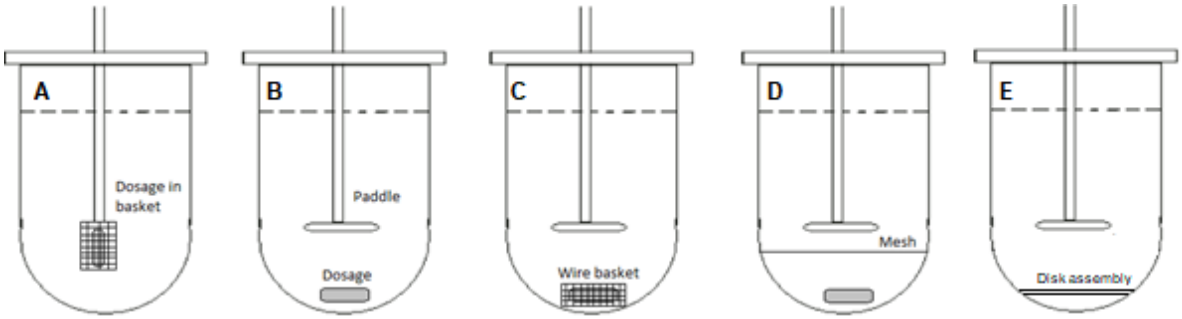

Figure 8. Schematic representation of the USP A) apparatus 1, B) apparatus 2, modified versions of apparatus 2 with C) wire basket and D) mesh, and E) apparatus 5.

1.6.1.3 Reciprocating cylinder dissolution apparatus

The reciprocating cylinder dissolution test method (USP apparatus 3) was designed based on the drawbacks encountered with the USP apparatuses 1 and 2 [76]. The USP apparatus 3 consists of a set of cylindrical, flat-bottom glass outer vessels, with a set of glass reciprocating inner cylinders that resides within the outer vessels, attached with non-sorbing and non-reactive mesh screens made to fit at both ends to contain the dosage form (

Figure **9** A). Upon operation, the vessel is filled with dissolution media at a controlled temperature of 37 ± 0.5 °C, with a motor and drive assembly used to operate the reciprocating cylinders vertically at a specified agitation rate (dips per minute). On the upward stroke, the bottom mesh in the inner cylinder moves upward to contact the dosage form, and on the down stroke the product leaves the mesh and floats freely within the inner tube, creating a moving medium. At specified time interval specified, a portion of the solution is withdrawn from a zone midway between the surface of the dissolution media and the bottom of each vessel.

1.6.1.4 Flow-through cell dissolution apparatus

The flow-through cell (USP apparatus 4) consists of an input pump for the dissolution medium, a flow-through cell compartment where the dosage form resides, and a filtration system which removes undissolved drug prior to sampling (

Figure **9** B). The bottom cone of the flow-through cell is filled with small glass beads and one larger bead positioned at the apex to prevent the drug from descending into the inlet tubing.

Upon operation, the dosage form is clamped onto the dosage holder unit and the pump forces the dissolution medium through the flow-through cell at a constant flow rate.

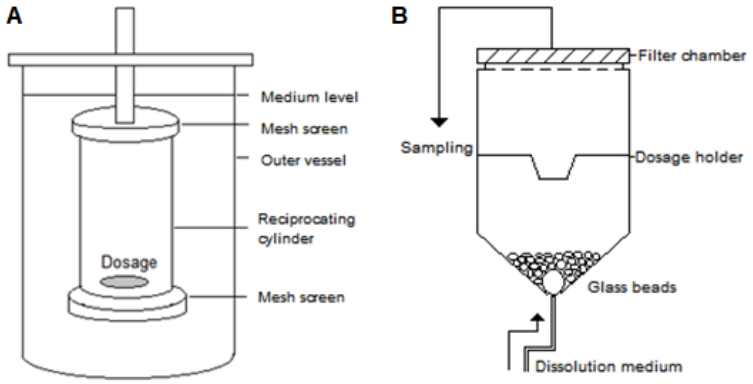

Figure 9. Schematic representation of the USP apparatuses A) 3 and B) 4.

1.6.2 Developing suitable dissolution test apparatuses for orally inhaled products

As the aforementioned apparatuses were developed to assess *in vitro* dissolution and drug release characteristics for oral solid dosage forms and transdermal systems, several limitations exist when translating such methods to evaluate inhalation products. Firstly, as the therapeutic efficiency of inhaled products is linked to the distribution and extent of lung deposition, it is intuitive to study the dissolution profiles of the portion of dose that deposits in the lung, not the whole dose that resides in the device.

To address this issue, Davies and Feddah established a novel hybrid *in vitro* approach to evaluate the effect of dissolution media composition on the dissolution rates of poorly-soluble inhaled glucocorticoid, administered as powdered aerosols [72]. To resemble the portion of the dose entering the lung, Davies and Feddah placed a glass filter downstream of the USP/Ph. Eur IP, attached to the ACI and sampled the emitted dose under routine compendial procedures.

The collected dose was then transferred to a modified flow-through cell dissolution apparatus, and dissolution assessment was conducted as recommended by the guidelines [72]. Although this approach provided sufficient discriminatory power between different drug substances and dissolution media, the size fractionation capabilities of the ACI was not fully exploited; hence, such reported dissolution data may not necessarily reflect the dissolution profiles of fine particles which may be expected to deposit in the lung. To address this issue, Copley Scientific Limited have launched the world's first commercially available NGI dissolution cup apparatus specifically designed for dissolution testing of inhaled drug formulations, a concept developed by Professor Jason McConville and his team at the College of Pharmacy, University of Texas. This test apparatus is identical to a standard compendial NGI cup, except that it has a removable insert in the impaction area [74]. The removable insert was designed to collect aerosols of known aerodynamic size, and the drug-deposited inserts are then transferred onto the disk assembly position in the USP apparatus 5 for routine dissolution assessment. Simple to use and now commercially available, the integration of the NGI dissolution cup for *in vitro* assessment of inhaled aerosols has provided better representation in terms of dissolution testing for OIPs.

1.7 Cell- and tissue-based methods

Up to this point, *in vitro* dissolution profiles for OIPs have been assessed using hybrid approaches, providing valuable insight on drug solubility characteristics and/or release profiles of deposited aerosols within the "respirable-size" range [72, 74]; however, the major limitation is the overall simplicity with such approaches when attempting to match *in vitro* data to clinical outcomes. Firstly, the non-representative large dissolution media used upon testing, i.e., USP

apparatuses 1 and 2 may involve dissolution volumes up to 900 ml to simulate dissolution of tablets in the stomach; however, for dissolution of inhaled aerosols at the airway epithelia, this large volume is not reflective of the small volume of the airway epithelium. Additionally, such small amounts of liquid within a stagnant environment (as opposed to the agitated environment established within dissolution apparatuses) may possibly inhibit drug dissolution of deposited aerosols, particularly for poorly water-soluble glucocorticoid drugs [72, 77]. Furthermore, the exact composition of the aqueous fluids and surfactant, cells and tight junctions present at the airway epithelia are not accurately known, and are absent during compendial USP dissolution testing, since synthetic, semi-permeable membranes are used as physical barriers of the epithelia within these test apparatuses.

To address the aforementioned limitations, the development of *in vitro* cell-based models has been proposed to characterise drug permeation, absorption and cellular transport behaviour of deposited aerosols [78, 79]. Such cell-based models have been used to study the effects of gas concentrations, exposure time, biophysical stress, and biological agents on human airway epithelial cells [80].

Typically, this approach involves placing fresh cell culture media at the basolateral compartment and apically culturing cells directly onto synthetic semi-permeable membranes until a confluent monolayer is developed (Figure 10). Different cell-lines require different culturing conditions. Ideally, cell-lines should produce features of differentiated, functional human airway epithelial cells and contain relevant proteins and surfactants. Hence, it is up to the analyst to ensure and validate cultured cell lines are representative of human epithelial cells. Several cell-based models have been thoroughly investigated to better understand and improve the efficacy of treatment regimens [29, 78].

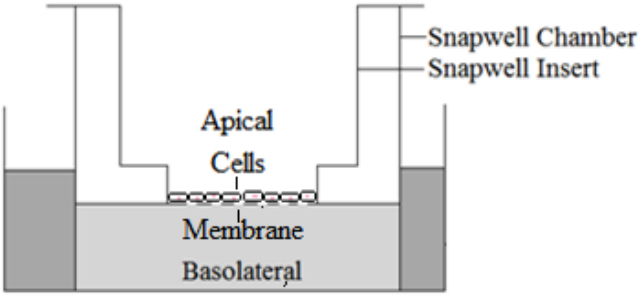

Figure 10. *In vitro* experimental set up of the call-based model cultured on Snapwell inserts used to assess drug permeation and absorption behaviour.

1.8 Summary

In vitro tests are used extensively within pulmonary drug delivery research and are recognised as a fundamental aspect in the development and regulation of OIPs. Currently, head-to-head comparison of OIPs for regulatory intentions is performed *in vitro* primarily by quantifying the total emitted dose and the generated aerodynamic size distribution using compendial impactors or impingers, in which derived parameters can be used to make statements on likely airway deposition pattern.

The link between *in vitro* characteristics of OIPs and their clinical performance is rather complex. To date, the focus on developing good IVIVCs has been achieved by integrating patient-related factors into *in vitro* testing, i.e., realistic airway models and representative inhalation manoeuvres. However, when no correlations are established, this gives rise to validity issues pertaining choice of test apparatus and testing conditions. Principle decisions and adaptions to current methods to find a suitable *in vitro* tool for the question being asked are required (either for regulatory purposes or predictive estimates of total lung deposition), as well as their associated advantages and limitations do need to be addressed. Fundamentally, if

reliable IVIVCs could be established, then such methods could be used to simplify and cheapen the drug development process.

CHAPTER 2

Development and Modification of the Virginia Commonwealth University Realistic Upper Airway Model

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This chapter describes the method development and modification of the medium-sized VCU MT – TB realistic upper airway model, intended for *in vitro* post airway deposition evaluation. The TB model was modified to accommodate two Snapwell® inserts above the first TB bifurcation region, for the collection of aerosolised drug in a more clinically-relevant airway model. As a proof of concept study, an in-house, high-dose aqueous solution of ciprofloxacin, termed Free Ciprofloxacin for Inhalation (FCI) was used as the model drug and delivered into the VCU MT – TB airway model using the PARI LC Sprint® jet nebuliser. Modifications to the TB model resulted in a minor increase in the airway deposition for nebulised FCI drug; however, such an increase is anticipated to lie within the expected lung deposition variation seen *in vivo*. Overall, the aerosol drug delivery system produced reproducible droplet sizes suitable for inhalation and enough drug was collected on the membrane inserts within the modified airway model for post airway deposition characterisation. Chapter 3 will describe the application of this modified airway model to characterise *in vitro* drug permeation behaviour following realistic airway deposition.

2.1 Introduction

Compendial methods regarding *in vitro* tests for OIPs have relied, until present, on cascade impactors and impingers to measure the aerodynamic sizing properties and emitted dose characteristics. Data produced from these apparatuses is used to assess the delivery performance and provide inferences pertaining delivery performance of OIPs [81, 82]. Yet, deposition in the respiratory tract is the first in a series of events for a therapeutic effect to occur, hence relying on routine impaction procedures may not necessarily provide direct measurements of clinical response. There has been an increasing interest to better understand the pulmonary fate of inhaled aerosols following airway deposition [69-71]. That is, how do inhaled aerosols interact with the lung cells once deposited on the airway epithelia? How do deposited aerosols transport across the epithelial barrier and penetrate deep into the lung tissue? Unfortunately, there are currently no established compendial *in vitro* test methods for such characterisation. Recent attempts have modified the cascade impactor by introducing integrated epithelial cell monolayers, which have been cultured onto Snapwell® or Transwell® inserts of semi-permeable material to collect respirable-sized aerosols directly onto the cells in order to model drug transport behaviour *in vitro* [83, 84]. However, the main drawback with this hybrid impactor-cell approach is that these nozzle-based impactors are not surrogates of the respiratory tract, and therefore may not necessarily reflect airway deposition, drug dissolution and transport behaviour *in vivo* [85].

There has been a growing impetus to improve on the clinical realism in OIP testing. For instance, the USP/ Ph Eur induction port, which is a straight 90º bend serves as the standard throat model used during routine testing of OIPs, have been shown to underpredict *in vivo* oropharyngeal deposition and failed to provide an accurate realisation of air flow through the upper respiratory tract [55, 56]. To address this issue, researchers at VCU developed and validated physical airway models and established *in vitro* test methods, to predict and capture

in vivo lung deposition for a range of commercial OIPs. By coupling realistic upper airway models with appropriate representative inhalation profiles, they were able to show a good one-to-one IVIVC, in terms of total lung deposition for a range of commercial OIPs and were able to capture deposition variations seen *in vivo* [86, 87]. Intuitively, lung deposition and dosimetry of inhaled aerosols should predict their beneficial clinical effects, since these drugs act locally on the airway surface, hence assessing the rate and extent of drug absorption at specific sites *in vitro* could theoretically give inferences regarding clinical outcomes. With this logic, it would be interesting from a research perspective, to take these established, representative airway models a step further and integrate cell-cultured epithelial monolayers to model *in vitro* the transport behaviour of deposited pharmaceutical aerosols. Therefore, the focus of this chapter describes the method development and validation procedure in modifying the characteristic medium-sized VCU TB airway model with cavities for the placement of Snapwell inserts intended to study *in vitro* the pulmonary fate of inhaled aerosols.

There are three main objectives in this chapter; the first is to select a suitable drug delivery system that can reproducibly generate aerosols within the respirable-size range. The second is to evaluate if any modifications towards the VCU airway model would result in any significant changes in airway deposition behaviour. As previous work from Longest *et al*. have suggested less than 3 % of the total delivered dose was observed to deposit within the VCU TB airway model [88, 89], the last objective of this chapter is to assess if the selected drug delivery system can generate enough drug to be collected onto the semi-permeable inserts for drug quantification, and therefore be used to assess post airway deposition events. Overall, the development of a modified characteristic airway model is proposed and if successful, will enable researchers in the field of aerosol science to better measure deposition behaviour in a more representative manner and consequently understand the pulmonary fate of inhaled pharmaceutical aerosols *in vitro*.

2.2 Materials and Methods

2.2.1 Preparation and delivery of Free Ciprofloxacin for Inhalation to the realistic airway model

2.2.1.1 Realistic airway model construction

Stereolithography files of the characteristic medium-sized VCU MT and TB upper airway models were provided by the School of Engineering, Faculty of Mechanical and Nuclear Engineering at VCU. Airway models were manufactured as split models, constructed from a transparent acrylic photopolymer resin (FLGPCL02) at a layer thickness of 0.025 mm (Formlabs Inc, MN, USA). The MT model was made to snap fit on top of the TB model (Figure 11).

2.2.1.2 Delivery of drug to the airway model

A high-dose, in-house aqueous FCI solution (MP Biomedical Australasia Pty Limited, NSW, Australia) of concentration 20 mg/mL was formulated by dissolving ciprofloxacin hydrochloride drug into deionised water and was used as the model drug. The selected drug delivery system was the PARI LC Sprint® nebuliser, used in conjunction with the PARI TurboBOY S compressor, supplied by PARI Medical Holding GmbH (Starnberg, Germany). A high-dose, continuous output aerosol drug delivery system was selected to ensure sufficient FCI drug collected on the Snapwell inserts for drug quantification and analysis. A custom-made adaptor was made to attach the mouthpiece of the PARI nebuliser directly onto the opening of the VCU MT model. To deliver the solution into the VCU MT – TB airway model, the TB airway model was housed in a custom-built acrylic housing-assembly as shown in Figure 11 (inner diameter and height of 14.5 and 10.5 cm, respectively). An external filter (Suregard®, Bird Healthcare, VIC, Australia) was attached at the base of the assembly,

connected to a vacuum pump source (Westech Scientific Instruments, Bedfordshire, UK) to draw ambient air at the inlet of the airway model, at a controlled volumetric airflow rate of 15 L/min for 90 seconds.

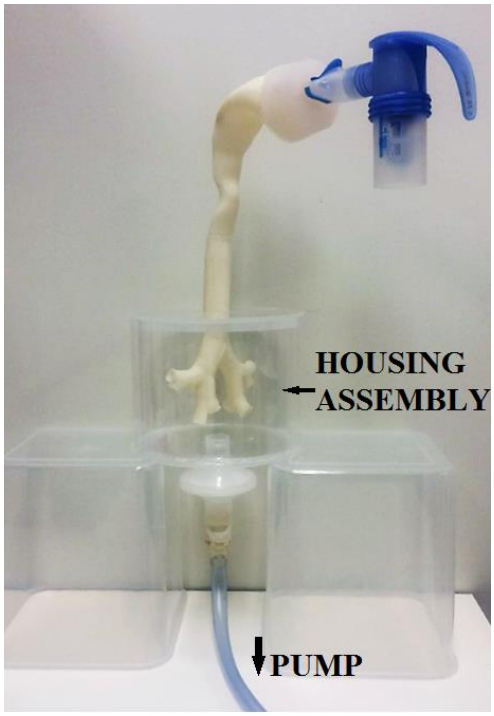

Figure 11. Physical test set-up used to assess *in vitro* realistic airway deposition. The medium-sized Virginia Commonwealth University mouth-throat and tracheal-bronchial airway model displayed in this figure was constructed of acrylonitrile butadiene styrene plastic for visualisation purposes [90].

2.2.2 Aerodynamic size determinations of nebulised Free Ciprofloxacin for Inhalation

2.2.2.1 Aerodynamic size characterisation using the Next Generation Impactor

Aerodynamic size characterisation of nebulised FCI drug exiting the PARI nebuliser was determined using the compendia Next Generation Impactor (NGI) (Westech W7, Westech Scientific Instruments, Bedfordshire, UK), with the standardised USP/Ph Eur induction port

replaced with the medium-sized VCU MT model (Figure 12). Two millilitres of the aqueous solution were loaded in the reservoir of the PARI nebuliser and delivered into the NGI, at a volumetric flow rate of 15 L/min for a total duration 90 second. Individual NGI stages and VCU MT model were left uncoated. An external filter (Suregard®, Bird Healthcare, VIC, Australia) was connected to the outlet of the NGI to ensure complete capture of ultra-fine droplets less than 0.98 µm in aerodynamic diameter from penetrating the final the micro-orifice collector (MOC) stage as recommended by Marple *et al* [52]. Prior to each experiment, the vacuum pump was left running for 5 seconds to ensure that test conditions were established and stabilised within the NGI before conducting size determinations. The NGI apparatus was dissembled after each experiment. For mass recovery assay, the device, mouth-piece adaptor, VCU MT model, all stages of the NGI and external filter were rinsed using known volumes of deionised water and analysed using a validated ultra-fast liquid chromatography (UFLC) method discussed in Section 2.2.3. The MMAD, FPF (in this case, defined as droplets less than 5.0 µm in aerodynamic diameter) and the geometric standard deviation (GSD), were determined using linear interpolation of the percent cumulative frequency profiles.

The effect of droplet evaporation within the NGI were also studied by storing the NGI in the refrigerator overnight [91], and results compared with ambient NGI determinations (NGI not refrigerated). Triplicate runs were performed for both ambient and refrigerated conditions, and the mean was calculated for each condition.

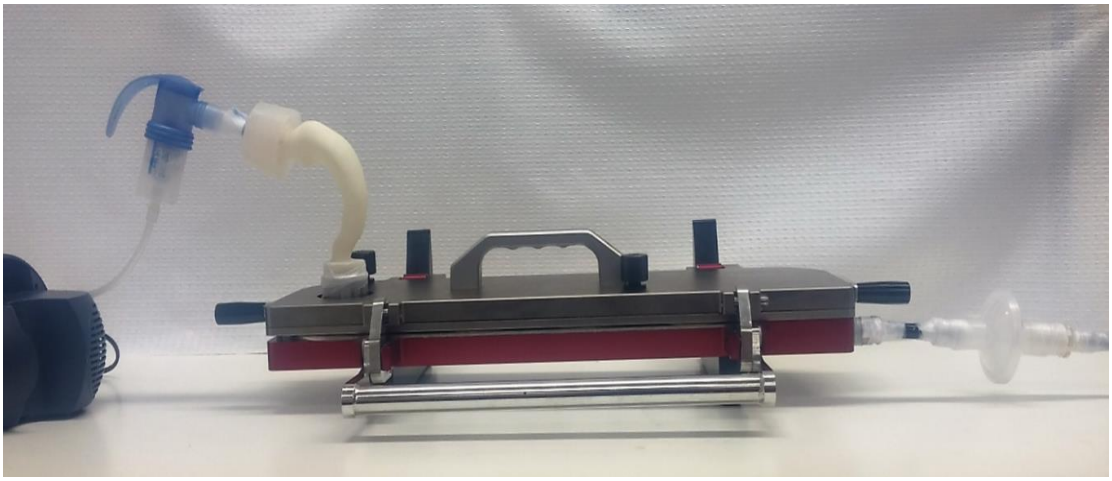

Figure 12. Physical set-up of the Next Generation Impactor, with the USP/Ph Eur induction port replaced with the medium-sized Virginia Commonwealth University mouth-throat model.

2.2.2.2 Droplet size distribution using the laser diffraction

The droplet size distribution of nebulised FCI solution exiting the characteristic medium-sized VCU MT model was determined using Malvern Spraytec® laser diffraction (Malvern Instruments Ltd., Malvern, UK) to observe if droplet measurements, assuming generated droplets are spherical and approach unit density, were comparable with NGI sizing data. The Spraytec apparatus was setup in a vertical configuration, with the VCU MT model attached directly onto the sheath flow collar, to minimise the distance between the exit of the MT model and the measurement zone within the Spraytec apparatus, and therefore any potential droplet evaporation according to previous test protocols [92]. The entire assembly was a set up as a closed system, with a vacuum pump attached downstream to draw a controlled volumetric airflow rate of 15 L/min at the entrance of the MT model. Triplicate runs were performed. The volumetric median diameter (VMD), GSD, and corresponding FPF were determined using linear interpolation of the percent cumulative frequency profiles.

2.2.3 Drug quantification and data analysis

Drug quantification of ciprofloxacin hydrochloride drug was performed using the Shimadzu Prominence UFLC system (Shimadzu Corporation, Kyoto, Japan). The system consisted of an SPD-20A UV-vis detector, LC-20AD liquid chromatography, SIL-20A HT Autosampler and Luna C-18 (2) 100A column (3µm, 150 x 4.6 mm) (Phenomenex Pty, Ltd, Lane Cove, Australia). Chromatography conditions were conducted using a mobile phase composition of methanol and 0.1 M sodium dihydrogen phosphate at a 30:70 (v/v) ratio, with pH adjusted to 3.30 with phosphoric acid. The sampling flow rate was set to 0.8 mL/min and 20.0 µL of each sample was injected into the column, with the column temperature set to 40°C. Ciprofloxacin was detected at a wavelength of 275 nm and linearity was obtained between 0.1 and 50.0 µg/mL (R^2 >0.999) at a mean retention time of 6.55 minutes.

2.2.4 Development and modification of the realistic airway model

To determine the most suitable region to place the inserts within the TB model, a clear transparent model of the airway was 3-D printed and the internal surface of the model was uniformly coated with Sar-Gel® paste (Spill Crew Corporation, NSW, Australia); a paste which turns purple on contact with water. Deposition of FCI droplets were carried out using the same set-up as shown in Figure 11. Again, 2.0 mL of FCI solution was loaded into the reservoir of a PARI nebuliser and delivered into the airway model at a flow rate of 15 L/min. Deposition patterns and regions of intense colourisation, which provided cues of deposition "hot-spots" within the characteristic airway model, were assessed by visualisation and considered as potential regions to accommodate the Snapwell inserts. Following the Sar-Gel experiments, the 3-D printed TB model was modified to include cavities to accommodate the Snapwell inserts.

2.2.5 *In vitro* realistic airway deposition studies

Realistic airway deposition experiments using the unmodified and modified airway models including Snapwell inserts were carried out using the set up as shown in Figure 11. Three millilitres of FCI solution was loaded into the reservoir of a PARI nebuliser and nebulised into the airway model at a controlled volumetric airflow rate of 15 L/min for a total duration of 5 minutes. Recovery of FCI drug in the PARI nebuliser, airway model, housing-assembly and filter was determined using known volumes of deionised water and quantified using the UFLC method as discussed in Section 2.2.3. Triplicate experiments for each airway model were performed.

2.3 Results and Discussion

2.3.1 Aerodynamic size determinations of nebulised Free Ciprofloxacin for Inhalation

Device retention and mass determinations of deposited FCI drug in the mouthpiece adaptor, MT, individual NGI stages, MOC and external filter in terms of percent total mass recovered collected in the ambient and refrigerated NGI are illustrated in Figure 13. Mass balance (> 95.0 %) was obtained in all determinations. As discussed by Marple *et al* [52], the collection efficiency of stage 7 when operating at 15 L/min failed to collect some of the finer droplets that penetrated beyond stage 7, resulting in mass retained on the external filter. Comparable mass deposition of FCI drug on the VCU MT model and each consecutive NGI stage were observed between the refrigerated and ambient groups, indicating no droplet evaporation of the solution within the NGI apparatus ($p>0.05$, two-tailed t-test).

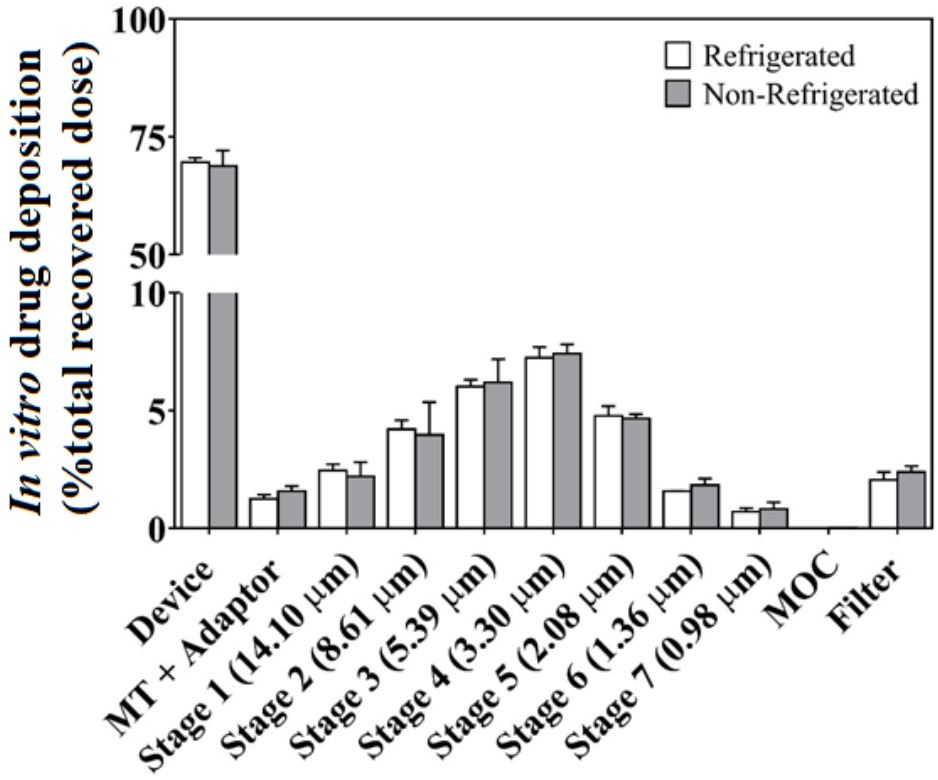

Figure 13. *In vitro* determinations of device retention and nebulised ciprofloxacin-hydrochloride drug deposited on the Virginia Commonwealth University mouth-throat model and adaptor, and individual Next Generation Impaction stages with corresponding cut-off diameters. Data expressed as total percent recovered within the refrigerated and non-refrigerated impactor, operating at an ambient airflow rate of 15 L/min for a total nebulisation duration of 90 seconds (n=3; mean + [SD]) [90].

The percent cumulative frequency profiles of nebulised FCI droplets entering the NGI, tested at refrigerated and ambient conditions, were plotted as a function of midpoint aerodynamic diameters for the individual stages of the NGI, as well as the equivalent volumetric diameter distribution collected from the Spraytec diffraction data, assuming droplets are spherical in nature and approaching unit density. Such data is displayed in Figure 14. Derived sizing parameters, that is, MMAD, GSD and corresponding FPF are tabulated in Table 2. No droplet evaporation was evident when operating the NGI at ambient conditions, with derived metrics

proved to be statistically comparable between the two NGI groups ($p>0.05$, two-tailed t-test). Furthermore, the sizing profile obtained from the Spraytec apparatus were superimposed with NGI profiles (Table 2), with corresponding parameters being statistically comparable between the three groups ($p>0.05$, one-way ANOVA, Tukey's Post Hoc test). Overall, both *in vitro* test apparatuses revealed droplets being generated from the PARI nebuliser being suitable for inhalation purposes, with mean MMAD and GSD values being 4.77 μm and 2.25, respectively. Furthermore, the aerosol drug system could generate a high FPF of about 52.0 %, which is significantly higher than most conventional aerosol delivery systems [93, 94].

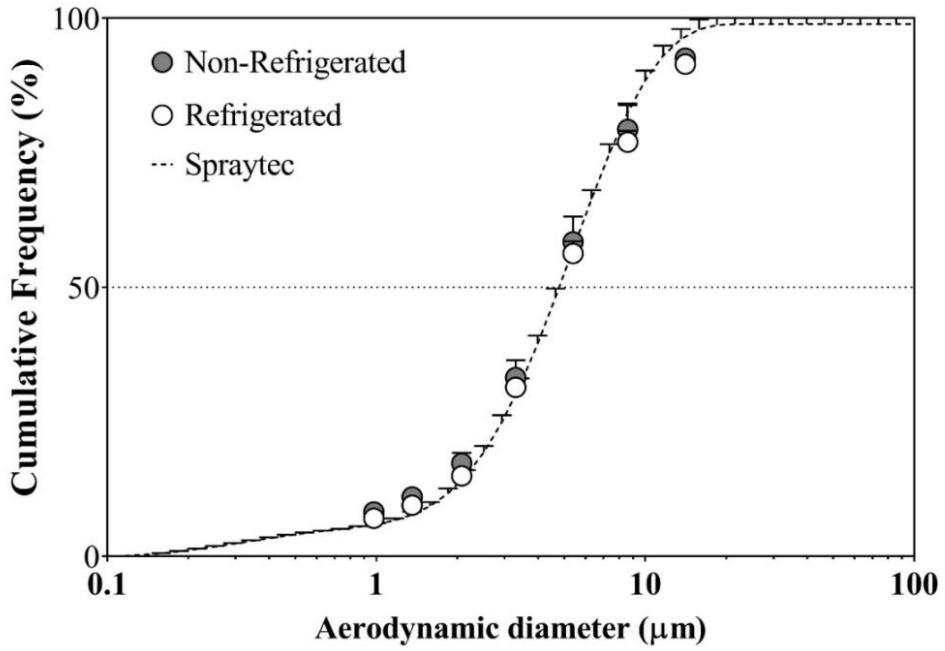

Figure 14. *In vitro* aerodynamic size characterisation of nebulised FCI droplets exiting the medium-sized Virginia Commonwealth University mouth-throat model, determined using Spraytec laser diffraction apparatus and Next Generation Impactor when operating at 15 L/min for a total nebulisation duration of 90 seconds (n=3; mean + [SD]). Volumetric size data were converted to aerodynamic size data assuming aqueous droplets are spherical and approach unit density [90].

Table 2. Derived parameters from the Next Generated Impactor and Spraytec laser diffraction test apparatuses (n=3; mean ± [SD]).

Sizing Apparatus	MMAD (µm)	GSD	FPF (%)
Ambient NGI	4.71 ± 0.36	2.24 ± 0.16	53.73 ± 4.42
Refrigerated NGI	4.87 ± 0.18	2.29 ± 0.03	51.65 ± 2.13
Spraytec [a]	4.74 ± 0.07	2.21 ± 0.03	52.44 ± 1.58

a - droplets from nebulisation are assumed to be spherical and approach unit density. Therefore, VMD equals MMAD

2.3.2 Development and modification of the realistic airway model

2.3.2.1 Sar-Gel® observations

Figure 15 shows the close-up, time-lapse deposition behaviour of nebulised FCI droplets depositing onto the Sar-Gel coated VCU TB airway model. Initial deposition sites first developed in the upper region of the TB model, after 10 seconds of nebulisation. Droplets were then observed above the first bifurcation region at the 30 second mark, with complete deposition in the airway by 60 seconds. The Sar-Gel deposition patterns of nebulised FCI drug revealed a similar *in silico* deposition trends, with most deposition occurring at the bifurcation region due to inertial impaction from a range of aerosol drug delivery systems [88, 89]. Following Sar-Gel experiments, the models were left in the laboratory for 30 minutes to test if the surrounding environment conditions, ~ 25 ºC at 30 – 40 % relative humidity (RH), could result in further colourisation within the airway model. No further colourisation was observed, nor any dripping of the droplets following deposition.

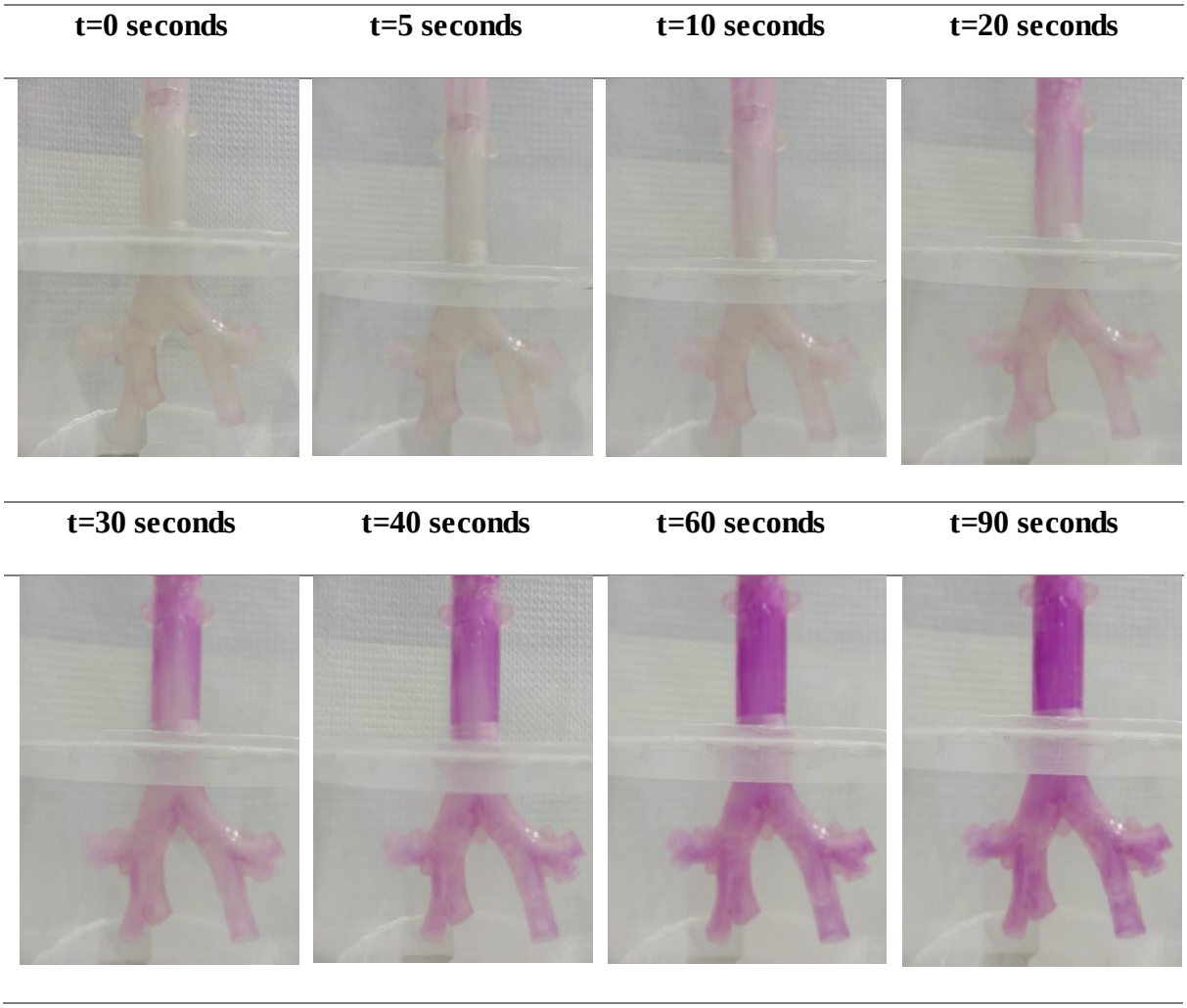

Figure 15. Close-up, time-lapse deposition patterns of nebulised FCI droplets deposited on the uniformly-coated Sar-Gel® medium-sized Virginia Commonwealth University tracheal-bronchial airway model, delivered from the PARI LC nebuliser at 15 L/min [90].

2.3.2.2 Physical airway modifications

Due to the inherently low aerosol deposition fraction previously reported in the medium-sized VCU TB airway model [88, 89], the choice in selecting Snapwell (Corning Life Sciences, polyester, pore size 0.4 µm, MA, USA) over Transwell inserts was due to the larger surface area (1.13 cm^2 for the Snapwell inserts, compared to 0.33 cm^2 for the Transwell inserts), which would increase the likelihood of collecting more drug for post airway deposition analysis. Since deposition hot-spots occurred at the first bifurcation region in the TB airway (Figure 15), the

TB airway model was modified by placing a cavity above the first bifurcation region at the front and back, allowing for ease of insertion and removal of the Snapwell inserts (Figure 16B). The unmodified and modified TB airway models are shown in Figure 16.

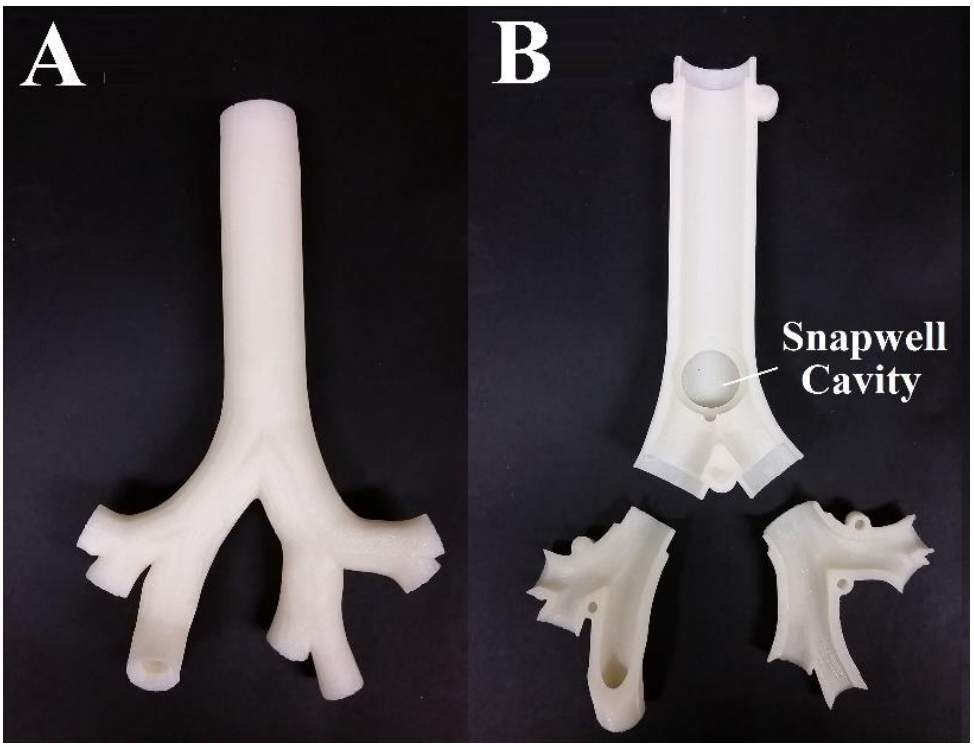

Figure 16. The medium-sized TB airway model: A) Unmodified and, B) Split modified version used in this study, with one cavity at the first bifurcation region (one at the front and one at the back) for the insertion and removal of the Snapwell inserts. The medium-sized TB airway models displayed in this figure were constructed of acrylonitrile butadiene styrene (ABS) plastic using fused deposition modelling 3-D printing technology (Dimension Elite, MN, USA) for visualisation purposes.

2.3.3 Realistic airway deposition studies

Mass quantification, expressed as percent total recovered dose of FCI drug retained in the PARI nebuliser, deposited on the MT and TB models, and ex-TB (determined by the drug recovered

from the housing chamber and filter) using the modified and unmodified airway models are shown in Figure 17. Most of the delivered dose was collected in the housing chamber and filter (ex-TB). No significant differences in MT deposition was observed in the unmodified and modified airway models, resulting in mean ± SD drug deposition of 2.89 ± 0.06 and 3.13 ± 0.14 % of the total recovered dose, respectively ($p>0.05$, two-tailed t-test). An increase in deposition was observed in the modified TB model from 1.20 ± 0.075 to 2.18 ± 0.188 % ($p<0.05$, two-tailed t-test) of the total recovered dose. It is important to note that the modified TB model comprises of two cavities to house the inserts within the model and were not completely flush with the surface, most probably affecting the overall deposition behaviour as observed in this study. However, it is important to realise that large variations in whole lung deposition have been reported in healthy human subjects, even for the same test inhaler, with gamma scintigraphy studies revealing high variations ranging from 20.0 – 60.0 % of the total metered dose for a single test inhaler [61]. Furthermore, work from Delvadia *et al*. [86, 87] has effectively captured this large variation seen *in vivo* by scaling the medium-size VCU MT-TB upper airway model to generate small and large versions, and coupling these representative models with clinically-relevant inhalation manoeuvres; therefore, it could be concluded that the observed 1.0 % increase in TB deposition in this study lies within the expected variation observed *in vivo*. Large variations seen *in vivo* are likely due to the range of airway geometries and differences in inspiratory manoeuvres between patients. The variations observed in this chapter are likely due to the structural changes within the modified TB airway model. Nevertheless, it is important to reinforce that the main aim of this study was to develop and modify the realistic characteristic medium-size VCU MT-TB upper airway model with Snapwell inserts. For our experiment, a sufficient amount of FCI drug was successfully collected when nebulised for a total of 5 minutes, equating to 8.2 ± 1.7 and 13.2 ± 2.2 µg of FCI drug deposited on the front and back Snapwell inserts, respectively (data not shown).

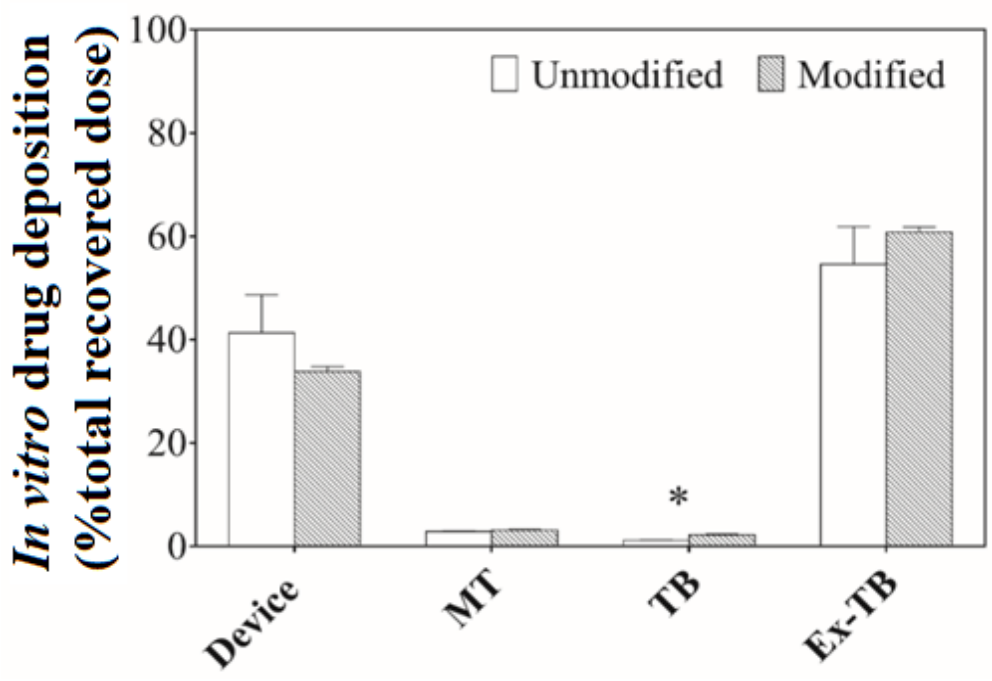

Figure 17. *In vitro* deposition results of nebulised FCI drug in the unmodified (white bars) and modified (grey bars) airway models for a total nebulisation time of 5 minutes (n=3; mean + SD). Results expressed as percent total recovered dose. Ex-TB represents dose exiting the tracheal-bronchial airway model and is the amount collected on the housing chamber and filter. Asterisks (*) indicates a statistically significant difference at p = 0.05 [90].

2.4 Summary

This chapter describes the method development and modification of the established medium-sized VCU TB realistic upper airway model with semi-permeable Snapwell inserts, intended for *in vitro* post airway deposition characterisation. The selected aerosol nebulisation system generated respirable-sized droplets, suitable for inhalation, resulting in sufficient FCI drug deposited onto the Snapwell inserts following realistic airway deposition.

Based on these results, it is possible to conclude that the use of this modified realistic airway model for *in vitro* testing of OIPs could potentially be advantageous in allowing aerosol researchers to better understand the pulmonary fate of inhaled drug in the airway. Chapter 3 will discuss the next steps in utilising this modified airway model for post airway deposition characterisation.

CHAPTER 3

Development and Validation of an *In Vitro* Hybrid Test Method used to Study Drug Permeation across Synthetic Membranes

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This chapter describes the development and validation of a novel *in vitro* test method intended to assess the permeability behaviour of deposited FCI drug across synthetic membranes, when nebulised into the medium-sized VCU MT and modified TB realistic upper airway model, as described previously in Chapter 2. Following realistic airway deposition, drug permeation behaviour of deposited FCI droplets were carried out using the Snapwell® test system to mimic the limited lining fluid volume on the lung surface. The Snapwell test system demonstrated reproducible and discriminatory drug permeation profiles for already-dissolved and nebulised FCI drug permeating across a range of synthetic membranes under different *in vitro* test conditions. The rate and extent of drug permeation depended on the synthetic membrane used, presence of a stirrer in the basolateral compartment and most importantly, the drug collection method. Although the synthetic membranes do not resemble the complex structure of the epithelial airway barrier, inferences regarding drug permeability behaviour for aerosolised drug within a representative physical airway model can be made using this novel *in vitro* set up. This approach holds great potential to evaluate other post airway deposition characteristics, such as particle dissolution and epithelial transport behaviour. Chapter 4 will further expand this approach by integrating Calu-3 monolayers on the semi-permeable inserts prior to airway deposition to model epithelial transport behaviour of inhaled deposited aerosols *in vitro*.

3.1 Introduction

Although recent IVIVCs for OIPs have been established for lung dose estimations [86, 87], predicting the fate of deposited drug in the lung is rather complex [31]. Following inhalation, the deposited drug must dissolve onto the airway lining fluid, permeate and be transported across the lung tissue to achieve a clinical effect [95]. Typically, a drug effect is related to its concentration at the deposited site, hence it would be advantageous to monitor this concentration *in situ*; however, sampling of the lung tissue for direct measurements is not practical. Therefore, establishing robust and representative *in vitro* test methods to characterise the rate and extend of drug dissolution and epithelial transport behaviour for OIPs is highly valuable not only from design and formulation development standpoint, but also for toxicity and quality control purposes.

To date, there are currently no standardised methods, nor is there a single, ideal test apparatus appropriate for such *in vitro* determinations [31, 70]. Over the past years, *in vitro* dissolution studies using compendial dissolution test apparatuses originally designed for orally-administered solid dosage forms, namely the flow-through cell and rotating paddle apparatuses have been integrated and modified to study drug dissolution behaviour for OIPs [96]. For instance, Davis *et al*. have incorporated the flow-through cell apparatus, which consists of a reservoir and a pump that pushes the dissolution media through a flow-through cell enclosing the sample dose of interest, to study the effect of dissolution media composition on dissolution rates for a range of poorly water-soluble glucocorticoid drugs [72]. In their study, the ACI was used to collect aerosolised drug powder onto a glass pre-filter positioned downstream of the USP/ Ph Eur induction port. Although their test apparatus was able to discriminate between different powder formulations providing good reproducibility, their sampled dose was characterised as the portion of the emitted dose collected downstream from the USP/Ph Eur.

induction port, which is not reflective of the dose that is deposited and distributed within the airway. To overcome this, Son *et al*. took on a similar approach and placed polycarbonate filter membranes onto each stage of the NGI to collect aerosolised powder of relevant, pre-defined particles in the respiratory size range, followed by dissolution testing using the compendial rotating paddle apparatus [74]. As this approach allowed researchers to assess the effect of particle size and mass loading on drug dissolution kinetics, the main drawback with this approach is the significantly higher amount of dissolution media used in their study, which does not reflect the limited epithelial fluid lining volume *in vivo* [31, 69, 97].

Recent efforts by Arora *et al*. have addressed this issue by substituting the rotating paddle apparatus with the Transwell® system, a system intended for cellular uptake studies, to model and mimic particle dissolution onto, and drug permeation across the limited epithelial airway lining [97]. As these current and emerging methods heavily rely on cascade impactors to collect aerosolised drug that are within the "respirable-size" range for subsequent dissolution evaluations, it is important to realise that from a realistic view point, these nozzle-based impactors are not surrogate models of the airway, and therefore do not represent deposition behaviour of aerosolised drug following inhalation. With this logic, it would be intuitive to directly collect drug from a physical, representative airway model, followed by post airway characterisation using the Transwell system as a means to model dissolution onto, and drug permeation across the limited epithelial airway lining.

Before any conclusive findings can be reached through *in vitro* methods, it is essential that the underlying methods are thoroughly characterised and assessed in terms of equipment design and experimental protocol. Hence, as a proof of concept study, this chapter provides a validation protocol of a novel, hybrid *in vitro* approach which integrates the VCU MT and modified TB realistic upper airway described previously in Chapter 2 to collect drug aerosols in a representative model, used in conjunction with the Snapwell test system to model and

mimic post airway deposition behaviour for inhaled, deposited pharmaceutical aerosols. There are two main objectives in this Chapter; the first is to evaluate the feasibility in adapting the Snapwell system by assessing the permeation characteristics of already-dissolved FCI solution through a series of different synthetic permeable membranes, tested at static and non-static conditions. The second objective is to utilise the Snapwell test system to characterise and compare the permeation profiles of nebulised FCI droplets that have been collected onto the Snapwell inserts which have been placed within the modified VCU TB airway model. Overall, this Chapter describes the initial development and validation phases of a novel, hybrid *in vitro* approach intended to model and mimic post airway deposition behaviour in a more representative matter.

3.2 Materials and Methods

3.2.1 Preparation and delivery of Free Ciprofloxacin for Inhalation to the modified realistic airway model

As described previously in Chapter 2, a high-dose, in-house aqueous FCI solution of concentration 20 mg/mL was formulated by dissolving ciprofloxacin hydrochloride powder (MP Biomedical Australasia Pty Limited, NSW, Australia) into deionised water and was used as the model drug. The physical model used to collect drug was the modified VCU MT-TB upper airway model (Chapter 2), consisting of two Snapwell cavities located at the first bifurcation region to house two Snapwell inserts (Corning Life Sciences, MA, USA) for drug collection. The selected drug delivery system was the PARI LC Sprint® nebuliser, used in

conjunction with the PARI TurboBOY S compressor and were supplied by PARI Medical Holding GmbH (Starnberg, Germany).

Delivery of nebulised FCI droplets into the modified airway was achieved as described previously in Chapter 2. In brief, 3.0 mL of FCI solution was loaded into the reservoir of the PARI nebuliser, with the custom-made mouthpiece used to connect the PARI nebuliser to the VCU MT model. The TB airway model was housed in a custom-built acrylic housing-assembly, with external filter (Suregard®, Bird Healthcare, VIC, Australia) attached at the base of the assembly, connected to a vacuum pump source (Westech Scientific Instruments, Bedfordshire, UK) to draw ambient air at the inlet of the airway model at a controlled volumetric airflow rate of 15 L/min for a total duration of 5 minutes.

3.2.2 Snapwell test system for drug permeation characterisation

The Snapwell system consisted of a custom-made 3-D printed miniature chamber to accommodate a magnetic bar stirrer (8.0 x 3.0 mm, Westlab Group, VIC, Australia) in the basolateral compartment. The basolateral compartment was filled with 2.3 mL of pre-warmed phosphate buffer saline (PBS) (Gibco, Invitrogen, NSW, Australia) to ensure complete contact with the basolateral-side membrane surface. The whole system was placed in a water bath maintained at 37 ºC. A cross-section of the Snapwell apparatus is shown in Figure 18. Components of the system were manufactured from a clear acrylic photopolymer resin (GPLCL02) (Form 2 Desktop SLA 3D Printer, Formlabs Inc, MN, USA).

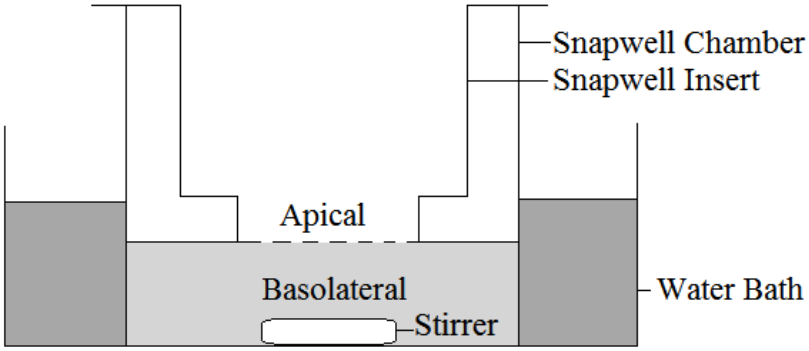

Figure 18. *In vitro* experimental set up of the Snapwell test system to assess drug permeation behaviour.

3.2.2.1 Suitability and discriminative ability of the Snapwell test system

To assess the feasibility of the *in vitro* Snapwell test system, the permeation characteristics of FCI solution was first assessed across the standard Snapwell polyester (PE) membrane. The Snapwell inserts were pre-soaked with PBS up to two hours prior to permeation experiments. A 0.1 mL aliquot of 0.1 mg/mL FCI solution was applied directly on the apical side of the PE Snapwell insert which was placed in the holder chamber at time = 0. A sample aliquot of 0.5 mL was withdrawn from the basolateral compartment at pre-determined time points over a period for 2 hours, with fresh pre-warmed PBS replaced immediately to maintain a constant volume in the basolateral chamber. At the end of each experiment, the membrane was washed with deionised water to recover any remaining FCI drug on top of the membrane. The influence of a stirred versus a non-stirred basolateral compartment on the permeation behaviour was investigated, with and without a magnetic stirrer. Permeation experiments were performed at ambient conditions (~ 25°C at 30 – 40 % relative humidity), and each test condition were performed in triplicate.

To assess the discriminatory ability of the proposed Snapwell system, several other synthetic membrane materials were selected. Polyamide (PA) membranes (Sartorius™, 0.45 μm pore size, Göttingen, Germany) and regenerated cellulose dialysis membranes (Cellu-Sep® T2, 6-8 kDa molecular weight cut-off, TX, USA) were the synthetic semi-permeable membranes used in this Chapter. Modified Snapwell inserts were created by removing the PE membrane from the insert and attaching either PA or a layer of a regenerated cellulose dialysis membrane onto the Snapwell insert. A 3-D printed ring was manufactured to attach the membrane onto the Snapwell insert, using a similar approach to that reported by May *et al*. [98]. Membranes were pre-soaked in PBS for two hours prior to experiments and 0.1 mL of 0.1 mg/mL FCI solution was applied directly onto the membrane of the modified Snapwell insert. Experiments were tested with and without a stirrer in the basolateral compartment and tested in triplicate.

3.2.2.2 Permeation characteristics of nebulised Free Ciprofloxacin for Inhalation collected on the modified airway model

To assess the permeability behaviour of nebulised FCI droplets, two standard PE or modified-Snapwell inserts (PA and dialysis membrane inserts) were placed within the previously modified TB airway model prior to realistic airway deposition experiments, to collect deposited nebulised FCI droplets onto the synthetic membrane inserts. Prior to airway deposition experiments, the inner walls and internal surface of the Snapwell inserts were covered with a removable thin 3-D printed sheet (Form 2 Desktop SLA 3D Printer, Formlabs Inc, MN, USA) as recommended by May *et al*. [98], to allow for more accurate quantification of drug deposited onto the synthetic membranes. Airway deposition experiments were carried using the same conditions as described in Section 3.2.1. Following deposition, the two inserts were transferred onto the Snapwell test system to assess the permeation characteristics across the different membranes, with a magnetic stirrer placed in the basolateral chamber. Work from Arora *et al*.

placed additional dissolution medium on top of the drug-deposited membrane to further facilitate the dissolution and permeation of the collected corticosteroids particles across permeable membranes [99]. Henceforth, an additional subset of experiments was conducted to assess if the permeation of nebulised FCI droplets can be further facilitated through the membrane by adding 0.1 mL of deionised water on top of the drug-deposited membrane. Permeation experiments for each test condition were performed in triplicate.

3.2.3 Drug quantification and data analysis

Mass quantification of FCI drug was determined using the validated UFLC methodology previously described in Chapter 2. The amount of CIP-HCL drug permeating into the basolateral compartment was calculated by the following equation:

Equation 1: $m_{t=i} = C_{t=i}.V_{medium} - \left[C_{t=i-1}.\left(V_{medium} - V_{sampling}\right)\right] + m_{t=i-1}$

Where V_{medium} is the volume of PBS buffer in the basolateral compartment,

$V_{sampling}$ is the sampling volume,

$C_{t=i}$ and $C_{t=i-1}$ are the drug concentration of FCI detected from UFLC at times *i* and *i-1*, respectively, and

$m_{t=i}$ and $m_{t=i-1}$ are the amount of mass released at times *i* and *i-1*, respectively.

The total amount of FCI drug collected was calculated as the cumulative amount collected in the basolateral compartment at the end of 2 hours, plus the amount remaining on top the membrane.

3.3 Results and Discussion

3.3.1 Suitability and discriminative ability of the Snapwell test system

The percent cumulative mass of FCI solution permeating across the PE, PA and dialysis membranes into the basolateral compartment, tested under static (no stirrer) and agitated (stirrer) conditions are shown in Figure 19 A, B and C, respectively. It was apparent that the permeation behaviour of already-dissolved FCI solution differed considerably across each membrane. For PA, there was fast initial permeation with complete transfer of FCI observed after 20 minutes when tested at stirred conditions, yet slower and complete transfer was observed for the other membranes. This can be explained by the differences in membrane structure and physical interactions between the aqueous solution and the material surface. The aqueous solution has a better affinity to the most hydrophilic PA membrane, resulting in complete wetting of the solution onto the whole surface area, whereas with the tissue culture-treated PE membrane and dialysis membranes, poorer surface wettability behaviour was observed at the beginning of the experiments when the solution was loaded directly on top of these more hydrophobic membranes, which may explain the slower and more gradual permeation of FCI solution across these membranes. Similarly, May *et al*. reported slower drug permeability behaviour from the PE membrane for a range of drug substances, with scanning electron microscopy images showing PE being less porous and having fewer permeation areas [98]. As expected, stirring in the basolateral compartment provided adequate mixing of FCI solution and PBS in the compartment, resulting in smaller experimental variability [100].

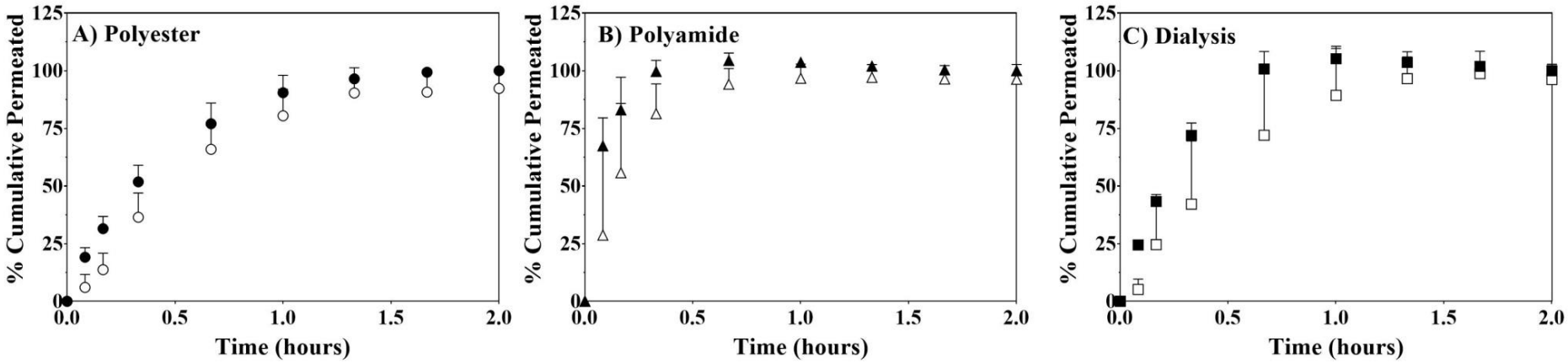

Figure 19. Percent cumulative mass of FCI solution permeating across the: A) Polyester, B) Polyamide, and C) Dialysis membrane inserts into the basolateral compartment, tested at static (empty symbols) and agitated (filled symbols) conditions (n=3; mean (error bars are + [SD])) [101].

3.3.2 Permeation characteristics of nebulised Free Ciprofloxacin for Inhalation collected on the modified airway model

The permeation behaviour of nebulised FCI droplets, collected on Snapwell inserts located at the front and back regions in the modified TB airway model, with or without additional water added, post deposition, on the PE, PA and dialysis membranes are shown in Figure 20A, B and C, respectively. Approximately 50 % of the deposited nebulised FCI drug permeated across all the tested membranes without any additional water after 2 hours of sampling, perhaps indicating that drug deposited as droplets on the membrane surface became dried following evaporation of water. As expected, direct application water following deposition onto the membranes increased both the rate and extent of the permeation behaviour, with approximately 75 – 90 % of FCI drug permeating across the membrane by the end of the experiment. It is thought that adding water on top of the membrane enhanced the increased dissolution on the donor compartment and spread of FCI drug onto the surface membrane, increasing the surface area coverage onto the porous areas, hence facilitating the permeation into the basolateral chamber. As such, the altered rate and extent of permeation may reflect a dissolution step for the deposited nebulised droplets on the membranes.

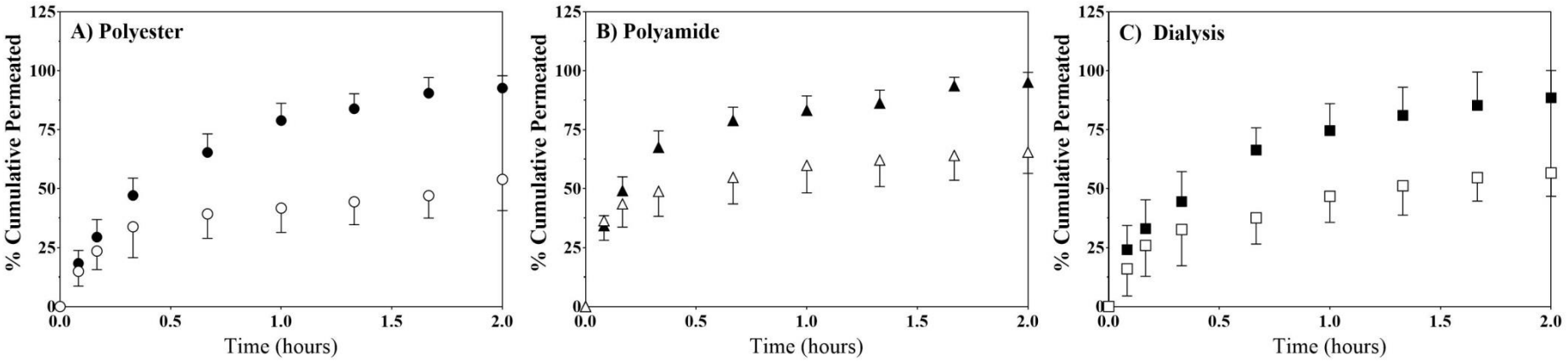

Figure 20. Percent cumulative mass of FCI drug permeating across the front and back membranes of the: A) Polyester, B) Polyamide, and C) Dialysis inserts into the basolateral compartment, with (filled symbols) and without (empty symbols) additional water (n=6; mean (error bars are + [SD])) [101].

3.4 Summary

In conclusion, the development and validation of a novel, hybrid *in vitro* test method intended to characterise post airway deposition behaviour for OIPs was assessed in terms of equipment design and experimental protocol. As a proof of concept study, the modified, medium-sized VCU MT-TB airway model was used to collect nebulised FCI droplets onto semi-permeable Snapwell inserts within a representative airway model to mimic deposition behaviour *in vivo*, followed by drug permeation characterisation across a range of synthetic membranes. Although the membranes used in this Chapter do not resemble the complex structure of the epithelial airway barrier, the findings have illustrated how experimental factors, including, but not limited to the choice of permeable membrane material, presence of a stirrer and the aerosol collection method can significantly influence drug permeation behaviour observed *in vitro*.

Chapter 4 will focus on expanding this hybrid *in vitro* approach to a cell-based set up, by integrating epithelial cell monolayers onto Snapwell inserts prior to airway deposition experiments.

CHAPTER 4

In Vitro Characterisation of Drug Permeation and Calu-3 Transport Behaviour for Free and Liposomal Ciprofloxacin Drug

The development of physiologically-relevant *in vitro* test methods used to characterise the pulmonary fate of OIPs is required. This Chapter describes the development and application of an *in vitro*, cell-based representative upper airway model intended to study *in vitro* epithelial transport behaviour of two nebulised ciprofloxacin formulations: free and liposomal ciprofloxacin solution. Prior to realistic airway deposition, Calu-3 cell-line monolayers were cultured at air-liquid interface (ALI) conditions onto semi-permeable Snapwell inserts placed within the medium-sized VCU MT and modified TB upper airway model, as described previously in Chapter 2. Following airway deposition, the drug-deposited inserts were transferred to an *in vitro* Snapwell test system to mimic drug dissolution onto and epithelial transport into the limited airway fluid lining. *In vitro* drug permeation across semi-permeable synthetic membranes were also investigated and compared with reported transport profiles. The rate and extent of ciprofloxacin drug permeation and Calu-3 transport showed to be greatly dependent on the drug mass-concentration, formulation type, and most importantly the selected physical barrier used to partition the apical and basolateral compartments. The development of more physiologically-relevant upper airway models intended to study *in vitro* realistic airway deposition and post deposition events is vital not only from a formulation design and development, but also important as a tool to better understand the complex mechanisms underlying the pulmonary absorption of inhaled, deposited pharmaceutical aerosols.

4.1 Introduction

The airway epithelium in the upper respiratory tract, together with the mucus layer coating its surface, lies at the interface between the host and the external environment, forming a physicochemical protective barrier against inhaled microbes, allergens and foreign particulate matter [66, 67]. A number of factors that help orchestrate this protective layer are the mucociliary escalator [102], presence of tight and adherens complex junction layers that regulate and control paracellular diffusion of ions and certain small molecules, associated transport processes via the intracellular pathway [103], and the presence of antimicrobial agents [104, 105]. Under the same principle, the airway epithelium may entrap inhaled therapeutic aerosols. For example, drugs intended for local or systematic delivery may be subjected to numerous clearance mechanisms such as mucociliary escalator, cough clearence, phagocytosis by macrophages, absorption across the epithelium and metabolism imto non-active related substances, all possibly reducing the overall efficacy and therapeutic ratios of the treatment regimen [1, 68].

For instance, low tissue permeability is typically necessary for locally-acting drug intended to treat respiratory disorders in the upper respiratory tract, since rapid absorption into the bloodstream will reduce the drug's ability to render a therapeutic effect at the targeted site, potentially resulting in unwanted systemic side effects if absorbed readily [106]. Longer lung retention of inhaled drug may also be desirable for slow, controlled-release formulations, i.e., inhaled antibiotics where the therapeutic dose may be lowered compared to oral or parenteral doses, therefore potentially minimising drug resistance [107] and the time at which an minimimum bacterisocidal concentration locally is increased. On the other hand, the therapeutic profile for inhaled drugs intended for systematic delivery (for example in some peptides and proteins) is dictated by the absorption characteristics at the epithelia. Drug absorption behaviour depends on an array of factors: type of formulation (solution or

suspension), droplet or particle size properties, mass-concentration at the deposited site, and the physicochemical properties of the molecule (i.e. intrinsic solubility, LogP, acidic and basic nature) and the physical state upon contact with the airway epithelium [95, 108]. For instance, inhaled drugs that are in complete solubilised form, will passively diffuse across the epithelia intracellularly, or be transported paracellularly, becoming available for absorption [109]. Conversely, poorly water-soluble drugs may experience incomplete dissolution onto the epithelial liquid lining, and are more likely to be retained in the lung for longer periods; therefore having a greater chance of being removed from the lungs by mucociliary clearance or cough [110]. As such, the development of representative *in vitro* epithelial barrier models intended to mimic these complex interactions between drug carriers, active ingredients at the epithelia will help researchers understand the complex mechanisms underlying the pulmonary absorption of pharmaceutical aerosols.

To date, the development and use of *in vitro* cell-based culture models as reconstructed barriers of the epithelium offers greater simplicity, reproducibility, and better control over experimental parameters compared to isolated perfused organs and use of animal tissue [111-113]. When developing *in vitro* epithelial cell-based culture models, the selection of either primary cells or immortalised cell-lines is presented, each with their associated strengths and weaknesses [114, 115]. Typically, primary cell cultures endure major limitations due to lack of availability or access to normal human airway tissue by researchers, long and complicated cell culture processes, and large variations between donors. Immortalised cell-lines on the other hand have been manipulated to undergo 'indefinite' cellular division in culture, making them more reproducible, homogeneous and readily available [116]. Derived from bronchial adenocarcinoma tissue, the immortalised Calu-3 cell-line is a well-differentiated and characterised *in vitro* cell model and that is considered to be representative of the bronchial epithelium in terms of expression of tight intercellular junctions and secretory proteins. These

cells have been extensively used to study *in vitro* pulmonary absorption for a range of OIPs [29, 78]. To create a representative barrier of the epithelia, Calu-3 cells can be cultured onto semi-permeable Transwell or Snapwell cell-culture inserts to form tight, confluent monolayers [79, 116, 117]. There are two methods to culture confluent Calu-3 monolayers onto these membranes, either in an air interface culture (AIC) or liquid covered culture (LCC) condition. for aerosol/particle related studies AIC is generally considered more relevant and is morphologically representative of the airway epithelium [29, 118].

For this reason, the overall aim of this Chapter, is to extend the *in vitro* hybrid methodology previously described in Chapter 3 to a representative bronchial epithelial cell-based culture model, intended to mimic and study realistic deposition, drug dissolution into and consequent Calu-3 transport behaviour across the confluent monolayer within a physical realistic airway model. This was achieved by first culturing Calu-3 cells onto semi-permeable Snapwell cell culture inserts under ALI conditions, which were then placed in the modified medium-sized VCU TB airway model prior to realistic airway deposition studies.

Overall, four study aims are presented in this Chapter:

(1) The first aim was to evaluate and compare *in vitro* Calu-3 transport behaviour of two nebulised ciprofloxacin formulations: a controlled-released encapsulated liposomal nanoparticle solution and an aqueous, free solution of ciprofloxacin drug. As ciprofloxacin is a well-established broad-spectrum fluoroquinolone antibiotic used to treat infectious bacteria [119, 120], the encapsulation of ciprofloxacin drug to treat respiratory infections in the lungs could potentially be more advantageous compared to unencapsulated free drug, since drug release profiles are slower and sustained at deposited targeted sites, resulting in safer and lower dosing frequencies and therefore reducing overall systematic side effects. As such, the first aim was to assess how a different formulation type may influence *in vitro* drug transport behaviour.

(2) The second aim was to evaluate the effect of drug mass-concentration on *in vitro* drug transport behaviour, by varying the total nebulisation time to deposit different amounts of ciprofloxacin drug onto the Calu-3 monolayer. Extending this aim, it was hypothesised that the dynamic airflow within the airway model may potentially induce shearing stresses on the Calu-3 monolayer, which could affect the barrier integrity and tight junction functionality of the cell-line monolayer.

(3) Following on from aim two, the third aim was to characterise any changes in the barrier integrity and tight junction properties by assessing the trans-epithelial electrical resistance (TEER) and apical-to-basolateral flux of the paracellular tracer molecule Fluorescein-Sodium (Flu-Na) following Calu-3 transport of both formulations at different mass-concentrations.

(4) Lastly, drug permeation behaviour for both formulations was assessed across different semi-permeable synthetic membranes and compared to reported Calu-3 transport behaviour.

Overall, this Chapter describes the extension and application of a novel, morphologically representative *in vitro* cell-based model intended to better understand the complex mechanisms underlying the pulmonary absorption of OIPs.

4.2 Materials and Methods

4.2.1 Preparation of Free and Liposomal Ciprofloxacin solution formulations

As described previously in Chapter 2, a high-dose, in-house aqueous FCI solution of concentration 20 mg/mL was formulated by dissolving ciprofloxacin hydrochloride powder (MP Biomedical Australasia Pty Limited, NSW, Australia) into deionised water and was used as the model drug used in this Chapter. For comparison purposes, Lipoquin®, termed

Ciprofloxacin for Inhalation (CFI) is a controlled-released liposomal formulation consisting of ciprofloxacin nanoparticles encapsulated in unilamellar liposomes, and was kindly provided by Aradigm Corporation (Hayward, CA, USA). In brief, multilamellar liposomes were formed by completely dissolving a mixture of hydrogenated soy phosphatidylcholine and cholesterol with mixture of t-butanol, ethanol and water, followed by mixing with ammonium sulphate [119]. This liposomal mixture was then extruded through 80 nm filters to yield unilamellar liposomes, and ciprofloxacin drug was actively loaded to the unilamellar liposomes [119, 121]. Unencapsulated ciprofloxacin drug was removed, resulting in an encapsulation efficacy of ciprofloxacin drug greater than 99.0 %, at a total target concentration of 50 mg/mL [119]. For comparison purposes, CFI was diluted from 50 mg/mL in sodium acetate buffer (25 mM sodium acetate, 145 mM sodium chloride at adjusted pH of 4.0) to a final concentration of 20 mg/mL per protocol [122].

4.2.2 Physical barriers used to model *in vitro* drug transport and permeation

4.2.2.1 Calu-3 air-liquid interface cell-line for in vitro drug transport studies

The immortalised bronchial adenocarcinoma Calu-3 cell-line was purchased from American Type Culture Collection (ATCC HTB-55, Manassas, VA, USA) and was cultured onto semi-permeable Snapwell cell culture inserts (1.13 cm^2, polyester membrane, 0.4 μm pore, Corning Life Sciences, MA, USA) to model epithelia transport behaviour *in vitro*. Calu-3 cells were used between passage 40-43 and cultured in 75 cm^2 tissue culture flasks (Corning® Costar®, Corning Life Sciences, MA, USA), cultured in Dulbecco's Modified Eagle's medium: Nutrient Mixture F-12 (DMEM:F12, Sigma-Aldrich, Sydney, NSW Australia) without phenol red and L-glutamine, including sodium bicarbonate and 15 mM HEPES, and supplemented with 10 % (v/v) fetal calf serum, 1 % (v/v) non-essential amino acid solution, and 1 % (v/v) L-glutamine

solution (200mM) (Invitrogen, Sydney, Australia). Calu-3 cells were grown at 37°C in 5% CO_2 and media was changed every 2 days until confluence was reached. Calu-3 monolayers were grown on the apical side of the Snapwell inserts at seeding density 1.65 x 10^6 cells/well in 200 µL apical and 2 mL of DMEM:F12 media in the basolateral compartment. The apical media was removed after 2 days to establish ALI conditions, with media changed every second day. The monolayers were allowed to differentiate under ALI conditions until confluency reached at day 11.

4.2.2.2 Synthetic membranes for *in vitro* drug permeation studies

Since the amount collected into the basolateral compartment must first be transported across the Calu-3 monolayer and then permeate across the semi-permeable synthetic polyester membrane of the Snapwell insert, separate *in vitro* drug permeation experiments, for both FCI and CFI formulations, were repeated without culturing the Calu-3 cell-line on the membrane, to account for any membrane effect on *in vitro* transport behaviour.

Additionally, since the polyester membrane contain pore sizes of 0.4 µm in diameter, to model the release of encapsulated drug nanoparticles from the liposomes (approximately 80 nm in diameter [119]), followed by permeation of the released encapsulated nanoparticles across the synthetic membrane, additional permeation experiments were repeated across a layer of a regenerated cellulose dialysis membrane (Cellu-Sep®, Regenerated Cellulose Tubular Membrane, 6-8 kDa molecular weight cut-off). The polyester membrane was replaced with a dialysis membrane layer by mounting the dialysis membrane onto the insert using a custom-made 3-D printed ring as previously described in Chapter 3.

4.2.3 Delivery of Free and Liposomal ciprofloxacin to the modified realistic airway model

The physical airway model used to deliver both ciprofloxacin formulations was the modified medium-size VCU MT-TB upper airway model previously described in Chapter 2. This airway model was modified at the first TB bifurcation region (front and back regions) to accommodate two cavities were Snapwell inserts were located. The MT and TB airways were made to snap-fit together, and the TB model was housed in a custom-built acrylic housing-assembly, with an external filter (Suregard®, Bird Healthcare, VIC, Australia) attached at the base of the assembly, connected to a vacuum pump (Westech Scientific Instruments, Bedfordshire, UK) to draw ambient air at the inlet of the airway model, at a controlled volumetric airflow rate of 15 L/min.

The aerosol drug delivery system used in this Chapter was the PARI LC Sprint® nebuliser, used in conjunction with the PARI TurboBOY S compressor, supplied by PARI Medical Holding GmbH (Starnberg, Germany). Prior to airway deposition, Calu-3 cell-culture inserts or synthetic inserts were placed within the modified TB airway model, using the experimental set-up as described in Chapter 3. In brief, 3.0 mL of either FCI or CFI solution was loaded into the reservoir of the PARI nebuliser using a custom-made mouthpiece to connect the PARI nebuliser to the opening of the VCU MT model. To deposit different amounts of ciprofloxacin onto the inserts, the total nebulisation times investigated were 1 minute and 3 minutes to assess the effect of drug mass-concentration on *in vitro* drug transport and permeation behaviour across the cell-line.

4.2.4 Drug permeation and transport following realistic airway deposition

Following airway deposition of FCI and CFI solution into the modified airway model, the nebulised drug was left to settle within the model and housing chamber up to 2 minutes before detachment to prevent any loss of drug to the surrounding environment. The modified TB airway model, which either housed the Calu-3 cultured Snapwell inserts or the synthetic inserts, was disassembled and the drug-deposited inserts transferred to the standard Snapwell system (Corning® Costar®, Corning Life Sciences, MA, USA), without a stirrer in the basolateral chamber to mimic drug dissolution into and permeation and transport of deposited droplets across the airway liquid lining, as opposed to methodology described in Chapter 3. Given the small clearance between the cell monolayer and base of the chamber, the stirrer was removed in this experimental set up as it may result in contact with the monolayer, possibly disrupting the physical barrier. The basolateral compartment was filled with 1.0 ml of either pre-warmed Hank's Balanced Salt Solution (HBSS) buffer (Sigma-Aldrich, Sydney, NSW Australia) for drug transport studies, or PBS buffer (Sigma-Aldrich, Sydney, Australia) for drug permeation studies. The whole Snapwell system was placed in a well-controlled incubator maintained at 5% CO_2 at 37°C to mimic stagnant humid *in vivo* conditions. Frequent aliquots of 200 µL were sampled from the basolateral compartment at pre-determined time points over 4-hours, with fresh, pre-warmed PBS/HBSS replaced immediately to maintain a constant volume. For CFI studies, complete recovery of encapsulated ciprofloxacin drug was achieved by diluting the sample aliquot with 80:20 (v/v) methanol:water as described by Ong *et al* [123]. For drug permeation experiments, the apical compartment was washed with water to recover any remaining drug at the end of 4 hours, or in the case with CFI, further diluted with 80:20 (v/v) methanol:water. For drug transport studies, the monolayer was firstly washed with known volumes of HBSS buffer to quantify the remaining drug left on the cell surface, and in the case

with CFI, the sample was further diluted with 80:20 (v/v) methanol:water. To quantify the amount of drug remaining intracellularly, the monolayer were removed and trypsinated from the membrane using trypsin-EDTA solution (2.5 g/L trypsin, 0.5 g/L EDTA, Invitrogen, Australia) and left on the monolayer for 5 minutes, subsequently lysed in CelLytic™ M Cell Lysis reagent in the presence of 1% (v/v) protease inhibitor cocktail, on ice according to a published protocol from Ong *et al* [84]. Lysates were centrifuged at 10,000 g for 10 minutes at 4°C [84], and the supernatant was aspirated and diluted to an appropriate volume for drug quantification using a validated UFLC methodology as described in Section 4.2.5.

4.2.5 Drug quantification and data analysis

The chromatography conditions for mass quantification of ciprofloxacin-hydrochloride drug was determined as described in Chapter 2. Calculations in the amount of drug being transported and permeated into the basolateral compartment is similarly determined in Chapter 3. The initial mass of drug deposited on the Snapwell inserts was calculated by determining the cumulative mass of drug collected in the basolateral compartment at the end of 4-hours, plus the amount remaining on the apical compartment. Drug transport or permeation profiles of both FCI and CFI were described as cumulative mass of drug being transported across the Calu-3 monolayer, or permeated across the synthetic membrane, into the basolateral compartment as a function of time. The statistical profile comparison employed either unpaired, two-tailed t-test or one-way analysis of variance (ANOVA), where results were found to be significantly different based upon 95% probability values.

4.2.6 Barrier integrity and tight junction functionality of the calu-3 monolayer

4.2.6.1 Trans-epithelial electrical resistance measurements of Calu-3 monolayers post-transport studies

To assess if the dynamic airflow exposure to cells following 1-minute and 3-minutes nebulisation within the airway model induced any shearing stresses onto the Calu-3 monolayer, the barrier integrity and tight junction functionality following post 4-hour transport studies of FCI and CFI deposition was assessed by TEER measurements using an epithelial voltohmmeter (EVOM) with chopstick electrodes (World Precision Instruments, Sarasota, FL, USA). TEER readings were measured by the electrical resistance across the monolayer, which is an indirect measure of tight junction functionality, where higher TEER readings indicate tighter junctions. For TEER measurements following transport studies, the drug-deposited Snapwell inserts were transferred to a new Snapwell system which contained fresh, pre-warmed HBSS buffer in the basolateral compartment. Fresh 200 µL of HBSS buffer was then added on the apical compartment, and the system was equilibrated for 30 minutes in a humidified atmosphere at 5% CO_2 at 37°C. TEER measurements for each Snapwell were measured at 3 different locations. Raw TEER data were corrected by subtracting measurements with the average TEER measurements from blank polyester Snapwell insert and multiplied by the surface area of the membrane (1.13 cm^3). TEER measurements for control (without nebulisation) were carried out for comparison purposes.

4.2.6.2 Paracellular permeability of fluorescein-sodium across Calu-3 monolayers

The apical-to-basolateral flux of the tracer molecule Flu-Na (molecular weight 0.367 kDa, May & Baker Ltd. Dagenham, England) across the Calu-3 monolayer dictate the movement of small molecules via the paracellular route [124]; hence, flux comparison of Flu-Na across the

monolayer following 4-hour drug transport studies were carried out and compared to control to assess any real differences in permeability behaviour as a result of nebulisation. Following TEER measurements, the HBSS buffer on the apical compartment was removed and 200 µl of 2.5 mg/mL Flu-Na solution was applied directly onto the apical compartment. At pre-determined time points, 100 µL aliquots were sampled directly from the basolateral compartment over 1 hour, with fresh HBSS replaced to maintain a constant volume in this compartment. Sample aliquots were diluted into HBSS and fluorescence of Flu-Na was measured using a black 96-well plate via SpectraMax M2 microplate reader (POLAR star Optima, VWR International, Leicestershire, UK) at excitation and emission wavelengths of 485 nm and 520 nm according to Ong *et al* [115]. The apparent permeability coefficient (P_{app}) was calculated according to the following equation:

Equation 2: $$P_{app} = {}^{V}/_{AC_o} . {}^{dC}/_{dt}$$ d

where V is the volume of HBSS buffer in the basolateral chamber

A is the surface area of the Snapwell membrane (1.13 cm^2)

C_0 is the initial concentration of Flu-Na in the apical compartment (µg/mL) and

dC/dt is the flux (rate of change in Flu-Na concentration measured in µg/mL/sec)

Units for P_{app} coefficient are in cm/sec.

Standards of Flu-Na solution were confirmed between 0.125 and 12.5 µg/mL with a coefficient of determination greater than 0.999.

Results and Discussion

4.2.7 Barrier integrity and tight junction functionality of the calu-3 monolayer

Mean (SD) of reported TEER measurements for both nebulised groups exposed to 1-minute and 3-minutes nebulisation of FCI and CFI drug, and the corresponding P_{app} of Flu-Na following 4-hour drug transport studies, are illustrated in Figure 21 A and B, respectively. With respect to the control group, a significant decrease in TEER was observed when nebulising both solutions into the airway model for a duration of 3 minutes, irrespective of the formulation type (Figure 21 A) ($p<0.05$, one-way ANOVA, Dunnett's Post Hoc test), resulting in a decrease in TEER measurements from 974.6 ± 187.5 $\Omega.cm^2$ (control group) to 445.9 ± 210.6 and 462.3 ± 55.98 $\Omega.cm^2$ for FCI and CFI, respectively. No significant decreases in TEER was observed when nebulising both ciprofloxacin formulations for 1 minute, compared to control (600.5 ± 110.0 and 612.6 ± 57.1 $\Omega.cm^2$ for FCI and CFI, respectively; $p>0.05$, one-way ANOVA, Dunnett's Post Hoc test). Furthermore, no significant differences in TEER measurement were also observed between both formulations when tested at both nebulisation conditions ($p>0.05$, unpaired, two tailed t-test), thus indicating that the nebulisation duration being the stronger variable influencing TEER measurements in comparison to formulation type.

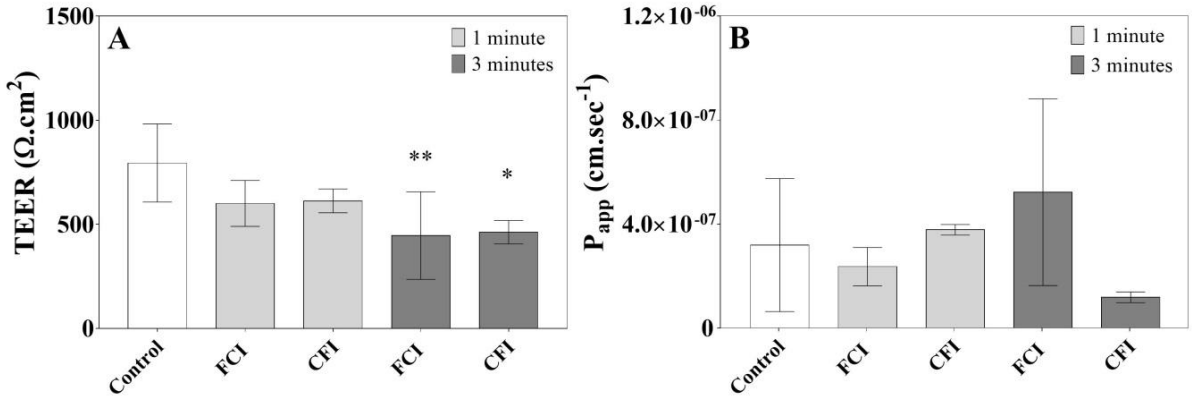

Figure 21. *In vitro* results showing: A) Trans-epithelial electrical resistance (TEER) measurements and, B) derived P_{app} coefficients of Flu-Na flux permeating across the Calu-3 cell monolayer post 4-hour transport studies following 1-minute and 3-minutes nebulisation of Free Ciprofloxacin for Inhalation (FCI) and Ciprofloxacin for Inhalation (CFI) formulations ($n \geq 3$; mean ± [SD]); Statistical analysis was performed using one-way ANOVA, Dunnett's Post Hoc test; * $p < 0.05$; ** $p < 0.01$.

Calculated P_{app} values showed no significant differences compared to the control group irrespective of nebulisation duration and formulation type ($p>0.05$, one-way ANOVA, Dunnett's Post Hoc test), indicating intact monolayers with functional tight junctions (Figure 21 B), despite the observed decrease in TEER measurements following 3-minutes nebulisation. However, it is important to realise that TEER is a measurement of electrical resistance across the cell monolayer, which is a simple and indirect method used to assess epithelial monolayer formation and integrity, rather than differences in paracellular permeability, or characteristic changes in pore sizes across cell-cultured monolayers using paracellular markers such as Flu-Na [125, 126]. To add to this argument, the total amount of drug deposited on the monolayer was hypothesised to be a confounding factor in this Chapter, as a two-fold increase in deposited mass was observed when nebulising for longer durations. However, a previous study from Ong

et al has illustrated that for both FCI and CFI solution formulations, no detrimental effects on the Calu-3 monolayer when ciprofloxacin droplets deposited Calu-3 monolayers, provided the total mass of ciprofloxacin drug collected was less than 20.0 µg [123]. In this Chapter, the total mass of ciprofloxacin drug deposited on the Calu-3 monolayer following 3-minutes nebulisation was less than 7.0 µg (as discussed below), substantiating that the drug mass-concentration due to total nebulisation duration does not have a confounding effect on cellular barrier integrity. With this logic, direct P_{app} measurements may be a more sensitive and reliable method used to confirm the integrity and permeability of deposited drug across the monolayer, despite observing a significant reduction in TEER when monolayers are exposed to the ambient air upon longer nebulisation times.

4.2.8 *In vitro* drug permeation and transport following realistic airway deposition

4.3.2.1 *In vitro* calu-3 transport behaviour of nebulised ciprofloxacin droplets

Cumulative mass profiles of both FCI and CFI droplets transporting across the Calu-3 cell monolayer following 1-minute (circle symbol) and 3-minutes (square symbol) nebulisation into the modified airway model are shown in Figure 22 A and B, respectively. Overall, faster drug transport was observed with increasing dose of both formulations, indicating that the drug mass-concentration was the rate-determining step for *in vitro* Calu-3 transport. For the FCI formulation, approximately 0.74 and 1.90 µg of ciprofloxacin drug was transported by the 4-hour mark when nebulised for 1-minute and 3-minutes, respectively, corresponding to approximately 43.3 and 47.5 % of the total mass collected, respectively. On the contrary, the CFI formulation resulted in significantly slower transport behaviour compared to FCI, with only 0.60 and 0.90 µg ciprofloxacin drug being transported by 4-hours when nebulised at 1-minute and 3-minutes, respectively, corresponding to 23.5 and 13.7 % of total mass collected.

The effect of drug mass-concentration was less evident for CFI, as the cumulative amount transported by 4 hours found to be comparable ($p>0.05$, unpaired, two tailed t-test). This slower rate of drug transport was expected for CFI, since encapsulated nanoparticles first must be released from the liposomes, and then be transported across the Calu-3 cell-line to be sampled from the basolateral compartment.

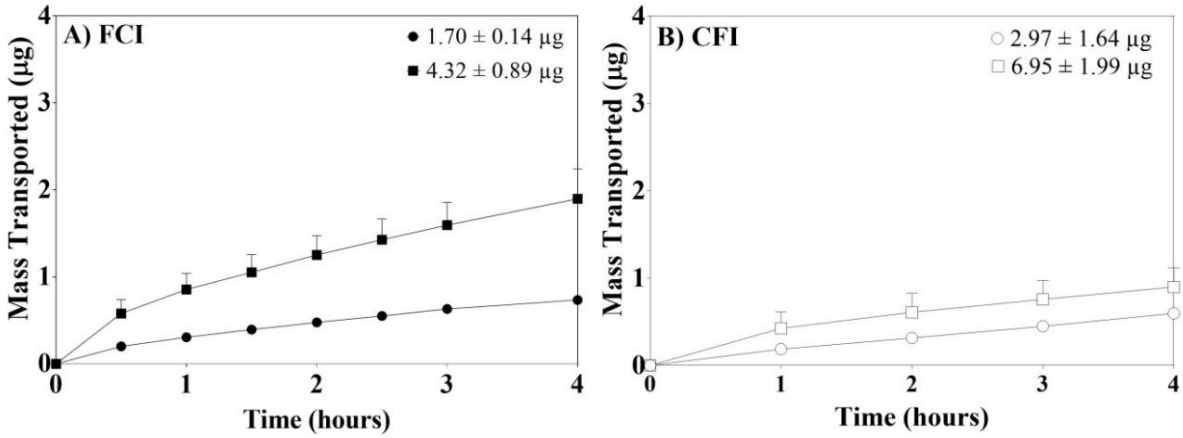

Figure 22. Cumulative mass transport profiles as a function of time following 1-minute (circle symbols) and 3-minutes (square symbols) nebulisation for: A) Free Ciprofloxacin for Inhalation (FCI) and, B) Ciprofloxacin for Inhalation (CFI) droplets transporting across the Calu-3 cell monolayer, respectively, collected within the modified Virginia Commonwealth University tracheal-bronchial airway model ($n\geq3$; mean + [SD]). Legend displays corresponding mean (±SD) masses collected at both nebulisation times.

A previous study by Ong *et al* were able to show using live-cell confocal microscopy imaging techniques an uneven distribution of nebulised CFI droplets across the Calu-3 monolayer and slower diffusional spreading compared to FCI, which additionally explains the slower transport behaviour of CFI reported in this Chapter [123]. For both formulations, faster transport of drug was observed with increasing mass and this was more apparent for FCI (Figure 22A), indicating

that the drug mass-concentration being the rate-determining step for *in vitro* drug transport behaviour. However, since monolayers are cultured onto synthetic PE membranes, to account for any membrane effect on *in vitro* transport behaviour, separate drug permeation experiments were repeated across the synthetic, semi-permeable polyester membrane of the Snapwell insert, tested under the same conditions.

4.3.2.2 *In vitro* drug permeation behaviour of nebulised ciprofloxacin droplets across synthetic membranes

Corresponding cumulative mass profiles of FCI and CFI droplets permeating across the polyester membrane following 1-minute (circle symbol) and 3-minutes (square symbol) are shown in Figure 23 A and B, respectively. Irrespective of total mass collected on the polyester membranes, cumulative mass profiles appeared to be comparable for FCI, resulted in approximately 2.10 and 2.12 µg of ciprofloxacin drug permeating across the polyester membrane by the end of 4 hours, when nebulised at 1-minute and 3-minutes, corresponding to 63.2 and 31.5 % of the total mass collected, respectively. This indicates that the synthetic polyester membrane, not the drug mass-concentration was the rate-determining step driving *in vitro* drug permeation of ciprofloxacin drug into the basolateral compartment. A similar effect was observed with CFI, with comparable amounts permeated by the end of 4 hours ($p>0.05$, unpaired, two tailed t-test). Approximately 2.0 and 2.6 µg of ciprofloxacin drug was permeated across the polyester membrane when nebulising CFI solution for 1-minute and 3-minutes, respectively, corresponding to 48.3 and 38.0 % of total mass collected).

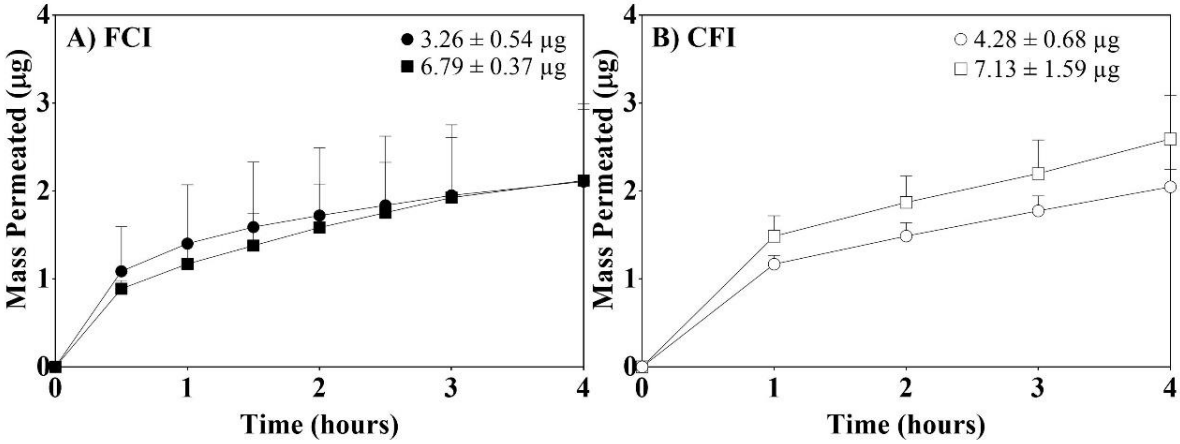

Figure 23. Cumulative mass permeation profiles as a function of time following 1-minute (circle symbols) and 3-minutes (square symbols) nebulisation, permeating across the synthetic polyester membrane for: A) Free Ciprofloxacin for Inhalation and, B) Ciprofloxacin for Inhalation droplets, respectively, collected within the modified Virginia Commonwealth University tracheal-bronchial airway model (n=4; mean + [SD]). Legend displays corresponding mean (±SD) masses collected at both nebulisation times.

Even though the use of synthetic membranes does not resemble the complex structure of the airway epithelium, inferences regarding *in vitro* drug release and permeability characteristics for slow, controlled release formulations can be inferred, depending on selected membrane. For instance, *in vitro* drug permeation for both FCI and CFI droplets appeared to be comparable across the polyester membrane, though a more discriminating capability, in terms of *in vitro* drug release and transport behaviour for both FCI and CFI formulations was observed across the Calu-3 monolayer (Figure 22). This lack in discriminative ability using the polyester membrane can be attributed to the relatively larger pore sizes, i.e., 0.4 µm, compared to reported unilaminar liposomes vesicle size ranges, i.e., 80-90 nm [122], potentially resulting in unilaminar liposomal vesicles which contain encapsulated ciprofloxacin nanoparticles permeating across the polyester membrane before the nanoparticles could be released from the

liposomes. As such, the polyester membrane proved to be inadequate to membrane model *in vitro* release behaviour of encapsulated drug from CFI in this Chapter.

To address this non-discriminating ability, further *in vitro* drug permeation experiments were carried out by replacing the polyester membrane with a layer of regenerated cellulose dialysis membrane, which partitions solutes based on their molecular cut off weight (6-8 kDa) instead of physical size. The cumulative mass profiles of FCI and CFI drug permeating across the dialysis membrane following 1-minute and 3-minutes nebulisation are illustrated in Figure 24 A and B, respectively.

In vitro drug release and permeation behaviour was more distinct between both formulations using the dialysis membrane, with a slower permeation behaviour seen with CFI as nanoparticles first must be released from the liposomes to be permeated across the dialysis membrane. For FCI, the drug mass-concentration appeared to be the rate-determining step for *in vitro* drug permeation, with approximately 1.42 and 2.44 µg of ciprofloxacin drug permeating across the dialysis membrane when nebulised at 1-minute and 3-minutes equivalent to 37.9 and 40.6 % of the total mass collected, respectively. This mass-concentration rate-determining effect was less evident for CFI drug, with comparable amount of ciprofloxacin drug being permeated by the end of 4 hours ($p>0.05$, unpaired, two tailed t-test), resulting to approximately 1.09 and 0.85 ug of drug permeated across the dialysis membrane when nebulised at 1-minute and 3-minutes, respectively, corresponding to 30.6 and 10.4 % of total drug collected.

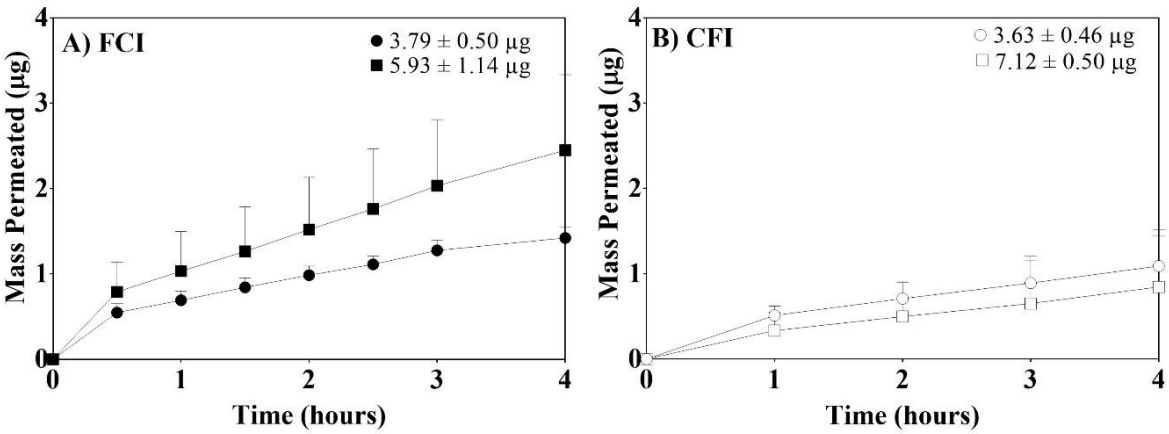

Figure 24. Cumulative mass permeation profiles as a function of time following 1-minute (circle symbols) and 3-minutes (square symbols) nebulisation, permeating across the dialysis membrane for: A) Free Ciprofloxacin for Inhalation and, B) Ciprofloxacin for Inhalation droplets, respectively, collected within the modified Virginia Commonwealth University tracheal-bronchial airway model (n≥3; mean + [SD]). Legend displays corresponding mean (±SD) masses collected at both nebulisation times.

Overall, the polyester membrane demonstrated comparable *in vitro* drug permeation profiles, irrespective of drug mass-concentrations and formulation type (Figure 23), whereas the regenerated cellulose dialysis membrane was able to differentiate between these variable factors (Figure 24), assuming the molecular weight cut off parameter is the sole determination feature for drug partitioning in this scenario. These apparent differences in permeation behaviour across the selected synthetic membranes can be attributed to differences in intrinsic properties of the membrane itself, i.e. membrane thickness, molecular-weight cut off range, nominal pore size and porosity.

Even though it is evident that the use of synthetic membranes as "representative" airway epithelial barriers can be used to collate useful information regarding drug release and permeability characteristics, it should be considered as a simplified approach with several limitations, i.e. lack in surface composition and lipophilicity properties. Specifically, of the two

synthetic membranes selected in this Chapter, the regenerated cellulose dialysis membrane better resembles *in vitro* Calu-3 transport profiles for both formulations (Figure 22), potentially being the more "representative" membrane out of the two for nebulised ciprofloxacin drug. This similarity in profiles could be due Calu-3 monolayers exhibiting tighter pore sizes, which may reflect the tighter partitioning behaviour when using the dialysis membrane.

Despite the use of a well-differentiated and characterised Calu-3 cell-line used to model epithelial transport behaviour *in vitro*, this set up does not take into account other clearance mechanisms such as mucociliary escalator, cough mechanisms, phagocytosis by macrophages, and metabolism; however from a research stand point, this novel cell-based upper airway model closely mimics the *in vivo* human physiology, compared to conventional cascade impactors and therefore allows more realistic information to be drawn regarding drug permeation and transport behaviour.

4.3 Summary

The successful application of a physiologically-relevant cell-based model used to mimic drug dissolution, epithelial transport and consequently absorption characteristics depends on how closely the model can mimic *in vivo* properties. This Chapter described the extension and application of a unique *in vitro* cell-based upper airway model intended to mimic the human environment and study the pulmonary fate of OIPs. Through this unique approach, *in vitro* drug permeation and transport of two nebulised ciprofloxacin formulations has shown to differ kinetically in terms of drug mass-concentration, formulation type and most importantly, the selected physical barrier used to partition the apical and basolateral compartment.

In conclusion, it is important to understand how these inhaled aerosols interact at the airway epithelia, especially for poorly soluble drugs or for complex, controlled-release dosage formulation and its role on the overall efficacy and therapeutic ratios of the treatment regimen.

CHAPTER 5

Summary and Future Directions

5.1 Summary

This thesis describes the development and validation of a new hybrid *in vitro* approach used to characterise realistic airway deposition and the events following deposition of aerosols at the epithelia. Deposition experiments were carried out in a modified version of the medium-sized VCU MT – TB airway model, initially developed by researchers from VCU using anatomical data of the respiratory tract of healthy normal adults. The airway model was intentionally modified to accommodate Calu-3 monolayers cells cultured onto Snapwell inserts within the model for the collection of aerosols.

Fundamentally, it was important to place the inserts at a region within the model to ensure sufficient drug collection for analysis, without causing significant changes to airway deposition behaviour. As described in Chapter 2, two cavities were placed at the first bifurcation region in the TB airway model, one at the front and one at the back. This has resulted in an increase of 1.0 % in total recovered dose in the TB model, in which we have concluded acceptable since this increase is expected to overlap with the large lung deposition variations seen in healthy patients.

Therefore, as a proof-of-concept study, since culturing cells onto the synthetic membranes is a time-consuming procedure, we initially wanted to verify and validate the modified airway, when coupled with the Snapwell test system can be used as a hybrid *in vitro* tool for drug permeation analysis. In Chapter 3, we explored this concept by assessing different permeable membrane materials and evaluated how different experimental parameters, i.e., aerosol

collection method and presence of a stirrer in the receptor compartment would influence the rate and extent of drug permeation behaviour *in vivo*. Although the synthetic membranes do not resemble the complex structure of the epithelial airway barrier, inferences regarding drug permeability behaviour for aerosolised drug within a representative physical airway model can be made using this novel *in vitro* set up. Overall, we have concluded that this *in vitro* hybrid approach proved to be feasible with a high-dose ciprofloxacin solution formulation, when nebulised for a total duration of 5 minutes.

This hybrid approach was then extended to assess *in vitro* drug dissolution into, and subsequent cellular transport behaviour across cultured Calu-3 monolayers of deposited aerosols collected in the proposed modified airway model. To assess the feasibility and discriminatory ability of this hybrid approach, two different ciprofloxacin formulations were used – an aqueous and a liposomal formulation. A compromise had to be made between total nebulisation duration and the overall integrity of monolayers following air exposure, since longer nebulisation times could potentially induce shear stresses onto the monolayer, compromising barrier integrity and tight junction properties, hence affecting drug permeation and transport behaviour. In Chapter 4, the total nebulisation duration was decreased from 5 minutes to either 3 minutes or 1 minute. Irrespective of nebulisation time, no apparent changes in P_{app} was observed when compared to untreated conditions, though a significant decrease in TEER measurements were observed when nebulising the two formulations at longer durations. Overall, we have concluded that TEER is a simple and indirect method used to assess epithelial monolayer formation and integrity, rather than differences in paracellular permeability, or changes in pore sizes. Interesting, the dialysis membrane acted as the "most representative" physical barrier to model *in vitro* transport and drug permeation behaviour of deposited ciprofloxacin droplets on the Calu-3 cell-line. Using representative membranes as a quick and reproducible screening tool would complement cellular uptake studies in future studies.

In conclusion, the number of *in vitro* tools used to investigate airway deposition and post-deposition behaviour of pharmaceutical aerosols is enormous and still increasing. With this thesis, new representative *in vitro* test methods and approaches have been proposed which have shown great promise as *in vivo* estimates. It is to be hoped that such can be used as potential screening tools for head-to-head comparative studies without resorting to expensive clinical trials and animal testing, as well as a foundation for the design of new inhalation platforms with improved performance efficiency and reproducibility.

5.2 Future Directions

One of the acknowledged limitations of this thesis is that the method of establishing and validating a simple yet practical approach used to characterise *in vitro* realistic deposition and the events following airway deposition have heavily relied on ciprofloxacin as the drug model, administered from the commercially-available PARI nebuliser. To further assess the method's robustness, it would be advantageous to evaluate other commonly-used drug candidates (i.e., poorly-soluble corticosteroids) delivered across different inhaler platforms (i.e., DPI, MDIs, and add-on devices such as spacers). In this framework, differences and/or similarities in *in vitro* airway deposition behaviour across different drug candidates and inhaler device platforms may be observed, as well as the mechanisms underlying drug dissolution and cellular transport profiles of inhaled, deposited aerosols. Leading to this, the developmental scientist may further refine the formulation excipients and/or via aerosol generation mechanisms to improve the overall performance efficiency of the treatment regime.

Another stimulating avenue for future investigation would be to assess different cell-line models to better understand the underlying mechanisms governing pulmonary absorption of inhaled aerosols. Up to this point, the Calu-3 cell-line was selected due to it being a similar

morphology to bronchial epithelial cells *in* vivo. Using different cell-lines (i.e., immortalised versus primary cells, healthy versus diseased cell models) which exhibit different polarised cell layers with different functional tight junctions and transporter proteins would be another means to assess the method's feasibility. Such approaches can be utilised to better understand lung retention properties and pulmonary half-life owing to the complex interactions with the deposited aerosols and airway epithelia.

As noted earlier, ciprofloxacin drug was nebulised into the modified airway model using ambient air at a constant flow rate of 15 L/min. Another potential area for future research is to test the hybrid model across a range of representative inspiratory flow rates, i.e., sampling at higher, as well as variable flow rates using a breath simulator at warm, humid conditions to mimic patient conditions. Such studies will provide insightful observations pertaining ranges in airway deposition behaviour across different air flow rates, as well as provide a better understanding in epithelial barrier dysfunction in response to dynamic sheer stresses from the air flow. Such testing regimes will provide further insight into the method's capability in terms of acceptable testing conditions (intensity and duration of flow rate).

Lastly, as differences in airway geometry would result in differences in drug deposition patterns, expanding this hybrid concept to the "large" and "small" VCU representative airway models (as shown in Chapter 1, Figure 6) would provide valuable information as to how structural differences in airway would affect how much drug could be collected onto the Snapwell inserts. Additionally, expanding the model to look at the lower region of the lungs is another interesting avenue to delve into; however, the challenging aspect to this is fitting the Snapwell insert at these small regions.

By acknowledging the existing limitations and examining future investigations to address these boundaries, it is hoped that the working foundations of this thesis can be used to better

understand airway deposition of inhaled pharmaceutical aerosols in a more realistic manner, and the events following airway deposition at the epithelial level.

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APPENDIX

Attachment 1: Conference paper in the Respiratory Drug Delivery, Europe 2017.

Validating a Realistic Physical Airway Model to Investigate *In Vitro* Drug Deposition, Dissolution, and Epithelia Transport of Orally Inhaled Products

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KEYWORDS: realistic physical models, total airway deposition, ciprofloxacin hydrochloride (CIP-HCL)

INTRODUCTION

Recent efforts to establish clinically-appropriate *in vitro* test methods to study total lung deposition for orally-inhaled products (OIPs) have been widely recognized [1, 2]. Yet, there is an increasing interest to study post-deposition events in the lung such as particle dissolution and epithelial transport of OIPs in a clinically-relevant manner [3]. Most of the current approaches to predict the dissolution and transport behavior of respirable-sized particles have been based on a combination of compendial impaction and dissolution methods [4]; however, these are not surrogates of the respiratory tract and may not necessarily represent deposition patterns or absorption processes in the lung [5]. The focus of this study is to develop and validate a realistic upper airway model by modifying the medium-sized Virginia Commonwealth University (VCU) mouth-throat (MT) and upper trachea-bronchial (TB) model to study post-deposition events in the lung for OIPs *in vitro*.

METHOD

As a proof-of-concept study, an in-house aqueous solution of 20 mg/ml ciprofloxacin hydrochloride (CIP-HCL) was formulated and delivered to the medium-sized VCU MT-TB airway model using the PARI LC® Sprint nebulizer (PARI Medical Holding GmbH, Starnberg, Germany), powered by the PARI TurboBOY® S compressor (PARI Medical Holding GmbH, Starnberg, Germany).

Aerodynamic and droplet size distribution characteristics of the nebulized droplets exiting the MT model were determined using the USP Apparatus 5 (Westech W7, Westech Scientific Instruments, Bedfordshire, UK) and the Spraytec apparatus (Malvern Instruments Ltd, Malvern, UK), respectively, sampled at 15 L/min for 90 seconds. The Spraytec inhalation cell was used in-line with the Spraytec apparatus, with a filter placed downstream to collect the nebulized droplets. Modified and unmodified versions of the TB model were constructed by rapid prototyping using acrylonitrile butadiene styrene (ABS) plastic (Dimension Elite, MN, USA). To visualize where CIP-HCL droplets deposit on the model, a transparent version was 3D printed using methacrylate photopolymer resin (Formlabs Inc, MN, USA). Before deposition experiments, the internal surfaces of the transparent model were coated with Sar-Gel® paste (Spill Crew Corporation, NSW, Australia), a paste that turns purple upon contact with water. The modified TB model was altered to accommodate two Snapwell inserts (1.12 cm^2 polyester, Corning® Costar®, MA, USA) at the first bifurcation in the TB region (front and back). Total airway deposition studies were performed by housing the airway model within a cylindrical chamber, with a filter attached at the base connected to a vacuum pump source as shown in Figure 1. A mouth-piece adaptor was created to attach the nebulizer directly to the entrance of the MT model. Experiments were performed at 15 L/min for a total nebulization time of 90 seconds. Drug quantification was determined by high performance liquid chromatography using a validated method [6].

Figure 1. Physical test apparatus used to assess total airway deposition.

RESULTS AND DISCUSSION

The aerodynamic and droplet size distributions, assuming a unit density value, of nebulized CIP-HCL exiting the MT model are shown Figure 2. A good correlation was observed between the aerosol deposition characteristics collected with the USP Apparatus 5 and Spraytec apparatus. The fine particle fraction (particles less than 5.8 μm) of nebulized CIP-HCL was calculated to be 64.0% and 62.0%, for the NGI and Spraytec data, respectively. Time-lapse images of nebulized CIP-HCL droplets deposited on the transparent unmodified TB model coated with Sar-Gel are shown in Figure 3. Deposition due to inertial impaction at the first bifurcation region became visible after 30 seconds of nebulization. Complete airway deposition was apparent after 60 seconds. No dripping of the Sar-Gel paste was observed during the experiments.

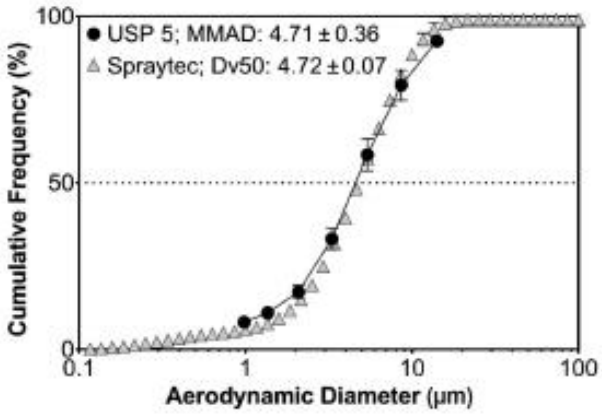

Figure 2. Droplet and aerodynamic particle size distribution of nebulized CIP-HCL exiting the VCU MT model (n = 3; mean ± SD).

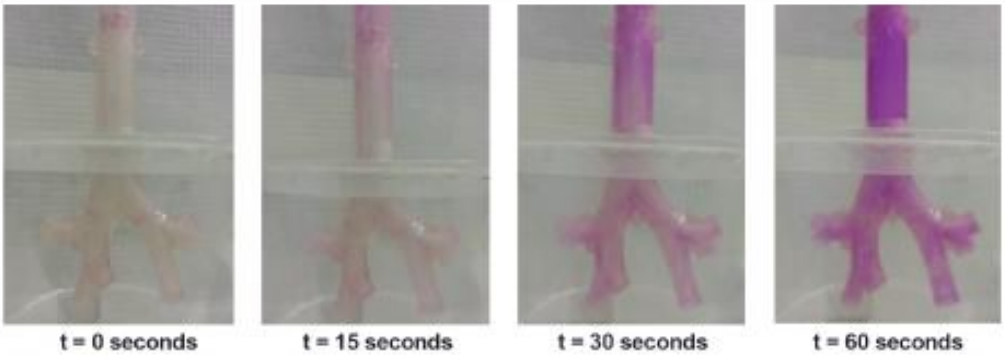

Figure 3. Deposition patterns of nebulized CIP-HCL in the TB model.

Quantification of CIP-HCL in the nebulizer, MT and TB models (modified and unmodified), collected on the cylindrical housing chamber and filter (which can be regarded as the pulmonary section) are illustrated in Figure 4. On average, 12.0 mg of CIP-HCL was delivered from the nebulizer, of which around 350 µg was deposited on the TB model, and approximately 10 µg was collected on each Snapwell insert when nebulized for 90 seconds. Total mass deposited in MT and TB for both modified and unmodified airway models were statistically comparable ($p>0.05$, unpaired two-tail t-test).

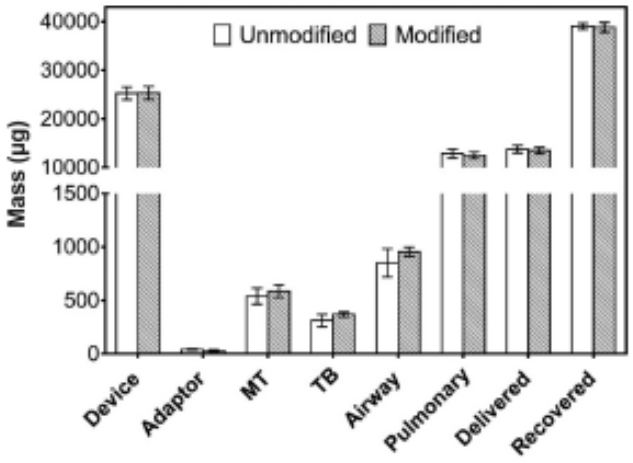

Figure 4. *In vitro* deposition results of nebulized CIP-HCL in the modified and unmodified airway models (n = 3; mean ± SD).

CONCLUSION

This proof-of-concept study fundamentally illustrates no significant changes in CIP-HCL airway deposition behavior when the medium VCU TB airway model was modified to accommodate two Snapwell inserts at the first bifurcation. Future work will investigate how this "realistic physical airway model" could enable researchers to examine post-deposition behavior of OIPs in the lung in a more clinically relevant manner.

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The Development and Validation of an *In Vitro* Airway Model to Assess Realistic Airway Deposition and Drug Permeation Behavior of Orally Inhaled Products Across Synthetic Membranes

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Abstract

Background: Current *in vitro* approaches to assess lung deposition, dissolution, and cellular transport behavior of orally inhaled products (OIPs) have relied on compendial impactors to collect drug particles that are likely to deposit in the airway; however, the main drawback with this approach is that these impactors do not reflect the airway and may not necessarily represent drug deposition behavior *in vivo*. The aim of this article is to describe the development and method validation of a novel hybrid *in vitro* approach to assess drug deposition and permeation behavior in a more representative airway model.
Methods: The medium-sized Virginia Commonwealth University (VCU) mouth–throat (MT) and tracheal–bronchial (TB) realistic upper airway models were used in this study as representative models of the upper airway. The TB model was modified to accommodate two Snapwell® inserts above the first TB airway bifurcation region to collect deposited nebulized ciprofloxacin–hydrochloride (CIP-HCL) droplets as a model drug aerosol system. Permeation characteristics of deposited nebulized CIP-HCL droplets were assessed across different synthetic membranes using the Snapwell test system.
Results: The Snapwell test system demonstrated reproducible and discriminatory drug permeation profiles for already dissolved and nebulized CIP-HCL droplets through a range of synthetic permeable membranes under different test conditions. The rate and extent of drug permeation depended on the permeable membrane material used, presence of a stirrer in the receptor compartment, and, most importantly, the drug collection method.
Conclusions: This novel hybrid *in vitro* approach, which incorporates a modified version of a realistic upper airway model, coupled with the Snapwell test system holds great potential to evaluate postairway deposition characteristics, such as drug permeation and particle dissolution behavior of OIPs. Future studies will expand this approach using a cell culture-based setup instead of synthetic membranes, within a humidified chamber, to assess airway epithelia transport behavior in a more representative manner.

Keywords: realistic physical airway models, drug permeation, ciprofloxacin-hydrochloride (CIP-HCL), *in vitro* test methods, synthetic permeable membranes

Introduction

DELIVERING LOCALLY ACTING DRUGS intended for the treatment of respiratory disorders through the pulmonary route has the potential to achieve higher regional drug concentrations while minimizing systemic exposure, resulting in fewer and less severe adverse effects.[1] As with most dosage forms, drug performance and quality assurance may be assessed through a range of *in vitro* test apparatuses and *in vivo* methods; however, the relationship between *in vitro*

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outcomes for orally inhaled products such as particle dissolution, drug permeation, and cellular transport behavior of locally acting drugs and their clinical performance is not straight forward.[2] After drug deposition onto the airway surface, the deposited drug must dissolve in the limited airway lining, and then permeate through the epithelia layer for subsequent drug absorption to render a therapeutic effect.[3]

To mimic *in vitro* absorption across the human airway epithelia, a number of researchers have modified the cascade impactor with integrated epithelial cell monolayers to deposit respirable-sized particles directly onto cells[4,5]; however, the main drawback in this approach is that these nozzle-based impactors may not necessarily represent the deposition behavior of inhaled drug *in vivo*.[6] To address this issue, a preliminary study by Huynh et al.[7] has evaluated the feasibility of modifying the medium-sized Virginia Commonwealth University (VCU) mouth–throat (MT) and tracheal–bronchial (TB) realistic upper airway model to accommodate Snapwell® inserts within the TB region, without any cells for drug collection and sampling. The airway model used in their study was a physical representation of the upper airway of an averagely sized human, which has provided excellent *in vitro* total lung dose predictions with *in vivo* outcomes.[8, 9]

As a proof-of-concept study, Huynh et al.[7] were able to show high drug deposition hot spots in the upper and first TB airway bifurcation regions when ciprofloxacin-hydrochloride (CIP-HCL) solution was nebulized into the airway model. Two Snapwell inserts were placed above the first TB bifurcation region by drilling two 14.0 mm diameter holes, one at the front and the other at the back, with the inserts placed from the outside of the model to ensure internal flush conditions with the inner tracheal walls.[7] *In vitro* deposition studies of nebulized CIP-HCL showed no significant changes in airway deposition behavior between the unmodified and modified prototype models.[7]

We hypothesis in this study that this initial prototype of the modified-drilled TB airway model could be further modified to accommodate two Snapwell inserts within the model at these regions of interest to evaluate postdeposition behavior *in vitro*. Therefore, the aims of this study are to (a) modify and validate the existing prototype of the medium-sized VCU MT-TB airway to accommodate Snapwell inserts within the model for drug collection, (b) evaluate the feasibility in adapting the Snapwell test system to assess the permeation characteristics of already dissolved CIP-HCL solution through a series of different synthetic permeable membranes, and (c) utilize the optimized Snapwell test system to characterize and compare the permeation profiles of nebulized CIP-HCL droplets collected onto the Snapwell inserts within the modified TB airway model.

Materials and Methods

Development and validation of the modified airway model

The TB airway model was modified by dividing it into two separate halves to allow the insertion and removal of the standard polyester (PE) membrane Snapwell inserts (Corning® Costar® Snapwell cell-culture inserts, 12.0 mm with 0.4 μm pore size; Corning Life Sciences, MA), and a cavity at the first TB bifurcation was incorporated as shown in Figure 1C (MOD#2). The unmodified and previously modified (MOD#1) models of the medium-sized TB airway model are also displayed in Figure 1. The medium-sized MT (not shown) and modified TB airway model used in this study were manufactured from a clear acrylic photopolymer resin (GPLCL02) at a resolution of 0.025 mm using 3D rapid prototyping (Form 2 Desktop SLA 3D Printer; Formlabs, Inc., MN).

In vitro *realistic airway deposition of nebulized CIP-HCL solution*

An in-house formulation of CIP-HCL aqueous solution, 20 mg/mL, was chosen as the model drug for this study. The formulation was prepared by dissolving CIP-HCL powder (MP Biomedical Australasia Pty Limited, NSW, Australia) in Milli-Q water. The MT model was made to snap fit on top of the TB model. Airway deposition of nebulized CIP-HCL solution onto the medium-sized MT and unmodified and modified (MOD#2) TB airway models were performed using the setup as shown in Figure 2.[7]

In brief, the TB airway region was housed in a custom-built housing assembly, with a vacuum pump attached at the base of the housing assembly, connected to a filter (Suregard®, Bird Healthcare, VIC, Australia) to draw ambient air through the airway model at 15.0 L/min. Three milliliters of CIP-HCL solution was loaded into the reservoir of a PARI LC Sprint® nebulizer (PARI Medical Holding GmbH, Starnberg, Germany) and delivered into the airway model by the PARI TurboBOY S compressor (PARI Medical Holding GmbH, Starnberg, Germany) for 5 minutes. Recovery of CIP-HCL in the PARI nebulizer, airway model, housing assembly, and filter was determined using known volumes of deionized water and quantified using ultra-fast liquid chromatography (UFLC) (See Drug quantification and data analysis section). Airway deposition experiments for each test condition were performed in triplicate.

Adapted in vitro *Snapwell system for drug permeation*

Suitability and discriminative ability of the Snapwell system for drug permeation. The Snapwell system consisted of a custom-made 3D-printed miniature chamber to accommodate a magnetic bar stirrer (8×3 mm, Westlab Group, Victoria, Australia) in the receptor compartment. The system was housed in a water bath at 37°C. A cross-section of the apparatus is shown in Figure 3. Components of the system were manufactured from a clear acrylic photopolymer resin (GPLCL02) (Form 2 Desktop SLA 3D Printer; Formlabs, Inc.).

To assess the feasibility of the Snapwell test system, the permeation characteristics of the CIP-HCL solution was first assessed across the standard Snapwell PE membrane without any cells. Although the synthetic membrane does not resemble the complex structure of the airway epithelia, inferences regarding membrane permeability behavior can be made using this simplified setup.

Snapwell inserts were presoaked with phosphate-buffered saline (PBS) (Sigma-Aldrich, Sydney, Australia) up to 2 hours before permeation experiments. A 100.0 μL aliquot of 100.0 μg/mL CIP-HCL solution was applied directly on top of the membrane of the Snapwell insert that was placed in the holder chamber at time = 0. The receptor compartment

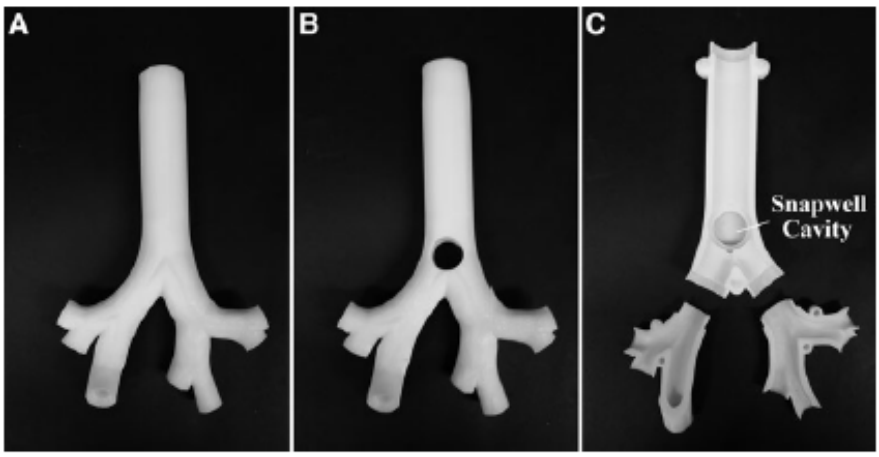

FIG. 1. The medium-sized TB airway model: **(A)** unmodified, **(B)** initial modified prototype (MOD#1) with two 14.0-mm diameter holes drilled at the first TB bifurcation region used in the previous study by Huynh et al.,[7] and **(C)** split modified version (MOD#2) used in this study, with one cavity at the first bifurcation region (one at the front and one at the back) for the insertion and removal of the Snapwell inserts. The medium-sized TB airway models displayed in this figure were constructed of ABS plastic using fused deposition modeling 3D printing technology (Dimension Elite, MN) for visualization purposes. ABS, acrylonitrile butadiene styrene; TB, tracheal–bronchial.

consisted of 2.3 mL of PBS at pH 7.4 and was filled to ensure complete contact with the receptor-side membrane surface. A sample aliquot of 500.0 μL was withdrawn from the receptor compartment using a syringe at different time intervals for a period of 2 hours, with fresh prewarmed PBS replaced immediately to maintain a constant volume of 2.3 mL in the receptor compartment.

At the end of each experiment, the membrane was washed with deionized water to recover any remaining CIP-HCL on top of the membrane. The total amount of CIP-HCL permeated was calculated by determining the cumulative amount collected in the receptor compartment after 2 hours, plus the amount remaining on top of the membrane. The influence of a stirred versus a nonstirred receptor on the permeation behavior was investigated, with and without a magnetic stirrer in the receptor compartment. Permeation experiments for each test condition were performed in triplicate.

To investigate the discriminatory ability of the proposed Snapwell system, modified Snapwell inserts were created by removing the PE membrane from the insert and attaching either a polyamide (PA) membrane (Sartorius™, 0.45 μm pore size) or a layer of a regenerated cellulose dialysis membrane (Cellu-Sep®, regenerated cellulose tubular membrane, 6–8 kDa molecular weight cutoff) onto the insert. A 3D-printed ring was manufactured (Form 2 Desktop SLA 3D Printer; Formlabs, Inc.) to hold the membrane onto the Snapwell insert, using an approach similar to that reported by May et al.[10] Membranes were presoaked in PBS for 2 hours before experiments and 100.0 μL of 100 μg/mL CIP-HCL solution was applied directly onto the membrane. Experiments were tested with and without a stirrer in the receptor compartment. Permeation experiments for each test condition were performed in triplicate.

FIG. 2. Physical setup of the modified airway model (MOD#2) used to assess *in vitro* airway deposition of nebulized CIP-HCL. CIP-HCL, ciprofloxacin–hydrochloride.

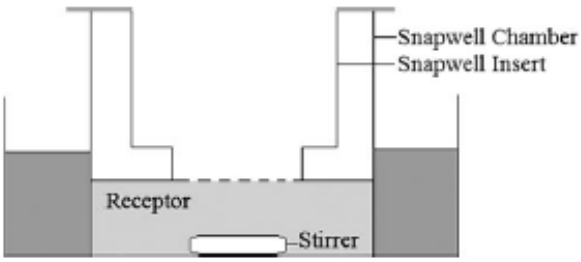

FIG. 3. *In vitro* experimental setup of the Snapwell test system

Permeation characteristics of nebulized CIP-HCL droplets collected on the modified model. To assess the permeability behavior of nebulized CIP-HCL droplets, two standard PE or modified Snapwell inserts (PA and dialysis membranes) were placed within the modified TB airway model (MOD#2) before realistic airway deposition experiments, to collect deposited nebulized CIP-HCL droplets onto the synthetic membrane inserts. Before airway deposition experiments, the inner walls and internal surface of the Snapwell inserts were covered with a removable thin 3D-printed sheet (Form 2 Desktop SLA 3D Printer; Formlabs, Inc.) as recommended by May et al.,[10] to allow for more accurate quantification of drug deposited onto the synthetic membranes. Airway deposition experiments were carried out using the same test conditions as described previously.

After deposition, the two inserts were transferred onto the Snapwell test system to assess the permeation characteristics through the different membranes, with a magnetic stirrer placed in the receptor compartment. Work from Arora et al. placed additional dissolution medium on the drug-deposited membranes to further facilitate the dissolution and permeation of the collected corticosteroid particles through the membrane.[11] Henceforth, additional experiments were conducted to assess whether the permeation of nebulized CIP-HCL droplets can be further facilitated through the membrane by adding 100.0 μL of deionized water on top of the drug-deposited membrane. Permeation experiments for each test condition were performed in triplicate.

Drug quantification and data analysis

Drug quantification of CIP-HCL was performed using the Shimadzu Prominence UFLC system (Shimadzu Corporation, Kyoto, Japan). The system consisted of an SPD-20A UV-vis detector, LC-20AD liquid chromatography, SIL-20A HT Autosampler, and Luna C-18 (2) 100A column (3 μm, 150 × 4.6 mm) (Phenomenex Pty, Ltd, Lane Cove, Australia). Chromatography conditions were conducted using a mobile phase composition of methanol and 0.1 M sodium dihydrogen phosphate at a 30:70 (v/v) ratio, with pH adjusted to 3.30 with phosphoric acid. The flow rate was set to 0.8 mL/min and 20.0 μL of each sample was injected into the column, with the column temperature set to 40°C and the detection wavelength of 275 nm. Linearity was obtained between 0.1 and 50.0 μg/mL ($R^2 > 0.999$) at a retention time of 6.55 minutes. All solvents used were of analytical grade (Chem-Supply Pty Ltd, South Australia, Australia).

The statistical profile comparison employed unpaired two-tail *t*-test, where $p < 0.05$ was considered to be significant.

Results and Discussion

In vitro *realistic airway deposition of nebulized CIP-HCL solution*

Mass quantification, expressed as percentage total recovered dose of CIP-HCL remaining in the PARI nebulizer device, deposited on the MT and TB models, and ex-TB (determined by the drug recovered from the housing chamber and filter) using the modified and unmodified airway models are shown in Figure 4. Collectively, deposition on the modified TB model was determined by the drug recovered on the TB model, Snapwell inserts, and 3D-printed sheets.

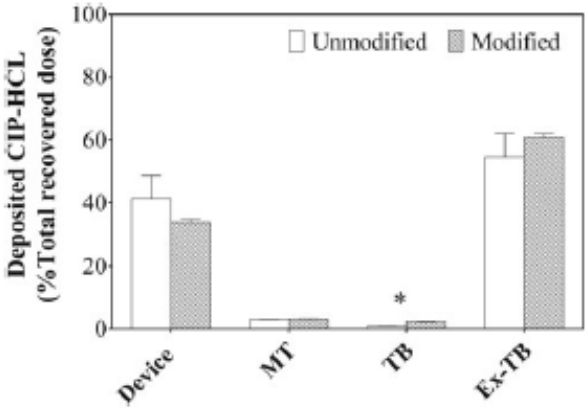

FIG. 4. *In vitro* deposition results of nebulized CIP-HCL in the unmodified (*white bars*) and modified (*gray bars*) airway models for a total nebulization time of 5 minutes ($n = 3$; mean [error bars are ± SD]). Results are expressed as percentage total recovered dose. Ex-TB includes amount collected on the housing chamber and filter. *Asterisks* (*) indicates a statistically significant difference at $p = 0.05$.

No significant differences in MT deposition was observed in the unmodified and modified airway models, resulting in mean (SD) CIP-HCL deposition of 2.89% ± 0.06% and 3.13% ± 0.14% of the total recovered dose, respectively ($p > 0.05$, unpaired two-tail *t*-test); however, an increase in deposition was observed in the modified TB model (MOD#2), from 1.20% ± 0.075% to 2.18% ± 0.188% ($p < 0.05$, unpaired two-tail *t*-test). This observation conflicted with our previous modified prototype (MOD#1), which illustrated no significant changes in TB deposition.[7] It is important to note that the MOD#1 prototype was modified by drilling two holes to place the Snapwell inserts on the outside for complete flush conditions and for quick mass quantification of drug collected at this region; whereas in this study, MOD#2 comprised two cavities to house the inserts within the model and was not completely flush, hence most probably affecting the overall deposition behavior.

It is important to realize that large variations in whole lung deposition have been reported in healthy human subjects with gamma scintigraphy studies revealing high variations ranging from 20% to 60% of the total metered dose for a single test inhaler[12]; furthermore, work by Delvadia et al.[8,9] has effectively captured this large variation seen *in vivo* by scaling the medium-sized VCU MT-TB airway model to generate small and large versions, and coupling these models with clinically relevant inhalation maneuvers; therefore, it could be concluded that the observed 1% increase in TB deposition lies within the expected variation observed *in vivo*. Although large variations seen *in vivo* are likely due to the range of airway geometries and differences in inspiratory maneuvers, the variations observed in this study are likely due to the structural changes within the airway model, that is, integration of the Snapwell inserts and 3D-printed sheets.

Nevertheless, the main aim of this study was to develop and modify the realistic medium-sized VCU MT-TB upper

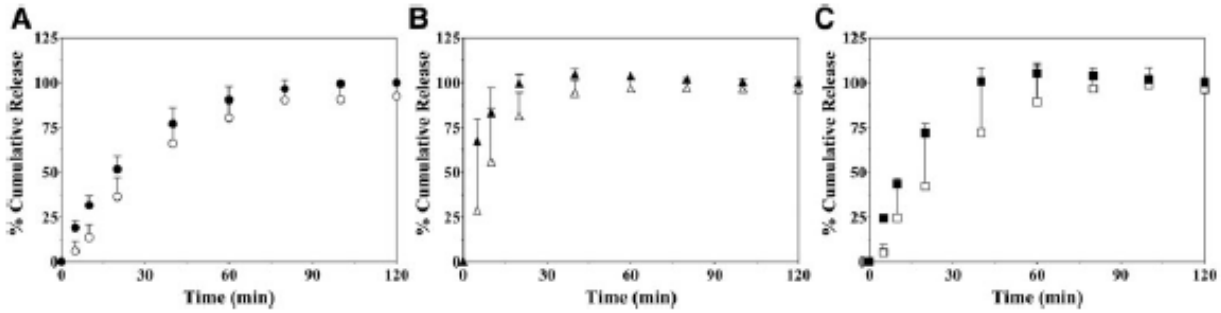

FIG. 5. Percentage cumulative mass release of CIP-HCL solution through the **(A)** polyester, **(B)** polyamide, and **(C)** dialysis membrane inserts into the receptor compartment, tested at static (*empty symbols*) and agitated (*filled symbols*) conditions ($n=3$; mean [error bars are + SD]).

airway model primarily to collect aerosolized drug onto Snapwell inserts in a more representative model for post-deposition evaluation. Successfully, 8.2 ± 1.7 and $13.2 \pm 2.2\,\mu g$ of CIP-HCL were deposited on the front and back Snapwell inserts, respectively, when nebulized for 5 minutes. Collectively, approximately 25.0 μg of CIP-HCL was collected on the individual 3D-printed Snapwell sheet covers.

Adapted in vitro *Snapwell test system for drug permeation*

Suitability and discriminative ability of the Snapwell system for drug permeation. The percentage cumulative mass of CIP-HCL solution permeating through the PE, PA, and dialysis membranes into the receptor compartment, tested under static (no stirrer) and agitated (stirrer) conditions is shown in Figure 5A–C, respectively. It was apparent that the permeation behavior of already dissolved CIP-HCL differed considerably through the selected membranes. For the PA membrane, there was fast initial permeation with complete transfer of CIP-HCL observed after 20 minutes when a magnetic stirrer was placed in the receptor compartment. Slower, yet complete transfer was observed for the other two membranes.

This can be explained by the differences in membrane structure and the physical interactions between the aqueous solution and the material surface. The aqueous solution has a better affinity to the most hydrophilic PA membrane, resulting in complete wetting of the solution onto the whole surface area, whereas with the tissue culture-treated PE membrane and dialysis membranes, poorer surface wettability behavior was observed at the beginning of the experiments when the solution was loaded directly on top of these membranes, which may explain the slower and more gradual permeation through these two membranes. Similarly, May et al.[10] reported slower drug permeability behavior from the PE membrane for a range of drug substances, with scanning electron microscopy images showing PE being less porous and having fewer permeation areas.

As expected, stirring in the receptor compartment provided adequate mixing of CIP-HCL solution and PBS in the receptor compartment, resulting in smaller experimental variability.[13]

Permeation characteristics of nebulized CIP-HCL droplets collected on the modified model. The permeation behavior of nebulized CIP-HCL droplets, which have been collected onto the front and back Snapwell inserts in the modified TB airway model, with or without additional water added on the PE, PA, and dialysis membranes is shown in Figure 6A–C, respectively. Only approximately 50.0% of the deposited nebulized CIP-HCL permeated through all the tested membranes without any additional water after a 2-hour sampling period, perhaps indicating that drug deposited as droplets on the membrane surface became dried after evaporation of water.

As expected, additional water on the nebulized droplets increased both the rate and extent of the permeation

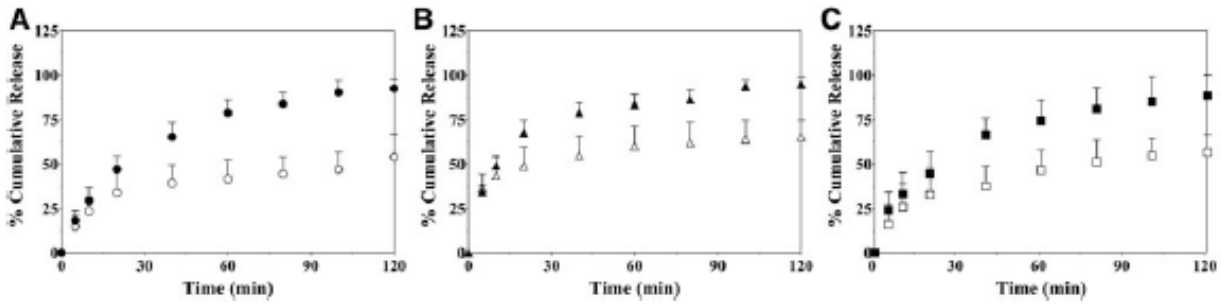

FIG. 6. Percentage cumulative mass release behavior of nebulized CIP-HCL droplets permeated through the front and back membranes of the **(A)** polyester, **(B)** polyamide, and **(C)** dialysis inserts into the receptor compartment, with (*filled symbols*) and without (*empty symbols*) additional water ($n=6$; mean [error bars are + SD]).

behavior, with approximately 75%–90% of CIP-HCL permeating through the membrane by the end of the experiment. It is thought that adding water on top of the membrane enhanced the increased dissolution on the donor compartment and spread of CIP-HCL onto the surface membrane, increasing the surface area coverage of CIP-HCL onto the porous areas, hence facilitating the permeation of CIP-HCL into the receptor compartment. As such, the altered rate and extent of permeation may reflect a dissolution step for the deposited nebulized droplets on the membranes.

In conclusion, a modified version of the existing medium-sized VCU MT-TB airway model (MOD#2) has been presented to assess realistic airway deposition and drug permeation behavior of the nebulized CIP-HCL drug across a range of synthetic membranes using the Snapwell test system. Although the synthetic membranes used in this study do not resemble the complex structure of the epithelial airway barrier, we were able to show how different types of membrane materials, presence or absence of a stirrer in the receptor compartment, and aerosol particle collection method can significantly influence drug permeation behavior. Furthermore, the feasibility in adapting the Snapwell test system have provided reproducible and discriminatory permeation profiles for the already dissolved and nebulized CIP-HCL solution.

Future work will focus on expanding this hybrid approach to a cell culture-based setup, by integrating epithelial cell monolayers onto Snapwell inserts before airway deposition experiments. To make *in vitro* testing more representative of *in vivo* conditions, the Snapwell system will be placed within a humidified chamber.

Author Disclosure Statement

The authors are faculty and students from the Woolcock Institute of Medical Research and the Virginia Commonwealth University. No conflicts of interest exist.

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