Development of mucus-penetrating antibiotic and quorum sensing inhibitor nanoparticle complex for bronchiectasis therapy

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**DEVELOPMENT OF MUCUS-PENETRATING ANTIBIOTIC AND
QUORUM SENSING INHIBITOR NANOPARTICLE COMPLEX
FOR BRONCHIECTASIS THERAPY**

TRAN THE THIEN

SCHOOL OF CHEMICAL AND BIOMEDICAL ENGINEERING

2020

**DEVELOPMENT OF MUCUS-PENETRATING ANTIBIOTIC AND
QUORUM SENSING INHIBITOR NANOPARTICLE COMPLEX
FOR BRONCHIECTASIS THERAPY**

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School of Chemical and Biomedical Engineering

A thesis submitted to the Nanyang Technological University, Singapore

in partial fulfilment of the requirement for the degree of

Doctor of Philosophy

2020

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Chapter 3 is published as **Tran TT**, Vidailac C, Yu H, Yong VFL, Roizman D, Chandrasekaran R, Lim AYH, Low TB, Tan GL, Abisheganaden JA, Koh MS, Teo J, Chotirmall SH, Hadinoto K. A new therapeutic avenue for bronchiectasis: Dry powder inhaler of ciprofloxacin nanoplex exhibits superior *ex vivo* mucus permeability and antibacterial efficacy to its native ciprofloxacin counterpart, *Int J Pharm.*, 2018; 547(1-2): 368-376. Permission has been granted by the licensed publisher “Elsevier” for utilization of the published content as a chapter in this thesis.

The contributions of the co-authors are as follows:

- A/Prof. Kunn Hadinoto and Asst/Prof. Sanjay Haresh Chotirmall raised funding for the project, supervised the project direction, reviewed and edited the manuscript drafts.
- I have done literature review, prepared methodology, and carried out investigation, validation, and writing –the materials and methods.
- Dr. Celine Vidailac and Ms. Valerie F.L. Yong have investigated the antimicrobial efficacy of the native CIP and the CIP nanoplex in DPI forms

against *Pseudomonas aeruginosa* biofilm. And, Dr. Celine has written the original manuscript draft.

- Dr. Yu Hong has investigated the optimal preparation condition for the spray-drying of the CIP nanoplex.
- Dr. Dan Roizman, Dr. Ravishankar Chandrasekaran, Dr. Albert Y.H. Lim, Dr. Teck Boon Low, Dr. Gan Liang Tan, Dr. John A. Abisheganaden and Dr. Mariko Siyue Koh have collected and supplied clinical strains of *P. aeruginosa* and clinical sputum from bronchiectasis patients.
- Dr. Jeanette Teo has isolated and supplied clinical strains of *P. aeruginosa*.

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ABSTRACT

Bronchiectasis is a chronic respiratory disease characterized by permanent and irreversible dilatation of the airways caused by a vicious cycle of infection and inflammation, leading to the loss of lung function, morbidity, and mortality. Early results suggested inhaled ciprofloxacin (CIP) as a promising therapeutic avenue for bronchiectasis. Herein our study aims i) to investigate the effectiveness of the dry powder inhaler (DPI) formulation of CIP nanoplex in the mucus environment of bronchiectasis evaluated against its native CIP counterpart, ii) to further evaluate the effectiveness of the DPI of CIP nanoplex in comparison with the DPI of CIP liposome, iii) to improve the mucus permeability of the CIP nanoplex by equipping it with the mucolytic papain (PAP) protease hydrolyzing the mucin matrix in sputum, and iv) to enhance the antimicrobial efficacy against *Pseudomonas aeruginosa* biofilm by developing the nanoplex of quercetin – an effective quorum-sensing inhibitor of *P. aeruginosa*.

The effectiveness of the DPI of CIP nanoplex was first evaluated in terms of (1) dissolution characteristics in artificial bronchiectasis sputum medium, (2) *ex vivo* mucus permeability in sputum from non-cystic fibrosis bronchiectasis (NCFB) and healthy individuals, (3) antibacterial efficacy in the presence of sputum against clinical *P. aeruginosa* strains (planktonic and biofilm), and (4) cytotoxicity towards human lung epithelial cells and so on. In our first evaluation, the DPI of CIP nanoplex exhibited superior mucus penetration and antibacterial efficacy to its native CIP counterpart. In the second evaluation, the DPI of CIP nanoplex was compared to the DPI of CIP liposome in the presence of NCFB sputum

environment. Both the CIP nanoplex and CIP liposome exhibited similar bactericidal activities against planktonic and biofilm *P. aeruginosa* even though the DPI of CIP liposome exhibited slightly superior mucus permeability to the DPI of CIP nanoplex. However, it was found that the CIP liposome possessed much lower CIP payload than the nanoplex (3.5% versus 84%), resulting in high lipid contents in its DPI formulation that led to higher cytotoxicity and lower aerosolization efficiency, indicating that the CIP nanoplex can be a highly promising CIP delivery therapy for NCFB owed to its simpler preparation, higher CIP payload hence lower dosage, better aerosolization, and lower cytotoxicity.

To further improve the mucus permeability hence the antibacterial efficacy, the CIP nanoplex was incorporated with a mucolytic enzyme papain (PAP) at the nanoplex formation step to produce the CIP-(DXT-PAP) nanoplex exhibiting built-in mucolytic capability. It was found that the CIP-(DXT-PAP) nanoplex exhibited ten-fold improvement in the mucus permeability compared to its CIP-DXT nanoplex counterpart, resulting in the former's superior bactericidal activity against clinical *P. aeruginosa* biofilm in the presence of mucus barrier. Nevertheless, the incorporation of PAP above a certain concentration threshold caused cytotoxicity towards human lung epithelium cells. Therefore, the optimal dosing of the CIP-(DXT-PAP) nanoplex must be carefully determined.

In the end, to further enhance the antibacterial efficacy and prevent the antibiotic resistant *P. aeruginosa* biofilm in the lung of bronchiectasis patients, the quercetin (QUE), a water-poorly soluble but effective quorum-sensing inhibitor against *P. aeruginosa* biofilm, was developed into the amorphous QUE nanoplex. It was found that the QUE nanoplex exhibited superior inhibition of *P. aeruginosa*

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LIST OF ABBREVEATIONS

AA	Acetic acid
ASM	Artificial sputum medium
Bc	Basolateral compartment
BCA	Bicinchoninic acid
C _{Sat}	Saturation solubility of the native CIP
CE	Complexation efficiency
CF	Cystic fibrosis
CFM	Confocal fluorescence microscope
CFU	Colony forming units
CHI	Chitosan
CIP	Ciprofloxacin
[CIP]	Ciprofloxacin concentration
[CIP] _{eq}	Equivalent ciprofloxacin concentration
d _A	Aerodynamic diameter of the inhalable powders
d _G	Geometric diameter of the inhalable powders
DLS	Dynamic light scattering
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DPI	Dry powder inhaler
DPTA	Diethylenetriamine pentaacetic acid
DXT	Dextran sulfate sodium

ED	Emitted dose
EDTA	Ethylenediaminetetraacetic acid
EE	Encapsulation efficiency
FDA	The United States Food and Drug Administration
FEV1	First forced expiratory volume
FPF	Fine particle fraction
HPLC	High performance liquid chromatography
HRCT	High resolution computed tomography
IP	Induction port
M _{CIP/liposome}	Molar ratio of CIP to liposome
MEM	Minimum Essential Medium with Eagle's salt
MHB	Mueller Hinton broth
MIC	Minimum inhibitory concentration
MLR _{Nanoplex}	Mannitol to lactose ratio for the CIP nanoplex
MLR _{Native}	Mannitol to lactose ratio for the native CIP
MMAD	Mass median aerodynamic diameter
NCFB	Non-cystic fibrosis bronchiectasis
NGI	Next generation impactor
OD ₅₅₀	Optical density at 550 nm
OD ₆₀₀	Optical density at 600 nm
PAP	Papain
PBS	Phosphate buffered saline
PDI	Polydispersity index
PET	Powder entrainment tube

PR	Permeability ratio
PS	Pre-separator
QUE	Quercetin
SFD	Spray freeze drying
UV–Vis	Ultraviolet–visible spectroscopy

CHAPTER 1. INTRODUCTION

1.1 Clinical significances of bronchiectasis

Bronchiectasis is a chronic respiratory disease characterized by abnormal and permanent enlargement of the lung airways. It is caused by a vicious cycle of pathological infection and inflammation, which subsequently leads to chronic cough production, lung functional disorder, morbidity, and mortality (Fig. 1-1) [1, 2]. Individuals with bronchiectasis had dehydration of airway, accumulation of mucus, and impaired clearance mechanism of respiratory systems, providing mucus-rich environment for the growth of bacterial pathogens. The most commonly isolated pathogens from bronchiectasis patients are *P. aeruginosa*, *Haemophilus influenza*, and less commonly *Streptococcus aureus* and *S. pneumonia* [3].

Figure 1-1: The vicious circle of bronchiectasis [2, 4].

Bronchiectasis is commonly categorized as cystic fibrosis (CF) and non-cystic fibrosis bronchiectasis (NCFB) [5]. CF is a genetic disorder caused by a mutation to the Cystic Fibrosis Transmembrane Conductance Regulator protein, which

regulates the transport of chloride ions. NCFB is referred for other etiologies (e.g. severe respiratory bacterial infections, allergic hypersensitivities, primary and secondary immunodeficiency and so on) and idiopathy [4, 6]. Although CF and NCFB are very similar in term of respiratory disease symptoms and progression, their pathogenesis are very different. CF patients early infected by *P. aeruginosa* could be cleared with antibiotics, but NCFB patients could not completely eradicate the pathogen with antibiotics [2]. NCFB has become a global burden associated with health, economy and society because NCFB patients have to suffer chronic recurrent infections, exacerbations and prolonged hospital admissions. The prevalence of NCFB in the United States is estimated at 139 cases per 100,000 persons, and increase with age, from 7 per 100,000 persons aged 18 – 34 years to 812 per 100,000 persons aged over 75 years [7, 8]. The management of NCFB requires a comprehensive therapeutic strategy which is capable of (i) airway mucus clearance, (ii) suppressing and eradicating bacterial airway colonization, (iii) reducing inflammation, and (iv) improving physical lung functions [9].

1.2 Antibiotic nanoparticles for bronchiectasis

Antibiotics play an important role in reducing the airway bacterial burden, in particular *P. aeruginosa*, which has been associated with more frequent exacerbations and an accelerated decline in the lung function in NCFB [10, 11]. Recently efforts showed that inhaled antibiotics has been recommended for the management of NCFB owed to its ability to deliver a high antibiotic concentration to the infected sites, resulting in high antibacterial efficacy, and limitation of systemic adverse side effects [12]. Nebulizers and dry powder inhalers (DPI) are

inhalation delivery systems widely used for the treatment of infected lung diseases. However, nebulizers exhibit some limitations, such as requirement of frequent administration, reconstitution of powders into solution for nebulization and cold chain storage. On the other hand, the DPI provides better physicochemical stability and deep deposition in the lung [13, 14]. The typical inhaled antibiotics for bronchiectasis consist of aminoglycosides (e.g. tobramycin, gentamicin), beta-lactams (e.g. meropenem, aztreonam), and fluoroquinolones (e.g. ciprofloxacin, levofloxacin). Ciprofloxacin (CIP), a broad-spectrum fluoroquinolone, has the highly proven antimicrobial efficiency against *P. aeruginosa*, and it is the most promising antibiotic for the management of NCFB. Recently efforts for NCFB showed that there are two inhaled CIP formulations undergoing Phase III clinical trials, namely (1) DPI of CIP betaine - the free base form of the native CIP (RESPIRE) [15, 16], and (2) the mixture of dual-release liposomal CIP and native CIP (ORBIT-3 and ORBIT-4) [17]. On the other hand, while we are waiting for the full results of the RESPIRE and ORBIT formulations of trials for NCFB, other inhaled antibiotic delivery systems that could overcome the mucus barrier and exhibit the high antimicrobial efficacy to biofilm pathogens should be continuously pursued.

There have been many antibiotic delivery systems, including liposomes, polymeric nanoparticles, polymeric lipid nanoparticles, nanoplex and so on. Among those systems, liposomes and polymeric nanoparticles are the two most widely studied formulations of nano-antibiotics. Liposomes encapsulating antibiotic had been paid much attention as they are able to control the sustained release above the MIC, leading to high antibacterial efficacy. However, the liposomal antibiotics are

usually weak in their physicochemical stability, resulting in antibiotic leakage and disintegration during drying and inhaled administration [18].

An alternative nano-antibiotic delivery system, polymer-based nanoparticles, exhibited better physicochemical stability and morphology control than liposomes. With their favourable characteristics, polymer nano-antibiotics were priorly investigated for overcoming the mucus barrier. However, the encapsulation efficiency of antibiotics in polymeric nanoparticles is quite low, with below 3% (w/w) [19]. As a result, it is required a large amount of nanoparticles to be inhaled for the treatment of infected sites, leading to a high risk of toxicity to cells.

Antibiotic nanoparticle complex, antibiotic nanoplex in short, prepared by electrostatically self-assembled complexation between antibiotic molecules and polymer chains, showed the excellent benefits in terms of enhancing the solubility of water-poorly soluble drugs, and drug loading (up to 80% w/w). The ciprofloxacin (CIP) nanoplex was specifically designed to overcome the mucus barrier. The CIP nanoplex has nanosize ($\approx 200 - 300$ nm) and anionic charge (zeta potential ≈ -30 to -50 mV), which enables it to permeate through the water channels of the mucus (typically ≈ 500 nm) [20], and minimizes the interaction to the polyanionic contents of the mucus (e.g. mucin, DNA) in bronchiectasis airways [21]. Moreover, the metastable amorphous state of the CIP nanoplex could generate a supersaturated CIP concentration in aqueous salt solution much higher than the concentration generated by the crystalline native antibiotics [22].

With the aims for bronchiectasis therapy, the CIP nanoplex has been developed for DPI formulation prepared by spray drying of the CIP nanoplex with mannitol [23]. Subsequently, the effectiveness of the DPI of CIP nanoplex was firstly

evaluated in the mucus environment of NCFB against its native CIP counterpart. The findings showed that the DPI of CIP nanoplex exhibited the superior mucus penetration and antibacterial efficacy against clinical *P. aeruginosa* in both planktonic and biofilm forms, indicating that the DPI of CIP nanoplex can be a highly promising CIP delivery system for the management of NCFB. And, to further evaluate the effectiveness of the DPI of CIP nanoplex, the CIP nanoplex was examined with a well established delivery system represented by the CIP liposome in the NCFB environment.

On the other hand, it is reported that the airway mucus in some bronchiectasis patients were remarkably thick and more viscous due to impaired mucociliary clearance in comparison with normal human [24]. And, papain (PAP), an industrial well-known proteolytic enzyme derived from the latex of papaya [25], has been conjugated into nanoparticles to facilitate their penetration through the mucus barrier [26]. As a result, to further enhance the mucus permeability of the CIP nanoplex, the CIP nanoplex was incorporated with the mucolytic PAP, and its mucus permeability and bactericidal efficacy was examined in the thick mucus environment.

In addition to the mucus barrier, it is reported that biofilm underlying the mucus layer is another barrier for treatment of NCFB. Biofilm are cell-to-cell communication systems known as quorum sensing (QS), which can regulate expression of virulence factors and antibiotic resistance [27]. It is detected that *P. aeruginosa* is the most common pathogen in the infected lungs, and it is associated with the accelerated damage of the lung function and frequent exacerbations in the lungs of patients. Quorum sensing molecules secreted from *P. aeruginosa* are

correlated with clinical status of CF patients [28]. Quorum-sensing inhibitor (QSI) based therapeutics has been developing to disable virulence factors and inhibit the antibiotic resistance without killing pathogens [29, 30]. Quercetin (QUE), a natural polyphenolic flavonoid extracted from plants, recently exhibited the effective inhibition of quorum sensing of *P. aeruginosa* biofilm and virulence factors [31, 32]. As a result, to further improve the antimicrobial efficacy of the CIP nanoplex and inhibit the bacterial antibiotic resistance, the CIP nanoplex can be co-delivered with the QUE nanoplex to completely eradicate *P. aeruginosa* for the management of NCFB.

1.3 Objectives

1. To investigate the effectiveness of the DPI of CIP nanoplex in the mucus environment of NCFB evaluated against its native CIP counterpart.
2. To further evaluate the effectiveness of the DPI of CIP nanoplex in comparison with the DPI of CIP liposome in the NCFB environment.
3. To improve the mucus permeability of the CIP nanoplex by equipping the CIP nanoplex with the mucolytic papain protease hydrolyzing the mucin matrix in sputum.
4. To enhance the antimicrobial efficacy of the CIP nanoplex and inhibit the bacterial antibiotic resistance by developing the quercetin nanoplex inhibiting quorum-sensing of *P. aeruginosa* biofilm.

CHAPTER 2. LITERATURE REVIEW

2.1 Antibiotic nanoparticle for bronchiectasis

Inhaled antibiotics have been recommended for the prevention of exacerbations and the management of NCFB because it can deliver a high concentration of antibiotics to infected sites with fewer adverse side effects [11]. It was reported that patients with NCFB inhaled antibiotics for 4 weeks could decrease 1000-fold bacteria load in sputum [33]. Tobramycin, a cationic aminoglycoside with highly proven antibacterial efficacy against *P. aeruginosa* biofilm, was one of the first inhaled antibiotics studied in bronchiectasis. And, its nebulization solution has been approved as clinically inhaled antibiotics used for treating *P. aeruginosa* in patients with chronic lung infections. However, it was reported that tobramycin exhibited short retention time in infected lungs (below 2 h) and bound to the mucin in the sputum, so it was required to be delivered twice daily at concentration ≥ 25 -fold MIC for effective killing of *P. aeruginosa* [34]. Also, its effectiveness in the long-term treatment was subsequently reduced over repeated uses due to sub-inhibitory antibiotic exposure. This caused heavy burdens for the patients and increased the risks of systemic toxicity [35], and could lead to the emergence of antibiotic resistance of *P. aeruginosa* biofilm in the infected lung [36, 37].

Ciprofloxacin (CIP) is one of broad-spectrum fluoroquinolone with well proven bactericidal activity against *P. aeruginosa* [38]. In comparison with tobramycin, the ciprofloxacin exhibited higher antimicrobial efficacy against *P. aeruginosa* [39]. Nevertheless, even though the CIP exhibited some improvements over tobramycin, the use of free-form antibiotics definitely delivers low antibiotic exposure due to the

lung epithelial cells 'rapid clearance mechanism and the presence of the physical mucus barriers, and the emergence of antibiotic resistant bacteria.

To enhance effectiveness of antibiotics for bronchiectasis, encapsulation of antibiotics into nanoparticles has been developed because of their mucus permeability and prolonged retention time in the lung. Among the drug delivery systems, namely polymer nanocarriers (e.g. polymeric nanoparticles, polymeric micelles), lipid nanocarriers (e.g. liposomes, solid lipid nanoparticles), and inorganic nanocarriers (e.g. gold, silica), the polymeric nanoparticles and liposomes are the most promising nanocarriers for delivery of antibiotics [40-43].

Polymeric nanoparticles (NPs) have been widely functionalized and developed for therapeutic applications as they possess high structural integrity, stability during storage, and controlled release capability. Polymeric NPs can be prepared by two main approaches, namely the polymerization of monomers, and a dispersion of preformed polymers. Both methods mostly use synthetic polymers (e.g. poly-lactic-co-glycolic acid (PLGA), poly(lactic acid) (PLA), stabilizers (e.g. poly(vinyl alcohol), and organic solvents, which resulted in toxicological problems of solvent residues from the products [44]. Moreover, polymeric NPs are limited to encapsulate water-soluble drugs due to rapid drug leakage from nanoparticles during the high-energy emulsification preparation step.

Liposomes have been paid much attention as the ideal drug delivery system because of their analogues of biological membranes, which can trap both hydrophobic and hydrophilic drugs. Their lipid bilayers can trap solvent-soluble drugs, whereas the aqueous core can trap water-soluble drugs [45]. Arikace^{TM}, the formulation of liposomes encapsulating amikacin, was approved by the FDA for the

treatment of CF [46], and the liposome encapsulating ciprofloxacin (Pulmaquin^{TM}) have reached Phase III clinical trials for the treatment of NCFB [17, 47]. However, antibiotic liposomes are limited by lack of structural integrity, leading to drug leakage and instability during preparation and storage [18].

Lipid–polymer hybrid nanoparticle (LPN), a new generation of therapeutic delivery system, combines the characteristics of both polymeric nanoparticle and liposome [48]. It is composed of a polymer core to encapsulate therapeutics, an inner lipid layer to envelope the polymer core to slow down the polymer degradation and drug leakage, and an outer lipid–(polyethylene glycol) (PEG) layer to prolong circulation time *in vivo*. With its core–shell structure, the LPN has been considered as an effective drug delivery system. In Cheow et al. study, the lipid layer was coated on PLGA nanoparticles encapsulating levofloxacin to form PLGA-PC nanoparticles, and the effect of lipid coat on antibiotic release and antibacterial efficacy against *P. aeruginosa* biofilm was investigated [49]. The results showed that the lipid layer slowed down the release of the highly water-soluble levofloxacin from LNPs over 24 h in comparison with the non-lipid coated NPs. Moreover, it was found that the LPNs exhibited superior antibacterial efficacy against *P. aeruginosa* biofilm 50-fold higher than their non-lipid coated counterparts. Although the LPNs exhibited many favorable characteristics, they are challenged by clinical translation, such as improving the drug loading efficacy, considerations of cost and quality for producing LPNs on large scales for industrial applications [50].

An alternative antibiotic nanoparticle complex, nanoplex in short, has been paid much attention on its simple, free-solvent, cost-effective preparation and high antibiotic loading [22]. The antibiotic nanoplex is simply formulated by mixing

oppositely charged antibiotics and polysaccharides solutions under ambient conditions [51, 52]. The complexation of antibiotics and polymers was generated by electrostatic interactions between the charge neutralization and the hydrophobic interactions, resulting in the precipitation of antibiotic nanoplex. In Cheow 's study, the nanoplex was generated by mixing the fluoroquinolones (i.e. levofloxacin, ciprofloxacin) and the polymer dextran sodium sulfate, resulting in above 80% w/w payload of drugs. It is implied that only a little demand of antibiotics nanoparticles needed to be delivered to targeted sites. In addition, the nanoplex possesses the characteristics ideal for mucus penetration because its mucus-inert surface (negative charges) diminishes the physical interaction with polyanionic components (i.e. mucin, DNA) of the mucus. Especially, metastable amorphous state of the nanoplex could generate faster dissolution rate and highly supersaturated drug solution in aqueous salt solution in comparison with the crystalline native antibiotics, which could enhance the bioavailability of poorly-water soluble antibiotics in the body [22, 53]. As a result, the antibiotic nanoplex is a greatly promising potential of inhalation therapy for bronchiectasis.

2.2 Mucus penetration of antibiotic nanoparticles for bronchiectasis

The airway mucus is a viscoelastic gel consisting of two layers, namely a periciliary layer with low viscosity overlying the epithelial cells and a mucus gel layer with higher viscosity (Fig. 2-1) [54, 55]. The airway mucus is composed of water, carbohydrates, DNA, proteins and lipids. Mucin is the main component of the mucus, which contains highly glycosylated regions (negatively charged). These regions contribute to the properties of mucus barrier for trapping positively charged

particles. In addition, mucins are continuously secreted by goblet cells and submucosal glands to form the gel layer, which is also continuously removed from the lungs via mucociliary clearance (MCC). The MCC of the gel layer is happened by freely moving of the hair-like cilia, resulting in removal of inhaled particles or microbes from the lung [55, 56].

Figure 2-1: The airway mucus of human primary bronchial epithelial cell cultures. It is composed of the periciliary layer (PCL) and the airway mucus gel layer [56].

In patients with bronchiectasis, the mucus layer is very concentrated due to hypersecretion of mucin and other biomacromolecules (e.g. DNA, filamentous actin...) caused by infection, inflammation and impaired lung function [57, 58]. These factors led to the mucus as the barrier for drug delivery to targeted sites. In addition to the mucus barrier, biofilm – a community of microorganisms–secreting matrix of extracellular polymeric substances (EPS) could resist antibiotics up to 1000 fold in comparison with the planktonic bacterial cells [59]. Nanoparticles with designed characteristics (i.e. size, charge, and surface modification) can improve their mucus penetration and deliver high drug concentration to infected sites [60]. Polymeric nanoparticles (NPs) are considered as the most promising drug delivery

system for penetrating through the thick mucus layer of bronchiectasis lung. The polymeric NPs possess the strong physicochemical stability to minimize antibiotic loss during preparation, and they can control sustained release of antibiotics, and prolong retention time in the lung [61].

Blank polystyrene (PS) nanoparticles without antibiotics were firstly used to investigate the mucus penetration. The first study showing the effect of different PS NPs sizes on the mucus penetration of CF sputum was described in Sanders et al.'s study [62]. It was found that the mass percentage of the nanoparticles with 120, 270, and 500 nm diffusing across the 220 μ m CF mucus was about 1.3, 6.8, and 42% lower than the ones diffusing the water, respectively. Another study also showed that the PS NPs with 200 nm penetrating the CF sputum was 25-fold lower in the mass percentage than the ones penetrating through water [63].

The charge of nanoparticles also had effects on its mucus permeability, it was reported that the polymeric nanoparticles with the neutral charge at the same size (below 200 nm) diffused through the mucus faster than the negatively charged ones [64]. And, the negatively charged PS NPs could be coated with polyethylene glycol (PEG) to produce neutrally charged NPs for enhancing mucus permeability. Also, it was reported that PEG-coated nanoparticles penetrating the CF sputum exhibited about 90-fold faster than the non-coated nanoparticles [20].

On the other hand, liposomes, one of the most widely studied nanocarriers, were investigated for the mucus penetration. In Meers' study, the mucus permeability of amikacin-encapsulated liposomes (i.e. $\approx$ 300 nm) were investigated on the sputum of CF patients [65]. It was found that liposomes penetrated through the sputum and were mainly accumulated at the bottom of sputum layer. To date, the liposomes

encapsulating amikacin (Arikace^{TM}) have been approved by the FDA for the treatment of CF [46].

On the other hand, to further enhance the mucus penetration of the polymeric nanoparticles, mucolytic agents, namely recombinant human DNase (rhDNase) and N-acetyl-L-cysteine (NAC), can be added to the sputum to decrease the mucus viscosity. RhDNase, one of commonly well-known mucolytic agents, can be used to break down the DNA in sputum [66]. In addition of rhDNase to the CF sputum showed that the mass percentage of the nanospheres diffusing across the rhDNase-added mucus was 3-fold higher than the one without rhDNase [62]. Guichard et al. have incorporated rhDNase with polyethylene glycol (PEG) and evaluated its mucolytic activity on CF sputum. The results showed that rhDNase-PEG was 25-time more stable than rhDNase in CF sputum [67]. However, rhDNase is considered to be contraindicated in NCFB as a trial reported deleterious effects on both lung function and exacerbation rate in adults [66]. For the addition of NAC in the CF sputum, it was found that the nanoparticles' diffusion velocity can improve about 13-fold compared to the non-added one [68].

Another alternative well-known mucolytics is papain (PAP), an industrial proteolytic enzyme derived from the latex of papaya (*Carica papaya*), could hydrolyze the mucus glycoprotein cross-linked networks [25]. Micro- and nanoparticles decorated with mucolytic papain (PAP) have showed to be able to cross the mucus barrier by cleaving peptide bonds of mucus glycoproteins [26].

2.3 Antibacterial efficacy of antibiotic nanoparticles for bronchiectasis

Use of inhaled antibiotics nanoparticles has been recommended for the management of NCFB because they can prolong retention time of antibiotics in the lung and penetrate through the mucus to reach the embedded pathogen biofilms, and control sustained release of the antibiotics, resulting in the high antibacterial efficacy [21]. Inhaled antibiotics nanoparticles have been widely delivered in two popular formulations, such as nebulizer and dry powder inhaler (DPI) [13]. Nebulizer is the traditional approach to deliver a high concentration of antibiotics to the infected sites in the lung. In Meers' study, the liposomal amikacin was examined in the antimicrobial efficacy against *P. aeruginosa* in the rat model with the lung infection but without incorporation of mucus. The results showed that the nebulized liposomal amikacin exhibited longer retention time (24 h half-life) in the lung and a superior antimicrobial efficacy ($3.2 \pm 0.4 \log_{10}$ CFU/lung) in comparison with the nebulized free amikacin (1.5 h half-life and $4.6 \pm 0.4 \log_{10}$ CFU/lung) [65]. Recent efforts showed that the nebulized solution of antibiotic nanoparticles is a great potential therapy for the treatment of bronchiectasis. For example, the nebulized solution of dual-release liposomal CIP and free native CIP are undergoing the phase III clinical trials (ORBIT-3 and ORBIT-4) [17]. In addition, in another study it was found that the PEG-PLGA nanoparticles encapsulating tobramycin (0.77 mg/L) could overcome the artificial mucus and exhibited the high antibacterial efficacy against the *P. aeruginosa* and *B. cepacia* biofilms (a reduction of 1–1.5 $\log_{10}$ CFU/mL and over 2 $\log_{10}$ CFU/mL, respectively) in comparison with 1000 mg/L of its free tobramycin counterpart [69].

Even though nebulized delivery of inhaled antibiotics showed such promising results, it still had many problems, such as time-consuming administration, complex operation, and a decreased efficacy of drug over time [33]. In comparison with nebulizers, antibiotic delivery by dry powder inhaler (DPI) showed great advantages, including (i) shorter administration time, (ii) higher effective delivery, (iii) higher portability, and (iv) higher stability [13, 70]. In Ungaro et al. study, the DPI of tobramycin-loaded PLGA nanoparticles exhibited antimicrobial efficacy against planktonic *P. aeruginosa*, even though their MIC was about ten-fold higher than the free antibiotics. Particularly, the DPI of tobramycin-PLGA nanoparticles coated with poly(vinyl) alcohol or chitosan could reach the deep lungs in healthy rat models [19].

In our previous study, the DPI of CIP nanoplex has been successfully developed by spray-drying of the CIP nanoplex with mannitol. It was found that the MIC of the DPI of CIP nanoplex against clinical *P. aeruginosa* similar to its native CIP counterpart, indicating that the preparation of the DPI of CIP nanoplex did not adversely affect its antibacterial activity [23]. Recently, the effectiveness of the DPI of CIP nanoplex in the mucus environment of NCFB has been evaluated against its native CIP counterpart [71]. The results showed that the DPI of CIP nanoplex exhibited superior mucus permeability (5 – 7 times higher) to its native CIP counterpart in *ex vivo* normal and NCFB sputum. And, the superior mucus permeability led to the DPI of CIP nanoplex 's superior antibacterial efficacy (> 3 log₁₀ CFU/mL reduction) to its native CIP counterpart against clinical strains of both planktonic and biofilm *P. aeruginosa*.

Last but not least, the DPI of CIP nanoplex was further evaluated with the well established drug delivery system represented by the CIP liposome [72]. The results showed that both DPI forms of the CIP nanoplex and the CIP liposome exhibited a fast penetration through the mucus barrier (50% permeation after 1 h) attributed to their nanosize and mucus-inert surface, leading to their similarly high antibacterial efficacy against clinical isolates of *P. aeruginosa*. These results showed that the DPI of CIP nanoplex can be a highly promising CIP delivery strategy for long-term management of NCFB.

2.4 Anti-quorum sensing of nanoparticles for bronchiectasis

Pseudomonas aeruginosa is an opportunistic Gram-negative human pathogen, which grows up to biofilm in the infected lung of bronchiectasis patients. In the biofilm state, cell-to-cell communication systems known as quorum sensing (QS) regulate expression of virulence factors and antibiotic resistance [27]. *P. aeruginosa* had three main QS pathways (Fig. 2-2) [73, 74]. The first two pathways are regulated by the synthases LasI and RhlI and transcriptional factors LasR and RhlR, respectively, which are required for the synthesis of autoinducers N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C₁₂-HSL) and N-butanoyl-L-homoserine lactone (C₄-HSL). The third QS system is regulated by the transcriptional factor PqsR for the synthesis of 2-heptyl-3-hydroxy-4-quinolone molecule that plays an important role in the connection between the Las and Rhl systems. The complexes of autoinducers and receptors activate the expression of genes required for biofilm formation and production of virulence factors (i.e.

pyocyanin, rhamnolipids, swarming motility, elastase and so on), which are crucial in establishing and maintaining an infection of *P. aeruginosa* [75].

Figure 2-2: Three main quorum sensing pathways of *P. aeruginosa*. AHLs: N-acylhomoserine lactones, AQ: alkylquinolines, 3OC₁₂-HSL: N-(3-oxo-dodecanoyl)-L-homoserine lactone; C₄-HSL: N-butanoyl-L-homoserine lactone; HHQ: 2-heptyl-4-quinolone; PQS: *Pseudomonas* quinolone signal [74].

It was found that *P. aeruginosa* associated with more frequent exacerbations in the lungs of patients, and quorum sensing molecules secreted from *P. aeruginosa* correlated with clinical status of CF patients [28]. Quorum-sensing based therapeutics have been developing to disable virulence factors and inhibit the antibiotic resistance without killing pathogens [29].

To date, various types of quorum-sensing inhibitors (QSIs) have been extracted from natural sources, or synthesized. However, these QSIs had low solubility and bioavailability, limiting their applications in clinical treatment. Therefore, various types of nanocarriers (i.e. metal nanoparticles, polymeric nanoparticles, liposome, solid lipid nanoparticles and so on) have been developed for encapsulation of QSIs to enhance their solubility and bioavailability [76].

Silver nanoparticles, one of most widely studied metal nanoparticles for anti-quorum sensing, were found to inhibit virulence factors in both Gram-positive and Gram-negative bacteria. In the study of Singh et al., the silver nanoparticles decorated on their surfaces with metabolites extracted from of a fungus *Rhizopus arrhizus* BRS-07 exhibited inhibition against ≈ 7 -93% of *P. aeruginosa* biofilm and production of virulence factors: 15–86% for LasA protease, 22–86% for LasB elastase, 18–96% for pyocyanin, 14–95% for pyoverdin, 10–92% for pyochelin, and 10–70% for rhamnolipid [77].

Polymeric nanoparticle, one of the most widely studied nanocarriers, has been developed for anti-QS in *P. aeruginosa*. In Lu et al. study, the various types of polymers (i.e. Polystyrene (PS), polylactide (PLA), polycaprolactone (PCL), and polypropylene oxide (PPO), and alpha tocopherol polyethylene glycol succinate (TPGS) based polyethylene glycol (PEG)) were used to encapsulate the synthesized QSI (V-06-018). The results showed that the nanocapsules and the free V-06-018 exhibited $\approx 90\%$ of pyocyanin production reduction of *P. aeruginosa*, indicating that the activity of the free V-06-018 and the nanocapsules were analogous and the encapsulation did not negatively affect V-06-018 activity [78].

Solid lipid nanoparticles (SLN) were developed to encapsulate the synthesized QSI (2-heptyl-6-nitro-4-oxo-1,4-dihydroquinoline-3-carboxamide) and investigated on the pyocyanin production of *P. aeruginosa* PA14. It was found that the QSI encapsulated-SLN did not only maintain the activity of the free QSI but also was superior to the free QSI in pyocyanin inhibition [79].

On the other hand, even though synthetic QSIs showed some promising results, some synthetic compounds exhibited the toxicity, limiting their use in mammalian

cells [80]. Therefore, QSIs from natural sources have been more attractive as their non-toxic and similar chemical structure to QS signal molecules, which facilitate degradation of the signal receptors [75, 81]. Flavonoids have been recently gained much attention due to their effective QS inhibitory activities [32]. And, quercetin (QUE), a natural polyphenolic flavonoid mainly found in vegetables and fruits, exhibited wide biological activities, such as anti-virulence or anti-biofilm formation, antioxidant and anticancer. Recently, quercetin has been paid much attention on its effective inhibition of quorum sensing of *P. aeruginosa* [31, 32]. QUE was found as a competitive inhibitor with the autoinducers by binding and forming the complex with LasR receptor protein [82]. In Ouyang's study, QUE significantly reduced the expression levels of *lasI*, *lasR*, *rhlI*, and *rhlR* to 34%, 68%, 57%, and 50%, respectively [31], indicating that QUE is an effective QS inhibitor of *P. aeruginosa*. However, QUE is a poorly-water soluble drug, so it is required to be encapsulated into nanoparticles to deliver a high concentration to targeted sites. Nguyen and co-workers have established quercetin-loaded chitosan/cyclodextrin/TPP nanoparticles, which exhibited highly effective inhibition of quorum-sensing (61.12%) and antimicrobial efficacy (88.32%) against *E. coli* [83]. However, this QUE encapsulation has not been tested with *P. aeruginosa*, and the QUE payload was very low with $\leq 3\%$ (w/w), limiting its application in clinical treatment.

In our study, the amorphous QUE-CHI nanoplex, which was prepared by the simple drug-polysaccharide complexation method, has been developed to enhance solubility of QUE and anti-biofilm and anti-quorum sensing (details showed in Chapter 6). The results showed that the QUE-CHI nanoplex exhibited the faster

dissolution rate and supersaturation solubility generation, resulting in a superior anti-biofilm and anti-quorum sensing efficacy against *P. aeruginosa* compared to its native QUE counterpart. With benefits of these obtained results, the QUE-CHI nanoplex would be a potential anti-biofilm and anti-QS formulation strategy for bronchiectasis therapy.

CHAPTER 3. THE EFFECTIVENESS OF THE DPI OF CIP NANOPLEX IN THE MUCUS ENVIRONMENT OF NCFB EVALUATED AGAINST ITS NATIVE CIP COUNTERPART

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3.1 Introduction

Bronchiectasis is a chronic respiratory disease characterized by permanent and irreversible dilatation of the airways caused by a vicious cycle of infection and inflammation, which eventually leads to the loss of lung function, functional disability, morbidity, and mortality [1]. Bronchiectasis is traditionally classified as either cystic fibrosis (CF) bronchiectasis or non-cystic fibrosis bronchiectasis (NCFB) [5]. Historically, the true prevalence of NCFB has been underestimated and consequently has received less attention than its CF counterpart [84, 85]. NCFB is now recognized as a major health, economic, and social burdens for its sufferers, who experience high risks of chronic recurrent infections, exacerbations and prolonged hospital admissions [7, 8].

The management of NCFB requires a comprehensive therapeutic strategy addressing its underlying cause (when known). This incorporates (i) airway mucus clearance, (ii) suppressing, eradicating, and preventing bacterial airway colonization, (iii) reducing inflammation, and (iv) improving physical functioning [9]. Antibiotics play an important role in the management of NCFB, both in acute exacerbations and in long-term intermittent therapy [33, 86]. The goal of the antibiotic therapy is to reduce the airway bacterial burden, in particular *Pseudomonas aeruginosa*, which has been associated with more frequent exacerbations and an accelerated decline in the lung function in NCFB [10, 11].

The use of inhaled antibiotics has been recommended for the management of NCFB owed to its ability to deliver a high antibiotic concentration localized at the site of infection, resulting in enhanced antibacterial efficacy, while at the same time reduces the risk of systemic adverse effects [12]. However, no inhaled antibiotics have been approved at present for NCFB due to a lack of long-term efficacy data [87]. Among the various antibiotics studied, inhaled ciprofloxacin (CIP) – a broad-spectrum fluoroquinolone with well proven bactericidal activity against *P. aeruginosa* – has shown the most promising clinical evidence for NCFB [38].

Two inhaled CIP formulations are currently under Phase III clinical trials specifically for NCFB, namely (1) dry powder inhaler (DPI) of CIP betaine - the freebase form of the native CIP - (RESPIRE) [15, 16], and (2) dual-release liposomal CIP delivered by nebulization (ORBIT-3 and ORBIT-4) [88]. The RESPIRE formulation attempts to solve the numerous problems associated with the nebulized delivery of native CIP solution, such as time-consuming administration, complex operation and cleaning, and low patient adherence [89]. The DPI delivery

mode in the RESPIRE formulation offers simplicity and convenience of using a user friendly, breath-actuated, and highly portable device.

The ORBIT formulation, on the other hand, attempts to address the problem of sub-inhibitory antibiotic exposures in the infected lungs when CIP is delivered in its native form, causing its diminished efficacy over time [90]. The sub-inhibitory concentration generated by the native CIP upon inhalation is caused by two factors, namely (1) its short half-life in the lungs due to rapid systemic absorption [91], and (2) the presence of a thick stationary mucus layer in the bronchiectasis airways, which acts as a physicochemical barrier that inhibits CIP diffusion to reach the embedded bacterial colonies [92]. The CIP encapsulation into liposomes results in the sustained release of CIP that enables the CIP concentration in the lungs to be maintained above the minimum inhibitory concentration (MIC) for a prolonged period. The prolonged high CIP exposure afforded by the ORBIT formulation leads to not only its higher antibacterial efficacy compared to the native CIP, but also minimizes the risk of emergence of antibiotic-resistant bacteria [42].

Therefore, while we await the full results of the RESPIRE and ORBIT trials, whose encouraging early results suggest inhaled CIP as a promising therapeutic avenue for NCFB, the development of inhaled CIP delivery vehicles that could overcome the mucus barrier and simultaneously enhance CIP exposure in the infected lungs should be continuously pursued. To this end, our group previously developed amorphous nanoparticle complex (or nanoplex in short) of CIP prepared by electrostatically driven self-assembly complexation between CIP and dextran sulfate (DXT) [22, 23].

First, the CIP nanoplex was specifically designed to overcome the mucus barrier, where the nanosize of the CIP nanoplex ($\approx 200\text{--}300$ nm) would enable it to permeate through the water channels of the mucus, whose size was typically ≈ 500 nm in bronchiectasis airways [20]. Moreover, the anionic charge and hydrophilic surface of the CIP nanoplex (zeta potential ≈ -30 to 50 mV) attributed to the presence of DXT on its surface would minimize the nanoplex binding to the hydrophobic polyanionic contents of the mucus (e.g. mucin, DNA) [21]. Second, its metastable amorphous state enabled the CIP nanoplex to generate a highly supersaturated CIP concentration upon dissolution in aqueous salt solution similar to biological fluids. The supersaturation generation by the CIP nanoplex, which was far above the concentration generated by the crystalline native CIP [22], should in turn translate to high CIP exposures in the targeted sites.

In previous studies, we had shown that the CIP nanoplex exhibited similar MIC as the native CIP against *P. aeruginosa* PAO1 indicating that the CIP complexation with DXT did not adversely affect its antibacterial activity [22]. We had also successfully developed DPI formulation of the CIP nanoplex [23] prepared by spray drying of the CIP nanoplex with mannitol – a mucolytic agent widely used in bronchiectasis therapy [92, 93]. However, the effectiveness of the CIP nanoplex in the mucus environment of NCFB, its bactericidal activity against clinical NCFB *P. aeruginosa* strains, and its cytotoxicity towards the lung cells had not been examined. In particular, how the presence of mucin as the major protein in the lung mucus and its possible interaction with the CIP nanoplex (if any) would affect the CIP nanoplex's mucus permeability, dissolution characteristics, and consequently antibacterial efficacy remained to be investigated.

Therefore, the objectives of the present works were to evaluate the DPI of CIP nanoplex in terms of its (1) mucin interaction (2) dissolution rate in artificial sputum medium (ASM), (3) mucus permeability in the sputum expectorated by healthy individuals and NCFB patients, (4) antibacterial efficacy in the presence of sputum against clinical isolates of *P. aeruginosa* (both planktonic and biofilm), and lastly (5) cytotoxicity towards human lung epithelial cells (i.e. A549 and 16HBE14o-). The mucin interaction, dissolution rate, *ex vivo* mucus permeability, antibacterial efficacy, and cytotoxicity of the DPI of CIP nanoplex were evaluated against the DPI of native CIP.

3.2 Materials and Methods

3.2.1 Materials

Materials for DPI of CIP nanoplex preparation: CIP, D-Mannitol, α -lactose monohydrate, and glacial acetic acid (AA) were purchased from Sigma Aldrich (Singapore), while DXT (5 kDa) was purchased from Wako Pure Chemical (Japan).

Materials for ASM preparation: Mucin from porcine stomach, salmon sperm DNA, HCl, and Tris base were purchased from Sigma Aldrich (Singapore). NaCl and KCl were purchased from VWR (Singapore). Diethylenetriamine pentaacetic acid (DPTA), egg yolk emulsion, and casamino acid were purchased from Alfa Aesar (Singapore), Oxoid (UK), and Merck Millipore (Singapore), respectively. Acetonitrile, methanol, and KH_2PO_4 for characterizing dissolution in ASM were purchased from Sigma Aldrich (Singapore).

Bacterial strains: *P. aeruginosa* PAO1 laboratory strain was purchased from ATCC (USA), whereas three clinical respiratory isolates of *P. aeruginosa* (i.e.

CS#1 – 3) were provided by the clinical microbiology laboratory at National University Hospital (Singapore) and Lee Kong Chian School of Medicine (Singapore). Mueller Hinton Broth (MHB), tryptic soy agar, and phosphate buffered saline (PBS, pH 7.4) for the bacterial cell culture were purchased from BD Diagnostics (Singapore), Gibco (USA), and Sigma Aldrich (Singapore), respectively.

Clinical sputum: Sputum spontaneously expectorated from both healthy individuals (n = 4) and NCFB patients (n = 7) were collected in sterile containers and transported on ice to the laboratory for experiments. The suitability of the sputum for inclusion in the study was confirmed by the criteria detailed in previous study [94]. This study was approved by the Institutional Review Boards of Nanyang Technological University (IRB-2016-01-031), the Centralized Institutional Review Board of SingHealth (CIRB C: 2016/2073), and National Healthcare Group's Domain Specific Review Board (DSRB).

Materials for cytotoxicity tests: A549 human carcinoma lung epithelial cells and 16HBE14o- human bronchial epithelial cells were purchased from ATCC (USA) and gifted by California Pacific Medical Center Research Institute, respectively. Penicillin–streptomycin, dimethyl sulfoxide (DMSO), and (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT, 98% purity) were purchased from PAA Laboratories (Austria), Sigma Aldrich (Singapore), and Alfa Aesar (UK), respectively. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were purchased from GE Healthcare Life Sciences (USA). Minimum Essential Medium with Eagle's salt (MEM), L-glutamine (200 mM), and 0.25%

trypsin ethylenediaminetetraacetic acid (EDTA) solution were purchased from Gibco (USA).

3.2.2 Methods

3.2.2.1 Preparation of DPI of CIP nanoplex and DPI of native CIP

The CIP nanoplex was prepared by the drug-polyelectrolyte complexation method described previously in Cheow 's study [22]. Briefly, CIP was dissolved at 10 mg/mL in 0.3% (w/v) aqueous AA solution after which it was mixed at equal volumes with 4.5 mg/mL aqueous DXT solution under gentle stirring. The resultant CIP nanoplex suspension then underwent two cycles of centrifugation (14,000×g for 5 min) and washing with deionized (DI) water to remove excess CIP and DXT that did not form the nanoplex.

The DPI formulation of the CIP nanoplex was prepared by spray drying at conditions detailed in Yu et al. study [23]. Either mannitol or lactose was used as the excipient at CIP nanoplex to excipient ratio = 1:1 (w/w). The morphology and aerosolization efficiency of the DPI of CIP nanoplex had been presented in [23] and not repeated here for brevity. The DPI of the native CIP was prepared by the same method at native CIP to excipient ratio = 1:1 (w/w). The CIP payload in the spray-dried powders was determined by dissolving a known mass of the powders in 0.3% (w/v) AA after which the CIP concentration in the solution was determined by UV–Vis spectrophotometer (UV Mini 1240, Shimadzu, Japan) at the optimal CIP absorbance wavelength of 324 nm.

3.2.2.2 Mucin interaction

The interaction between the DPI of CIP nanoplex and mucin was investigated using the dialysis-bag diffusion method following the protocols previously described in Huang et al. study [95] with a slight modification on the mucin concentration used. Briefly, DPI of CIP nanoplex was added at $[CIP] = 50 \mu\text{g/mL}$ to 1 mL PBS containing 6.5 mg/mL mucin representing the typical maximum concentration of mucin observed in bronchiectasis mucus [96]. The resultant mixture was immediately transferred to a dialysis bag after which the dialysis bag was incubated at 37 °C in 20 mL PBS placed in a covered petri dish. At specific time points over a 4 h period, 500 μL of the dialysate was withdrawn and replaced with fresh PBS of the same volume. The CIP concentration in the dialysate was determined by high performance liquid chromatography (HPLC) using Agilent 1100 (Agilent Technologies, USA). The HPLC analysis was performed in ZORBAX Eclipse Plus C18 column ($250 \times 4.6 \text{ mm}$, 5- μm particle size) at detection wavelength of 324 nm. Acetonitrile: methanol: 0.1 M KH_2PO_4 (15:25:60 v/v) was used as the mobile phase at 1.0 mL/min, resulting in CIP retention time of $\approx 3.6 \text{ min}$. A control experiment in which mucin was not added to the PBS was carried out to determine the mucin interaction.

3.2.2.3 Dissolution characteristics in ASM

The ASM was prepared according to the protocols described in Yang et al. study [97]. Briefly, 1 L ASM was prepared by dissolving 5 g mucin, 4 g salmon sperm DNA, 5.9 mg DPTA, 5 g NaCl, 2.2 g KCl, and 1.81 g Tris base in 0.9 L DI water. The pH was adjusted to 7.0 by adding 1 M HCl after which the solution was

autoclaved at 110 °C for 15 min. Next, 5 mL of egg yolk emulsion and casamino acid solution (0.5% w/v) were added to the solution. Two types of ASM, i.e. ASMs observed in normal and bronchiectasis sputum, which differed in their salt concentrations, were prepared by supplementing the ASM with NaCl and KCl. The salt concentrations representative of the normal and bronchiectasis sputum were $[\text{Na}^+] = [\text{Cl}^-] = 95 \text{ mM}$ and $[\text{Na}^+] = 115 \text{ mM}$ and $[\text{Cl}^-] = 130 \text{ mM}$, respectively [98].

The thermodynamic saturation solubility of the native CIP in the ASM (i.e. C_{Sat}) was determined by adding native CIP in excess (i.e. 20 mg) to 20 mL ASM maintained at 37 °C in a shaking incubator. After 96 h incubation, 2 mL aliquot was withdrawn and subsequently centrifuged (14,000 g for 5 min) and syringe filtered (0.2- μm pore size) to remove the undissolved CIP. The CIP concentration in the filtered solution was then determined by HPLC as previously described. The solubility of the CIP nanoplex in the ASM was similarly determined by adding the CIP nanoplex in excess (i.e. 15 mg) to 8.5 mL ASM maintained at 37 °C for 96 h in a shaking incubator. After 96 h incubation, 400 μL aliquot was withdrawn, centrifuged, and filtered as previously described. The CIP concentration in the filtered solution was subsequently determined by HPLC.

The dissolution rates of the DPI of CIP nanoplex and DPI of native CIP in the two types of ASM (i.e. normal and bronchiectasis) were determined under sink condition (i.e. 50 $\mu\text{g/mL}$) by adding the CIP powders to 50 mL ASM maintained at 37 °C in a shaking incubator. At specific time points over a 4-h period, 1 mL aliquot was withdrawn and the same volume of fresh ASM was immediately added

back to the dissolution vessel. The aliquot was centrifuged and filtered after which the CIP concentration was determined by HPLC.

3.2.2.4 Mucus permeability

The mucus permeability of the DPI of CIP nanoplex was characterized using a Transwell® membrane diffusion setup (Corning, USA) illustrated in Fig. 3-1. Briefly, 100 μL fresh undiluted sputum (either normal or NCFB blinded to the experimenter) was placed in the apical compartment of a Transwell® polycarbonate membrane inserts (12-mm diameter with 3- μm pore size) attached to a 12-well tissue-culture microplate, while the basolateral compartment was filled with 600 μL DI water. A control run without sputum in which 100 μL of DI water was placed in the apical compartment was carried out. The microplate was then incubated at 37 $^{\circ}\text{C}$ for 30 min after which 2 mg of the DPI of CIP nanoplex (which corresponded to ≈ 600 μg of CIP) was placed in the superior aspect of the sputum layer. At specific time points over 24 h, 50 μL aliquot was withdrawn from the basolateral compartment and replaced with the same volume of DI water. The CIP concentration in the aliquot was subsequently determined by HPLC as previously described.

Figure 3-1: Transwell® membrane diffusion setup used for the mucus permeability experiments.

The mucus permeability of the DPI of native CIP was characterized by the same procedures using 1.5 mg powders also corresponding to 600 µg of CIP. The difference in the mucus permeability between the DPI of CIP nanoplex and the DPI of native CIP was reported in terms of the permeability ratio (PR) defined in Eq. (1) (“bc” stood for basolateral compartment), where PR values larger than unity signified the superior mucus permeability of the CIP nanoplex. The effect of using mannitol or lactose as the excipient on the mucus permeability was characterized by calculating the mannitol-to-lactose ratio (MLR) defined in Eq. (2), where MLR higher than unity signified better mucus permeability when mannitol was used as the excipient.

$$PR = \frac{[CIP]_{bc} \text{ recovered from DPI of CIP nanoplex}}{[CIP]_{bc} \text{ recovered from DPI of native CIP}} \quad (1)$$

$$MLR_{\text{nanoplex}} = \frac{[CIP]_{bc} \text{ recovered from DPI of CIP nanoplex} - \text{mannitol}}{[CIP]_{bc} \text{ recovered from DPI of CIP nanoplex} - \text{lactose}} \quad (2a)$$

$$MLR_{\text{native}} = \frac{[CIP]_{bc} \text{ recovered from DPI of native CIP} - \text{mannitol}}{[CIP]_{bc} \text{ recovered from DPI of native CIP} - \text{lactose}} \quad (2b)$$

3.2.2.5 Antibacterial efficacy

The antibacterial efficacy of the DPI of CPI nanoplex against planktonic *P. aeruginosa* (PAO1 and CS#1-3 strains) in the presence of sputum was examined using the aforementioned Transwell® membrane diffusion setup. Briefly, 50 µL fresh undiluted sputum (either normal or NCFB blinded to the experimenter) was placed in the apical compartment of the Transwell® polycarbonate membrane

inserts attached to a 24-well tissue-culture microplate, while the basolateral compartment was filled with 600 μ L inoculum of *P. aeruginosa* having density of 1×10^6 colony forming units (CFU)/mL. The microplate containing the sputum and inoculum was then incubated at 37 °C for 30 min. Afterwards, 0.5 mg of the DPI of CIP nanoplex (which corresponded to ≈ 150 μ g of CIP) was placed in the superior aspect of the sputum layer. The amount of CIP used ensured that the CIP concentration in the basolateral compartment was above the MICs of the strains, which were in the range of 0.03–0.125 μ g/mL.

Next, 50 μ L aliquot was withdrawn after 1 h from the basolateral compartment and diluted in 450 μ L MHB, followed by filtration using 0.2- μ m membrane filter to prevent antibiotic carryover. The bacterial cells on the filter were then plated onto agar plates and the viable cells were enumerated after 24-h incubation at 37 °C. The antibacterial efficacy was reported as the residual bacterial cell count in $\log_{10}$ CFU/mL. Two control runs, i.e. (i) without antibiotic and (ii) without sputum, were carried out. The antibacterial efficacy of the DPI of native CIP was characterized by the same procedures using 0.4 mg powders corresponding to 150 μ g of CIP.

The antibacterial efficacy against *P. aeruginosa* biofilm was examined using similar procedures with the following modifications. In place of the planktonic suspension, *P. aeruginosa* biofilm was grown on sterile 3-mm glass beads after 24-h incubation using the method previously described in Barber et al. study [99]. The glass beads containing the biofilm were then suspended in 600 μ L MHB and subsequently placed in the basolateral compartment of the Transwell® membrane diffusion setup. After 2-h incubation at 37 °C, the glass beads were removed from the basolateral compartment and washed with PBS to remove the residual antibiotic

and any planktonic cells. The biofilm cells on the glass beads were then removed by three cycles of ultrasonication and vortexing after which the recovered biofilm cells were serially diluted in PBS and plated onto agar plates. The viable cells were enumerated after 24-h incubation at 37 °C.

3.2.2.6 Confocal fluorescence microscope

Overnight cultures of *P. aeruginosa* (CS#1-3 strains) adjusted to optical density $OD_{600} = 0.70$ in MHB were diluted to reach a starting inoculum of 1×10^6 CFU/mL. Subsequently, 200 μ L of the inoculums was placed in 8-well microscopic slides and incubated for 24 h at 37 °C. Afterwards, the biofilm formed in the wells was washed with PBS and MHB was added as fresh medium. Next, a Transwell® polycarbonate membrane inserts containing 100 μ L fresh undiluted normal sputum in its apical compartment was placed above the wells. Subsequently, 0.5 mg of the DPI of CIP nanoplex (or DPI of native CIP) was placed onto the superior aspect of the sputum layer.

After 2-h incubation at 37 °C, the inserts were removed and the biofilm in the wells was washed with PBS, followed by staining with LIVE/DEAD BacLight™ Bacterial Viability kit (SYTO9, propidium iodide) (ThermoFisher Scientific, USA). The stained biofilm was then imaged by Confocal Fluorescence Microscopy (CFM) (LSM710, Zeiss, Germany) in which fluorescent images of the live cells (green) and the dead cells (red) were acquired at 488 and 543 nm, respectively. The images were analyzed using COMSTAT 2 software (Technical University of Denmark) to determine the cells' Live/Dead ratios.

3.2.2.7 Cytotoxicity

The A549 cells were cultivated at the density of 10^5 cells/well in a 24-well tissue culture microplate containing DMEM supplemented with 10% (w/v) fetal bovine serum and 1% (w/v) penicillin-streptomycin solution. The 16HBE14o- cells were cultivated in MEM supplemented with 1% (w/v) L-glutamine (200 mM), 10% (w/v) fetal bovine serum, and 1% (w/v) penicillin-streptomycin solution. The cells were incubated for 24 h at 37 °C in 5% CO₂ incubator. Afterwards, the DPI of CIP nanoplex re-dispersed in DMEM was added to the cells at CIP concentrations in the range of 12.5–250 mg/L. This concentration range represented the typical range of CIP concentration recovered in the lungs (i.e. 33–237 mg/L) upon inhaled CIP delivery [42, 100].

The treated cells were then incubated at 37 °C for 24 h after which the culture media was replaced with serum-free DMEM containing 100 µL of MTT dye solution (0.5% w/v in PBS). Next, the cells were further incubated for 4 h. Afterwards, 2 mL DMSO were added to dissolve the formazan crystals generated by the viable cells. The formazan concentration was determined by UV–Vis spectrophotometry at 550 nm from which the cell survival was calculated by the ratio of OD₅₅₀ of the treated cells to OD₅₅₀ of the control cells (no treatment). The cytotoxicity tests were also performed for the DPI of native CIP and the non-active ingredients used in the CIP nanoplex powder preparation (i.e. mannitol, DXT, and AA).

3.3 Results and Discussion

3.3.1 Mucin interaction

The interaction between mucin and the DPI of CIP nanoplex was examined by comparing the CIP concentration recovered in the dialysate in the presence of mucin with the CIP concentration recovered in the control run (i.e. without mucin). The results in Fig. 3-2 showed that the CIP concentration recovered in the dialysate was slightly lower in the presence of mucin, particularly in the early period (< 120 min), suggesting the binding of the CIP nanoplex with mucin. A similar observation of mucin binding had been reported for the native CIP by Huang et al. [95].

Figure 3-2: Interactions between mucin and DPI of CIP nanoplex as characterized by the % CIP in the dialysate.

Specifically, the CIP concentrations expressed as the percentage of the total CIP concentration in the dialysis bag were found to be equal to $46 \pm 1\%$ (w/w) in the presence of mucin versus $57 \pm 2\%$ in the absence of mucin after 30 min. The CIP concentrations in the dialysate after 60 min were equal to $74 \pm 2\%$ and $82 \pm 2\%$ with and without mucin, respectively. The effect of the mucin binding became

negligible after 120 min as both CIP concentrations reached 100% indicating that the CIP nanoplex did not permanently bind with mucin. In other words, while the CIP nanoplex was hindered by mucin to a certain degree, the results showed that the CIP nanoplex was not mucoadhesive, therefore it should be suitable for NCFB therapy. The mucus permeability of the DPI of CIP nanoplex in *ex vivo* NCFB sputum was investigated next in Section 3.3.3.

3.3.2 Dissolution characteristics in ASM

Owing to the supersaturation generated by its amorphous state, the CIP nanoplex was found to exhibit a significantly higher solubility in the normal ASM than the native CIP with solubility equal to $(7.8 \pm 0.7) \times C_{\text{Sat}}$ in which C_{Sat} of the native CIP in the normal ASM was equal to $263 \pm 3 \mu\text{g/mL}$. The solubilities of the CIP nanoplex and the native CIP were found to be similar in the normal and bronchiectasis ASMs, despite the different salt concentration present in each ASM (data not shown). Importantly, the CIP nanoplex's solubility in the ASM was found to remain high after 96 h indicating the slow crystallization of the supersaturated CIP solution in the ASM, despite the absence of a known crystallization-inhibiting agent, such as hypromellose, in the ASM (Fig. 3-3A). In this regard, the presence of various biopolymers in the ASM likely contributed to the crystallization inhibition of the supersaturated CIP solution due to the well-established diminished nucleation rates in the presence of polymers [101].

Figure 3-3: (A) Supersaturation solubility of the CIP nanoplex in the normal ASM expressed as a multiple of the saturation solubility of the native CIP (C_{Sat}); (B) and (C) dissolution rates of the DPI of CIP nanoplex and DPI of native CIP prepared with mannitol as the excipient in the normal ASM and the bronchiectasis ASM, respectively.

It had to be pointed out, nevertheless, that the supersaturated solubility of the CIP nanoplex in the ASM eventually decreased back to C_{Sat} as time progressed (> 96 h) due to crystallization of CIP from the supersaturated solution. The prolonged high solubility of the CIP nanoplex boded well for its ability to potentially produce high antibiotic exposure in the infected lungs. Furthermore, the high solubility of the CIP nanoplex was achieved quickly in less than 1 h upon dissolution in the ASM denoting its fast dissolution rate (Fig. 3-3A). The fast dissolution rate of the CIP nanoplex in the ASM was attributed to the weakened electrostatic binding between CIP and DXT in the presence of high salt ion concentrations of the ASM.

Importantly, the fast dissolution rate of the CIP nanoplex was maintained upon its transformation to the DPI formulation. Specifically, for the DPI of CIP nanoplex prepared using mannitol as the excipient, $87 \pm 2\%$ (w/w) of the CIP payload was released in the normal ASM after 30 min (Fig. 3-3B). The fast dissolution rate exhibited by the DPI of CIP nanoplex signified that the use of mannitol as the drying adjuvant (in addition to its role as mucolytic agent) was appropriate to prevent irreversible agglomeration of the CIP nanoplex upon drying, which otherwise would lead to poor dissolution rates of the dry-powder form [102].

A similarly fast dissolution rate in the normal ASM was also observed for the DPI of native CIP with $88 \pm 5\%$ release after 30 min (Fig. 3-3B). It was worth pointing out, however, that the fast dissolution rates exhibited *in vitro* by the DPIs of CIP nanoplex and native CIP could be partly attributed to the sink condition used in the dissolution test, which might not be representative of the *in vivo* condition in which a limited amount of fluid in the lung lumen was available [103]. Therefore, while the present results showed that both the DPI formulations readily dissolved to

free CIP in a sputum-like environment, a further study using an animal lung model would need to be carried out in the future to determine their dissolution rates in a more clinically-relevant setting.

Similar dissolution time-profiles for both the DPIs of CIP nanoplex and native CIP were observed from the dissolution test performed in the bronchiectasis ASM denoting the minimal impact of the salt concentration (Fig. 3-3C). On this note, the CIP payloads of the DPI of CIP nanoplex and DPI of native CIP were equal to $31.5 \pm 1.0\%$ and $42.1 \pm 0.6\%$ (w/w), respectively, where the lower CIP payload of the former was caused by the presence of DXT in the CIP nanoplex. Even though both DPI formulations exhibited comparable CIP payload and similarly fast dissolution rates in the ASM, a higher CIP concentration would be delivered from a given dose by the DPI of CIP nanoplex owed to the higher ASM solubility of the CIP nanoplex.

3.3.3 Mucus permeability

3.3.3.1. Mannitol as excipient

The results showed that the DPI of CIP nanoplex exhibited significantly superior mucus permeability to the DPI of native CIP ($PR > 1$) in both normal sputum ($n = 4$) and NCFB sputum ($n = 7$) with $PR = 4.73 \pm 0.41$ and 6.13 ± 1.89 , respectively, after 1 h (Fig. 3-4A). The PR remained approximately at these values after 2, 3, 6, and 24 h denoting the DPI of CIP nanoplex remained superior even as the time for permeation was extended. For comparison, the control run without sputum, as expected, resulted in $PR = 1.06 \pm 0.10$ after 1 h denoting equal permeation rates between the DPIs of the CIP nanoplex and native CIP in the

absence of sputum. The different PR values observed in the normal and NCFB sputum, nevertheless, were found to be statistically insignificant indicating the minimal influence of the sputum heterogeneity on the mucus permeability of both the CIP powders.

Figure 3-4: Mucus permeability ratios (PR) of the DPI of CIP nanoplex and DPI of native CIP prepared using (A) mannitol and (B) lactose as the excipient in normal and NCFB sputum (NS = not statistically significant).

As both DPIs of the CIP nanoplex and the native CIP were previously established in Section 3.3.2 to readily disintegrate followed by dissolution to free CIP in a sputum-like environment of the ASM, the superior mucus permeability of the CIP nanoplex was attributed primarily to its ability to produce supersaturated CIP concentration in the sputum, resulting in higher CIP concentrations recovered in the basolateral compartment of the Transwell® diffusion setup. On this note, another factor that could plausibly contribute to the superior mucus permeability of the CIP nanoplex was its built-in physical characteristics that, as mentioned before, would enable the nanoplex to navigate through the mucus barrier prior to its disintegration. This contribution, nevertheless, would be challenging to characterize by standard particle tracking technique as the CIP nanoplex simultaneously disintegrated while it navigated through the sputum.

3.3.3.2. Lactose as excipient

To investigate whether the mucolytic activity of mannitol had any role towards the superior mucus permeability of the DPI of CIP nanoplex, the mucus permeability of the DPI of CIP nanoplex prepared using lactose as the excipient – a widely used excipient for DPI but not known for its mucolytic activity – was investigated next. The results showed that the DPI of CIP nanoplex remained superior to its native CIP counterpart with $PR = 6.32 \pm 1.65$ and 6.84 ± 2.45 after 1 h in normal and NCFB sputum, respectively (Fig. 3-4B). The PR was found to decrease with time, while remained above unity, suggesting that the mucus permeability of the native CIP became closer to that of the CIP nanoplex over time. Specifically, the PRs after 3 h were equal to 5.21 ± 1.29 and 6.58 ± 3.10 in normal

and NCFB sputum, respectively, after which they were reduced to 4.06 ± 1.01 and 5.65 ± 2.12 after 6 h, and to 2.82 ± 0.38 and 4.14 ± 0.66 after 24 h, respectively (Fig. 3-4B).

It was found that the DPI of CIP nanoplex prepared using lactose as the excipient exhibited a burst permeation time-profile as determined from $[CIP]_{bc}$, resulting in a rapid rise in the PR values after 1 h. The permeation rate of the CIP nanoplex then tapered off with time leading to the gradual decrease in the PR values. For comparison, the permeation rate exhibited by the DPI of CIP nanoplex prepared using mannitol was more gradual as a function of time likely caused by its slower disintegration in the ASM, leading to its relatively constant PR values over time.

Furthermore, similar to the earlier results with mannitol as the excipient, the different PR values observed in the normal and NCFB sputum were again found to be statistically insignificant when lactose was used as the excipient. On this note, while we examined the mucus permeability of the DPI of CIP nanoplex using only NCFB sputum, it had to be pointed out that there are other disease conditions in which inhaled antibiotic nanoplex exhibiting good mucus permeability could be useful, for example, in chronic rhinosinusitis therapy.

3.3.3.3 Mannitol versus lactose

The DPI of CIP nanoplex was demonstrated to exhibit superior mucus permeability to its native CIP counterpart independent of the excipient used. Comparing between mannitol and lactose, $MLR_{Nanoplex}$ after 1 h were calculated to be equal to 0.61 ± 0.22 and 0.71 ± 0.12 in normal and NCFB sputum, respectively,

which indicated slightly higher mucus permeability of the CIP nanoplex when lactose was used as the excipient due to the abovementioned fast permeation rate initially (Fig. 3-5A). Nevertheless, the $MLR_{Nanoplex}$ increased to approach unity after 6 h and 24 h in normal and NCFB sputum, respectively, indicating the reduced impact of the excipient type on the mucus permeability of the DPI of CIP nanoplex over time.

Figure 3-5: MLR ratios of (A) DPI of CIP nanoplex and (B) DPI of native CIP in normal and NCFB sputum (NS = not statistically significant).

For the DPI of native CIP, MLR_{Native} after 1 h were calculated to be equal to 0.81 ± 0.14 and 1.21 ± 0.56 in normal and NCFB sputum, respectively, indicating comparable mucus permeability of the native CIP using either mannitol or lactose as the excipient (Fig. 3-5B). The MLR_{Native} essentially remained approximately at these values with time as subsequent variations in the MLR_{Native} were not statistically significant. On the same note, the variations in both $MLR_{Nanoplex}$ and MLR_{Native} observed between the normal and NCFB sputum were not statistically significant either. In short, this analysis demonstrated the minimal impact of the excipient type on the mucus permeability of the DPIs of CIP nanoplex and native CIP. For this reason, the subsequent antibacterial efficacy and cytotoxicity studies were performed only for the DPI formulations prepared using mannitol as the excipient.

3.3.4 Antibacterial efficacy in the presence of sputum

3.3.4.1 Against planktonic *P. aeruginosa*

In the presence of NCFB sputum, both DPIs of CIP nanoplex and native CIP exhibited complete eradications of the planktonic PAO1 strain, which was not unexpected considering the high susceptibility of the PAO1 strain towards CIP [104] (Fig. 3-6A). Against the planktonic clinical strains (CS#1-3), however, only the CIP nanoplex exhibited bactericidal activities defined as $>3 \log_{10}$ CFU/mL reduction in the bacterial load compared to that of the non-treated control (Fig. 3-6A). Specifically, the mean reduction in the bacterial load afforded by the CIP nanoplex against the three clinical strains was equal to $4.48 \pm 0.62 \log_{10}$ CFU/mL, which was significantly higher than $2.76 \pm 0.54 \log_{10}$ CFU/mL exhibited by the

native CIP ($p \leq 0.05$). The superior antibacterial efficacy of the CIP nanoplex against the clinical strains was further reaffirmed by the visual observation of the agar plates (Fig. 3-6B).

Figure 3-6: Antibacterial efficacy of the DPI of CIP nanoplex and DPI of native CIP (mannitol as the excipient) against laboratory and clinical strains of planktonic *P. aeruginosa* in the presence of NCFB sputum, (A) residual bacterial cell counts and (B) visual observation of the agar plates (* $p \leq 0.05$).

Figure 3-7: Antibacterial efficacy of the DPI of CIP nanoplex and DPI of native CIP (mannitol as the excipient) against laboratory and clinical strains of planktonic *P. aeruginosa* in the presence of normal sputum, (A) residual bacterial cell counts and (B) visual observation of the agar plates (* $p \leq 0.05$, ** $p \leq 0.01$).

The same results were observed from the studies performed in the normal sputum in which the DPI of CIP nanoplex was shown to exhibit superior antibacterial efficacy to its native CIP counterpart against the clinical strains ($p \leq 0.01$ for CS#1-2 and $p \leq 0.05$ for CS#3) (Fig. 3-7). The lower antibacterial efficacy of the DPI of native CIP was caused by its aforementioned poorer mucus permeability, resulting in lower antibiotic exposure to the bacterial cells. For the

control run without sputum, both DPIs of CIP nanoplex and native CIP, as expected, produced similar reductions in the bacterial load (data not shown) as they produced similar antibiotic exposures in the absence of sputum attributed to their equal permeability through the Transwell® membrane.

3.3.4.2 Against *P. aeruginosa* biofilm

The superior antibacterial efficacy of the DPI of CIP nanoplex in the presence of sputum was also demonstrated against biofilm colonies of *P. aeruginosa*. Against PAO1 biofilm, the CIP nanoplex exhibited bactericidal activities with mean reduction in the bacterial load equal to $3.69 \pm 1.26 \log_{10}$ CFU/mL, which was significantly higher than $1.89 \pm 0.68 \log_{10}$ CFU/mL for the native CIP ($p \leq 0.001$) (Fig. 3-8A). A similar trend was observed against CS#1-3 biofilms. Specifically, the mean reductions in the bacterial load afforded by the CIP nanoplex were equal to 2.79 ± 0.20 , 3.53 ± 1.02 , and $3.84 \pm 0.34 \log_{10}$ CFU/ mL against CS#1, CS#2, and CS#3, respectively, which were significantly higher than 1.76 ± 0.65 ($p \leq 0.01$), 1.80 ± 0.88 ($p \leq 0.01$), and 2.45 ± 0.26 ($p \leq 0.001$) $\log_{10}$ CFU/mL, respectively, afforded by the native CIP (Fig. 3-8A).

The superior antibiofilm efficacy of the CIP nanoplex was reaffirmed by the CFM analysis showing a significantly higher presence of viable cells (as indicated by their green colors) in the biofilm colonies treated by the native CIP compared to those treated by the CIP nanoplex (Fig. 3-8B). The calculated mean Live/Dead ratios were equal to 0.99 ± 0.20 , 0.56 ± 0.10 , and 0.25 ± 0.11 for the non-treated control, and for the biofilms treated by the native CIP and CIP nanoplex,

respectively (Fig. 3-8C). The lower Live/Dead ratio signified the lower residual bacterial cell count resulted from the CIP nanoplex treatment ($p \leq 0.001$).

Figure 3-8: Antibiofilm efficacy of the DPI of CIP nanoplex and DPI of native CIP (mannitol as the excipient) against laboratory and clinical strains of *P. aeruginosa* biofilm in the presence of normal sputum, (A) residual bacterial cell counts, (B) visual observation by CFM, and (C) Live/Dead ratios (** $p \leq 0.01$, *** $p \leq 0.001$).

3.3.5 Cytotoxicity towards A549 and 16HBE14o- cells

Both DPIs of CIP nanoplex and native CIP exhibited negligible toxicity towards the A549 cells, where more than 95% of the cells remained viable upon exposure to 50 mg/L of CIP (Fig. 3-9A). At the highest CIP concentration investigated (i.e. 250 mg/L), the % cell survivals were equal to $77.9 \pm 2.4\%$ and $84.3 \pm 1.4\%$ for the CIP nanoplex and native CIP, respectively. The variations in the % cell survivals between the CIP nanoplex and native CIP were determined to be statistically insignificant, which suggested that the additional ingredients involved in the CIP nanoplex preparation (i.e. DXT, AA) did not lead to its higher cytotoxicity compared to the native CIP. The non-cytotoxicity of the non-active ingredients (i.e. DXT, AA, and mannitol) towards the A549 cells was later verified and presented in Fig. 3-10A.

Similar results were obtained for the cytotoxicity towards the 16HBE14o- cells, albeit at lower % cell survivals. At the highest CIP concentration investigated, the % cell survivals were equal to $53.7 \pm 9.8\%$ and $64.7 \pm 4.3\%$ for the CIP nanoplex and the native CIP, respectively (Fig. 3-9B), where again the variations in the % cell survivals between the CIP nanoplex and the native CIP were determined to be statistically insignificant. In this regard, the lower % cell survivals ought to be attributed to the inherent sensitivity of the 16HBE14o- cells towards CIP, as the non-active ingredients had been verified separately to possess minimal cytotoxicity towards the 16HBE14o-cells (Fig. 3-10B).

Figure 3-9: Cytotoxicity of DPI of CIP nanoplex and DPI of native CIP (mannitol as the excipient) towards (A) A549 and (B) 16HBE14o-cell lines (NS = not statistically significant).

Figure 3-10: Cytotoxicity of the non-active excipients (i.e. DXT, mannitol, and AA) present in the DPI of CIP nanoplex towards (A) A549 and (B) 16HBE14o- cell lines. Note: The concentrations of the non-active excipients used in the cytotoxicity study were expressed in terms of the equivalent CIP concentrations present in the DPI formulation at a given concentration of the excipient (NS = not statistically significant).

3.4 Conclusions

First, the DPI of CIP nanoplex was demonstrated to exhibit superior mucus permeability to its native CIP counterpart in *ex vivo* sputum, which was attributed to the CIP nanoplex's built-in ability to produce highly supersaturated concentration of CIP in the sputum environment. This was despite the fact that its dissolution was slightly hindered by the presence of mucin in the sputum. The superior mucus permeability of the CIP nanoplex was evident independent on whether or not mucolytic excipient was used. Second, the superior mucus permeability in turn led to the DPI of CIP nanoplex's higher antibacterial efficacy against clinical strains of *P. aeruginosa* (both planktonic and biofilm) in the presence of *ex vivo* sputum. Third, the DPI of CIP nanoplex exhibited similar *in vitro* cytotoxicity towards human lung epithelial cells as its native CIP counterpart denoting its low risk of toxicity. The results of our proof-of-concept study successfully established the DPI of CIP nanoplex as a highly promising CIP delivery strategy for long-term management of NCFB. The antibacterial efficacy of the DPI of CIP nanoplex against other pathogens clinically relevant to NCFB remained to be investigated in the future. Most importantly, the true therapeutic advantages afforded by the DPI of CIP nanoplex must be evaluated in the future from *in vivo* studies and subsequently in human clinical trials.

CHAPTER 4. THE EFFECTIVENESS OF THE DPI OF CIP NANOPLEX VERSUS THE DPI OF CIP LIPOSOME IN THE NCFB ENVIRONMENT

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4.1 Introduction

Individuals suffering from bronchiectasis are subject to a vicious cycle of airways inflammation, poor mucus clearance, and recurring infection that manifest in chronic cough and purulent sputum production, resulting in progressive lung damage [5]. A majority of bronchiectasis patients, with either cystic fibrosis (CF) or non-CF bronchiectasis (NCFB), experience frequent infective exacerbations causing further reduction in their quality of life, accelerated decline in the lung function, and consequently high risk of mortality [8, 105]. Therefore, the primary goal of bronchiectasis management therapy has been to reduce the frequency of exacerbations and slow down the disease progression [7, 9].

Inhaled antibiotics has been proposed to have a role in bronchiectasis management as a long-term intermittent therapy to reduce the airway bacterial burden [33, 106], particularly from *Pseudomonas aeruginosa*, where its colonization has been associated with increased frequency of exacerbations and

mortality [2, 10, 11]. Inhaled antibiotics has been the mainstay of treatment for CF with a number of clinically approved products, such as dry powder inhalers (DPI) of tobramycin (TOBI®) and colistin (Colobreathe®), nebulized aztreonam (Cayston®), and with several other products currently under late-stage clinical trials [107].

In contrast, currently there is not yet a clinically approved inhaled antibiotic product for NCFB therapy due to (1) a lack of long-term efficacy data and (2) the mixed outcomes in the clinical trials due to highly heterogeneous NCFB patient populations [87, 108]. Among the various antibiotics investigated, ciprofloxacin (CIP) – a broad-spectrum fluoroquinolone with proven bactericidal activity against *P. aeruginosa* – has been shown to possess the most promising clinical evidence for NCFB [38, 85]. At present, two inhaled CIP products, i.e. (1) nebulized liposome encapsulating CIP (ORBIT-3 and 4 trials) [17] and (2) DPI of native CIP (RESPIRE 1 and 2 trials) [15, 16] have reached Phase III clinical trials specifically for NCFB.

While the recently reported RESPIRE trials clearly suggest some benefits for NCFB, the development of innovative and improved formulations of inhaled CIP remains required due to the failure of RESPIRE from gaining approval by the United States' Food and Drug Administration [109]. One of the challenges faced in the development of inhaled CIP lies in the sub-inhibitory antibiotic exposure in the infected airways when antibiotics is delivered in its native form [90]. The sub-inhibitory antibiotic exposure in bronchiectasis lungs is caused primarily by the presence of a thick stationary mucus layer that limits antibiotic diffusion to reach the embedded bacterial colonies [110]. The problem is compounded by the rapid systemic absorption of those antibiotic molecules that have overcome the mucus

barrier [111]. The sub-inhibitory antibiotic exposure poses a serious problem as it not only leads to poorer therapeutic outcomes, but also increases the risk of the emergence of antibiotic-resistant bacterial strains [112].

In this regard, antibiotic nanoparticles with mucus-inert surface properties and sustained release profile have been widely accepted as a promising formulation strategy to improve the antibiotic exposure in the infected airways [21, 113-115]. For example, the nanoscale size of the CIP liposome in the ORBIT formulation enabled it to permeate through the aqueous pores of the mucus, while its anionic and hydrophilic surface minimized its binding with the polyanionic hydrophobic contents of the mucus (e.g. mucin, DNA) [42, 116]. This resulted in enhanced mucus permeability of the CIP liposome compared to the native CIP, hence improved the CIP exposure in the infected airway, resulting in higher antibacterial efficacy. Moreover, the CIP liposome also increased the CIP exposure in the infected airways by virtue of the prolonged lung residence time afforded by the slow release of the encapsulated CIP from liposome [117]. Besides CIP liposome, another example of CIP nanoparticle delivery system intended for bronchiectasis therapy was in the form of CIP-encapsulated polymer nanoparticles [118, 119].

Our group previously developed an alternative formulation of antibiotic nanoparticles in the form of CIP nanoparticle complex (or nanoplex in short) [22]. In addition to its mucus-inert surface and nanosize, the CIP nanoplex also exhibited a significantly higher apparent solubility than the native CIP in biological fluids attributed to its metastable amorphous state, which bodes well for its ability to enhance the CIP exposure in the airways. Indeed, our recent study showed that the DPI of CIP nanoplex exhibited higher *ex vivo* mucus permeability in NCFB sputum

than its native CIP counterpart which led to its higher antibacterial efficacy [71]. Moreover, the CIP nanoplex was shown to exhibit a similar cytotoxicity profile towards human lung epithelium cells as the native CIP, hence denoting its low risk of toxicity [71].

To further establish the CIP nanoplex as a viable inhaled antibiotic delivery system for NCFB management, a comparison with a more established delivery system represented by the CIP liposome is needed. The objectives of the present work therefore were to compare the DPI of CIP nanoplex against the DPI of CIP liposome in terms of their (1) physical characteristics (i.e. size, zeta potential, CIP payload, preparation efficiency), (2) *in vitro* aerosolization efficiency, (3) dissolution characteristics in artificial bronchiectasis sputum medium (ABSM), (4) *ex vivo* mucus permeability, (5) antimicrobial activity against *P. aeruginosa* (both planktonic and biofilm) in the presence of mucus barrier, and lastly (6) cytotoxicity towards human lung epithelial cells.

In this regard, unlike in the ORBIT formulation, we opted to use the DPI delivery mode for the CIP liposome over nebulization because of the many drawbacks of the nebulized delivery, such as complex and time-consuming administration, non-portability, meticulous cleaning, sterilization, and maintenance requirement. In contrast, the DPI delivery mode offered simple, short one-breath administration, no cleaning requirement, and full portability [89]. Moreover, the drawbacks of the nebulization delivery had been shown to result in compromised clinical outcomes due to a decrease in the drug delivery efficiency over time [120]. In place of spray drying used in Tran et al. (2018) [71], the DPI formulations of CIP nanoplex and CIP liposomes were both prepared in the present work by spray freeze

drying to minimize the product loss. D-Mannitol – a well-established mucolytic and cryoprotective agent [121, 122] – was used as the drying adjuvant.

4.2 Materials and Methods

4.2.1 Materials

Materials for CIP nanoplex and CIP liposome preparations: CIP, D-Mannitol, glacial acetic acid (AA), cholesterol, tert-butanol, ethanol, and ammonium sulfate were purchased from Sigma Aldrich (Singapore). Sodium dextran sulfate (DXT) (MW 5 kDa) and unsaturated soy L- α -phosphatidylcholine were purchased from Wako Pure Chemical (Japan) and Avanti Polar Lipids (USA), respectively.

Materials for ABSM preparation: Mucin from porcine stomach, salmon sperm DNA, hydrogen chloride (HCl), Tris base, sodium chloride (NaCl), and potassium chloride (KCl) were purchased from Sigma Aldrich (Singapore). Diethylenetriamine pentaacetic acid (DPTA), egg yolk emulsion, and casamino acid were purchased from Alfa Aesar (Singapore), Oxoid (UK), and Merck Millipore (Singapore), respectively. Acetonitrile, methanol, and potassium dihydrogen phosphate (KH_2PO_4) for the ABSM dissolution characteristics were purchased from Merck Millipore (Singapore).

Clinical sputum: The study was approved by the Institutional Review Board of Nanyang Technological University (IRB-2016-01-031), the Institutional Review Board of SingHealth (CIRB C:2016/2073) which includes cross recognition from the National Healthcare Group's Domain Specific Review Board (DSRB). The suitability of the sputum for inclusion in the study was confirmed by the criteria detailed in our previous work [94]. The sputum was spontaneously expectorated

from a deep cough from healthy individuals (n = 3) and NCFB patients (n = 2) at Tan Tock Seng Hospital (Singapore). The expectorated sputum was stored in sterile containers and immediately frozen at -80°C . The recruited healthy individuals were at least 21 years of age, had no history of any respiratory disease, and had never received regular medication in any form. The NCFB patients were at least 21 years of age, clinically stable at time of recruitment, had radiologically confirmed bronchiectasis by high resolution computed tomography (HRCT), and had first forced expiratory volume (FEV1) $\geq 30\%$ predicted. Patients with any of the following were excluded: other major respiratory diagnoses other than bronchiectasis, exacerbation or antibiotic use in the preceding four weeks prior to recruitment, use of regular corticosteroids in any form, pregnant or breastfeeding, active mycobacterial disease, or cancer treatment.

Materials for bacterial cell culture: *P. aeruginosa* PAO1 laboratory strain was purchased from ATCC (USA) and two clinical isolates of *P. aeruginosa* from NCFB patients were kindly provided by Lee Kong Chian School of Medicine (Singapore). Mueller Hinton Broth (MHB), tryptic soy agar, and phosphate buffered saline (PBS, pH 7.4) for the bacterial cell culture were purchased from BD Diagnostics (Singapore), Gibco (USA), and Sigma Aldrich (Singapore), respectively.

Materials for cytotoxicity tests: A549 human carcinoma lung epithelial cells and 16HBE14o- human bronchial epithelial cells were purchased from ATCC (USA) and kindly donated by California Pacific Medical Center Research Institute, respectively. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were purchased from GE Healthcare Life Sciences (USA). Minimum

Essential Medium with Eagle's salt (MEM), L-glutamine (200 mM), and 0.25% trypsin ethylenediaminetetraacetic acid (EDTA) solution were purchased from Gibco (USA). Penicillin-streptomycin, dimethyl sulfoxide (DMSO), and (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT, 98% purity) were purchased from PAA Laboratories (Austria), Sigma Aldrich (Singapore), and Alfa Aesar (UK), respectively.

4.2.2 Methods

4.2.2.1 Preparation and characterization of CIP nanoplex

The CIP nanoplex was prepared by electrostatically driven complexation with DXT as detailed previously in Cheow and Hadinoto (2012) [22]. Specifically, the protonated CIP molecules at acidic pH formed electrostatic binding with oppositely charged DXT, resulting in the formation of soluble CIP-DXT complex. The soluble complex then formed aggregates among themselves as a result of the hydrophobic interactions among the bound CIP molecules. Upon reaching a critical mass, the complex aggregates precipitated out of the solution to form the CIP nanoplex.

Briefly, CIP was dissolved at 10 mg/mL in 0.3% (w/v) aqueous AA solution and DXT was dissolved separately in deionized water at 4.5 mg/mL. Equal volumes of the CIP and DXT solutions were then mixed under gentle stirring. The resultant CIP nanoplex suspension was then washed by two cycles of centrifugation (14,000g for 5 min) to remove excess CIP and DXT that did not form the CIP nanoplex. Afterwards, the washed CIP nanoplex was re-dispersed in deionized water for characterizations. The size, polydispersity index (PDI), and zeta potential of the CIP nanoplex were characterized in triplicates by dynamic light scattering (DLS) after

100× dilution using Brookhaven 90 Plus Nanoparticle Size Analyzer (Brookhaven Instruments Corporation, USA).

The CIP payload, which was defined as the amount of CIP per unit mass of the CIP nanoplex, was determined in triplicates by dissolving a known mass of freeze-dried CIP nanoplex powders in 0.3% (w/v) AA solution. The amount of CIP in the AA solution was subsequently determined by UV–Vis spectrophotometer (UV Mini 1240, Shimadzu, Japan) at the optimal absorbance wavelength for CIP (i.e. 324 nm). The complexation efficiency (CE), which was defined as the ratio of the CIP mass that was transformed to the CIP nanoplex to the initial CIP mass added, was determined in triplicates by taking the difference between the initial CIP mass added and the CIP mass recovered in the supernatant after the first centrifugation of the CIP nanoplex suspension.

4.2.2.2 Preparation and characterization of CIP liposome

The CIP liposome was prepared by the active loading method driven by a lipid transmembrane pH gradient described previously by Ong et al. (2014) [117]. Briefly, soy L- α -phosphatidylcholine and cholesterol were dissolved at ratio of 7: 3 (w/w) in a mixture of tert-butanol: ethanol: water (49: 49: 2 v/v) under constant stirring at 70 °C for 1 h. The solvent was then removed by continuous evaporation at 70 °C, resulting in the formation of thin lipid film. The lipid film was subsequently hydrated with 0.5 M ammonium sulfate solution at 60 °C to form multilamellar liposomes, which were then extruded through a 0.2- μ m polycarbonate membrane syringe filter to produce unilamellar liposomes.

Aqueous CIP solution was then added to the unilamellar liposome suspension at the optimal CIP to liposome molar ratio ($M_{\text{CIP/liposome}}$) equal to 0.3 above which the encapsulation efficiency was lower. Afterwards, the CIP and liposome solutions were incubated at 50 °C for 2 h under gentle shaking to facilitate active loading of CIP into the liposome. The resultant CIP liposome suspension was then washed by centrifugation (14,000g for 1 h) at 4 °C to remove the non-encapsulated CIP. The size, PDI, and zeta potential of the CIP liposome were characterized in triplicates by DLS as described previously.

The CIP payload was determined in triplicates by dissolving a known mass of lyophilized CIP liposome powders in 80% (v/v) aqueous ethanol solution to liberate CIP from the liposome. The ethanol solution was subsequently diluted tenfold in 0.3% AA solution to dissolve the liberated CIP after which the CIP solution was centrifuged at 14,000g for 15 min to remove the precipitated lipids. The amount of CIP in the supernatant was determined by high performance liquid chromatography (HPLC) using Agilent 1100 (Agilent Technologies, USA). The HPLC analysis was performed in ZORBAX Eclipse Plus C18 column (250 × 4.6 mm, 5- μm particle size) at detection wavelength of 324 nm. Acetonitrile: methanol: 0.1 M KH_2PO_4 (15: 25: 60 v/v) was used as the mobile phase at 1.0 mL/min, resulting in CIP retention time of ≈ 3.6 min.

The encapsulation efficiency (EE), which was defined as the ratio of the CIP mass encapsulated into the liposome to the initial CIP mass added, was determined in triplicates by taking the difference between the initial CIP mass added and the CIP mass recovered in the supernatant after centrifugation of the CIP liposome

suspension. The amount of CIP in the supernatant was determined by HPLC as previously described.

4.2.2.3 DPI formulations

DPI formulations of the CIP nanoplex and the CIP liposome were prepared by spray freeze drying (SFD) as described previously by Yu et al. (2016) [23]. Prior to SFD, we had to assess whether the CIP nanoplex and CIP liposome possessed adequate physical integrity to undergo the freeze drying and SFD without significant adverse effects on their physical characteristics. For this purpose, the size, zeta potential, and CIP payload of the CIP nanoplex and CIP liposome were re-examined after undergoing freeze drying in the presence of D-Mannitol as the cryoprotectant at 1:1 mass ratio. The study was performed in Alpha 1–2 LD Plus freeze dryer (Martin Christ, Germany) operated at $-52\text{ }^{\circ}\text{C}$ and 0.05 mbar for 24 h, followed by rehydration of the freeze-dried powders in deionized water. The same examination was repeated for the nanoparticles after undergoing the SFD process.

Upon the completion of the physical integrity test, the SFD feed solution was prepared at 5.0% (w/v) solid concentration by adding D-Mannitol to the aqueous suspension of the CIP nanoplex (or CIP liposome) at 1:1 mass ratio. The feed solution was then sprayed to 400 mL liquid nitrogen placed in a large polypropylene vessel at feed flowrate and atomizing air flowrate of 0.25 L/h and 300 L/h, respectively. The distance between the atomizer and the liquid nitrogen container was fixed at 10 cm to maximize the production yield. The resultant frozen particles were immediately transferred to a freeze drying unit afterwards, the dry powders were stored in a dry cabinet for 48 h at $25\text{ }^{\circ}\text{C}$ prior to their use in the

biological and aerosolization experiments. The geometric size (d_G) and morphology of the DPI formulation were examined by scanning electron microscope (SEM) (6390LA, JEOL, USA) in which the size analysis was performed using ImageJ software (NIH, USA) with a minimum of 300 particle counts.

4.2.2.4 Aerosolization efficiency

The aerosolization efficiency of the DPI formulations was characterized following Yu et al. (2016) [23] in terms of the (1) emitted dose (ED), (2) fine particle fraction (FPF), and (3) mass median aerodynamic diameter (MMAD). The aerosolization test was carried out in triplicates in a seven-stage Next Generation Impactor (NGI) (Copley Scientific, UK) equipped with an induction port (IP), a pre-separator (PS), and a standardized powder entrainment tube (PET) used as the model inhaler. Following the compendial requirement of the European Pharmacopoeia (Ph. Eur) and United States Pharmacopoeia (USP) for proper assessment of DPI delivery performance [123], the inhalation flowrate in the NGI was set at 85 L/min to achieve the requirement of having 4.0 L of air drawn at 4 kPa pressure drop across the inhaler. At this flowrate, the cut-off aerodynamic diameter (d_A) at stages 1–7 of the NGI were equal to 6.7, 3.7, 2.4, 1.4, 0.8, 0.5 and 0.3 μm , respectively. On this note, inhaled powders with $d_A \leq 5.0 \mu\text{m}$ and MMAD between 2 and 6 μm were ideal for deposition in the upper respiratory tract [124].

The airflow duration was set at 2.8 s to simulate the volume of air drawn in inhalation of healthy humans (i.e. 4 L). The mass of the inhaled powders deposited in each stage was determined by HPLC as previously described. ED, which represented the mass percentage of the inhaled powders that were successfully

emitted off the inhaler, was determined from the mass ratio of powders recovered in the IP, PS, and stages 1–7 of the NGI to the initial mass placed in the inhaler (i.e. 10 mg). FPF, which was defined as the mass percentage of the ED that possessed $d_A \leq 5.0 \mu\text{m}$, was determined from interpolation of the cumulative mass d_A distribution curve between stage 1 ($d_A = 6.7 \mu\text{m}$) and stage 2 ($d_A = 3.7 \mu\text{m}$). Lastly, MMAD, which was defined as the median d_A of the inhaled powders recovered in stages 1–7, was taken from the 50% point of the cumulative d_A distribution curve.

4.2.2.5 Dissolution characteristics in ABSM

The artificial bronchiectasis sputum medium (ABSM) was used as the dissolution medium to provide a more clinically relevant environment that mimicked the mucus proliferated bronchiectasis lung via the inclusion of key components present in the bronchiectasis sputum (e.g. amino acid, free DNA, mucin, salts) in its formulation [125]. The ABSM was prepared by supplementing the artificial sputum medium's recipe presented in Yang et al. (2011) [97] with NaCl and KCl at concentrations of 115 mM and 130 mM, respectively, which were representative of the salt concentrations present in bronchiectasis sputum [98]. Briefly, 5 g mucin, 4 g salmon sperm DNA, 5.9 mg DPTA, 5 g NaCl, 2.2 g KCl, and 1.81 g Tris base were dissolved in 0.9 L DI water. The pH of the solution was adjusted to 7.0 by adding 1 M HCl after which the solution was autoclaved at 110 °C for 15 min. Afterwards, 5 mL of egg yolk emulsion and casamino acid solution (0.5% w/v) were added to the solution.

The apparent solubility of the CIP nanoplex in the ABSM was determined by adding the CIP nanoplex in excess (i.e. 15 mg) to 8.5 mL ABSM maintained at 37

°C in a shaking incubator. At specific time points over 96 h, 400 µL aliquot was withdrawn, centrifuged (14,000g for 5 min), and syringe filtered (0.2-µm pore size) to remove any undissolved CIP. The CIP concentration in the filtered solution was then determined by HPLC as previously described. The apparent solubility was reported as the ratio of the supersaturated CIP concentration generated by the CIP nanoplex to the thermodynamic saturation solubility of the native CIP in ABSM ($C_{\text{Sat}} = 263 \pm 3 \text{ µg/mL}$). The apparent solubility was determined in triplicates for both the aqueous suspension and DPI formulation of the CIP nanoplex.

The dissolution characteristics of the DPIs of CIP nanoplex and CIP liposome in the ABSM were determined in triplicates under sink condition ($1/4$ of C_{Sat}) by adding the CIP powders to 50 mL ABSM maintained at 37 °C in a shaking incubator. At specific time points over a 4-h period, 1 mL aliquot was withdrawn and the same volume of fresh ABSM was added back to the dissolution vessel. The aliquot was centrifuged and filtered after which the CIP concentration in the supernatant was determined by HPLC. The dissolution tests were also carried out for the aqueous suspension form of the CIP liposome and CIP nanoplex for comparison.

4.2.2.6 *Ex vivo* mucus permeability

The mucus permeability of the DPIs of CIP nanoplex and CIP liposome was characterized using a Transwell® membrane diffusion setup (Corning, USA) following Groo et al. (2014) [126] and Tran et al. (2018) [71]. Briefly, 100 µL fresh undiluted sputum from healthy donors was placed in the apical compartment of a Transwell® polycarbonate membrane inserts (12-mm diameter with 3-µm pore

size). A control run in which 100 μ L deionized water was placed in the apical compartment, in place of the sputum, was carried out. The membrane inserts were attached to a 12-well tissue-culture microplate with its basolateral compartment filled with 600 μ L DI water. After 30 min incubation of the sputum-containing microplate at 37 °C, approximately 2 mg of the DPI of CIP nanoplex was placed in the superior aspect of the sputum layer. This amount of powders corresponded to roughly 600 μ g of CIP.

At specific time points over 24 h, 50 μ L aliquot was withdrawn from the basolateral compartment and replaced with the same volume of deionized water. The CIP concentration in the aliquot was determined by HPLC as previously described. For the DPI of CIP liposome, a larger amount of powders (i.e. approximately 35 mg), which also corresponded to roughly 600 μ g of CIP, was used due to the lower CIP payload in the liposome. The mucus permeability was reported from three replicates as the ratio of the amount of CIP recovered in the basolateral compartment to the initial amount of CIP added. The mucus permeability test was also carried out for the aqueous suspension of the CIP nanoplex and CIP liposome for comparison.

4.2.2.7 Antimicrobial activity in the presence of mucus barrier

Against planktonic *P. aeruginosa*: 50 μ L fresh undiluted sputum from healthy donors was placed in the apical compartment of Transwell® polycarbonate membrane inserts attached to a 24-well tissue-culture microplate. The basolateral compartment was filled with 600 μ L inoculum of *P. aeruginosa* having density of 1×10^6 colony forming units (CFU)/mL as illustrated in Fig. 3-1. The microplate

containing the sputum and bacterial inoculum was then incubated for 30 min at 37 °C. Afterwards, 0.5 mg of the DPI of CIP nanoplex or 8.8 mg of the DPI of CIP liposome was placed in the superior aspect of the sputum layer. This amount of powders yielded CIP concentration of approximately 250 µg/mL that was significantly higher than the minimum inhibitory concentrations (MIC) of the PAO1 and clinical strains (i.e. 0.03–0.125 µg/mL).

After 1 h incubation, 50 µL aliquot of the bacterial cell suspension was withdrawn from the basolateral compartment and diluted in 450 µL MHB. The aliquot was subsequently filtered using 0.2-µm membrane filter to prevent antibiotic carryover. The bacterial cells deposited on the filter were then plated onto agar plates and the viable cells were enumerated after 24 h incubation at 37 °C. The antimicrobial activity was reported from three replicates as the residual bacterial cell count in log₁₀ CFU/mL. A non-treated control in which the CIP powders were not placed in the superior aspect of the sputum layer was carried out.

Against *P. aeruginosa* biofilm: *P. aeruginosa* biofilm was grown on sterile 3-mm glass beads following the protocols provided by Barber et al. (2014) [99]. The glass beads with the biofilm on the surface were then suspended in 600 µL MHB after which they were placed in the basolateral compartment of the abovementioned Transwell® setup. Undiluted sputum from either healthy or NCFB donors, and the CIP powders were then placed in the apical compartment as previously described. After 2 h incubation at 37 °C, the glass beads were lifted up from the basolateral compartment and washed with PBS to remove the residual antibiotic and any planktonic cells. The biofilm cells were detached from the glass beads by three cycles of ultrasonication and vortexing. Afterwards, the detached biofilm cells were

serially diluted in PBS and plated onto agar plates. The viable cells were enumerated after 24 h incubation at 37 °C from which the antibiofilm activity was determined in triplicates.

4.2.2.8 Cytotoxicity

The A549 cells were cultivated in a 24-well tissue culture microplate containing DMEM supplemented with 10% (w/v) fetal bovine serum and 1% (w/v) penicillin–streptomycin solution. The 16HBE14o-cells were cultivated in MEM supplemented with 1% (w/v) L-glutamin (200 mM), 10% (w/v) fetal bovine serum, and 1% (w/v) penicillin–streptomycin solution. Both cells were incubated for 24 h at 37 °C in 5% CO₂ incubator to produce cell density of 10⁵ cells/well. The DPIs of CIP nanoplex and CIP liposome were re-dispersed in DMEM after which they were added to the cells at CIP concentrations in the range of [CIP] = 12.5–250 µg/mL and 3.125–100 µg/mL for the A549 and 16HBE14o-cells, respectively. These [CIP] values were within the typical range of CIP concentration recovered in the lungs (i.e. 33–237 mg/L) upon inhaled CIP delivery [42, 100].

After the CIP treatment, both cells were incubated at 37 °C for 24 h after which the culture media was replaced with serum-free DMEM containing 100 µL of MTT dye solution (0.5% w/v in PBS). The cells were then incubated for further 4 h. Afterwards, 2 mL DMSO were added to dissolve the formazan crystals produced by the viable cells and the formazan concentration was determined by UV–Vis spectrophotometry at 550 nm. The cytotoxicity was reported from three replicates as the percentage of cell survival calculated from the ratio of OD₅₅₀ of the treated cells to OD₅₅₀ of the non-treated cells acting the control.

The cytotoxicity tests were also performed for the DPIs of blank nanoplex and blank liposome (i.e. without CIP) to examine the cytotoxicity of the various chemicals present in the DPI formulations (i.e. DXT, L- α -phosphatidylcholine, cholesterol, D-Mannitol). The cytotoxicity of the blank DPIs was investigated at the same concentrations as those used for the cytotoxicity tests of the DPIs of CIP nanoplex and CIP liposome, i.e. at equivalent CIP concentration ($[CIP]_{eq}$) of 12.5–250 $\mu\text{g/mL}$ and 3.125–100 $\mu\text{g/mL}$ for the A549 and 16HBE14o-cells, respectively. In addition, the cytotoxicities of the residual solvents (i.e. tert-butanol, ethanol) remaining after the CIP liposome preparation and the spray-freeze-dried D-Mannitol were also examined.

The residual solvents of ethanol and t-butanol were determined by gravimetric analysis following the protocols detailed in Vitor et al. [127]. Briefly, a measured amount of hydrogenated soy L- α -phosphatidylcholine and cholesterol was added at 7:3 (w/w) ratio to the mixture of tert-butanol/ethanol/water (49: 49: 2, v/v/v) with a known mass. Next, the solvents of the lipid solution were evaporated at 70 °C until a thin lipid film was formed on the glass tube wall. The mass of the glass tube containing the thin lipid film, which was later transformed to the liposome, was weighted to determine the residual amounts of solvents present in the thin lipid film. This amount of residual solvent present in the thin lipid film was assumed to be carried over to the liposome. The amount of residual solvent reported here was based on five independent replicates.

4.3 Results and Discussion

4.3.1 Physical characteristics of CIP nanoplex and CIP liposome

In their aqueous suspension form, the CIP nanoplex was larger with size of 306 ± 38 nm (PDI = 0.460 ± 0.006) compared to the CIP liposome at 196 ± 9 nm (PDI = 0.219 ± 0.004). Both of them thus possessed a size suitable for passive diffusion through the aqueous mucus pores, whose size was estimated to be in the range of 60–300 nm [20]. The CIP nanoplex exhibited a higher zeta potential than the CIP liposome at -23 ± 2 mV versus -10 ± 0.5 mV, respectively. The higher negative surface charge of the CIP nanoplex boded well for its ability to minimize interaction with the polyanionic mucus contents. For comparison, the CIP liposome in the ORBIT formulation exhibited a smaller size at ≈ 90 nm with CIP payload of approximately 33% (w/w) while its zeta potential was not reported [42].

In terms of the preparation efficiency, both CIP nanoplex and CIP liposome were prepared at high mass efficiency as reflected in the high CE and EE values at 78 ± 1 and $80 \pm 7\%$ (w/w), respectively, signifying that a large majority of CIP in the feed was successfully incorporated into the nanoplex and the liposome. As DPI was the intended delivery mode, the CIP payloads of the CIP nanoplex and CIP liposome were characterized in their dry-powder form after freeze drying. While the preparation efficiencies of the CIP nanoplex and CIP liposome were found to be relatively similar, the same trend was not observed for the CIP payload.

The CIP payload of the CIP nanoplex powder was more than twenty-fold higher at $84 \pm 6\%$ (w/w) compared to $3.4 \pm 0.2\%$ (w/w) for the CIP liposome powder. For comparison, at $M_{\text{CIP/liposome}} = 0.3$, the theoretical CIP payload of the CIP liposome in its aqueous suspension form was calculated from the EE to be equal to

approximately $24 \pm 3\%$ (w/w). This result hence indicated a significant loss of the CIP payload from the liposome upon drying as had been reported previously [128]. On this note, the CIP payload of the CIP liposome before freeze drying ($\approx 24\%$) was comparable to the CIP payload of the CIP liposome in the ORBIT formulation ($\approx 33\%$), where the lower CIP payload observed here was due to the lower EE achieved in the present work ($\approx 80\%$ versus 99%) [42].

Unlike its effect on the CIP payload, the freeze drying process had minimal impact on the size and zeta potential of the CIP liposome. Specifically, the size and zeta potential of the freeze-dried CIP liposome after re-hydration in deionized water remained at approximately ≈ 190 nm and -10 mV, hence they were similar to the values observed in the aqueous CIP liposome suspension before freeze drying (Table 4-1). The same trends in the size and zeta potential were observed after re-hydration of the spray-freeze-dried CIP liposome powders. The CIP payload of the CIP liposome after SFD remained at $\approx 3.5\%$, hence it did not decrease further from its value after freeze drying. These results demonstrated that (1) the CIP liposome powders readily re-dispersed back to the nanoscale liposome upon rehydration of the powders in aqueous environment, and (2) other than the reduction in the CIP payload, the CIP liposome's characteristics were not adversely affected by freeze drying and SFD.

Table 4-1. Physical characteristics of the CIP liposome after freeze drying and spray freeze drying (SFD)

CIP liposome	Size (nm)	Zeta potential (mV)	CIP payload (% w/w)
Before freeze drying	196 ± 9	-10.2 ± 0.5	24 ± 3
After freeze drying & re-hydration	189 ± 5	-9.7 ± 0.6	3.4 ± 0.2
After SFD & re-hydration	203 ± 5	-10.4 ± 0.4	3.6 ± 0.4

The CIP nanoplex, on the other hand, exhibited larger size (≈ 450 nm) and slightly lower zeta potential (≈ -20 mV) after freeze drying/SFD and re-hydration in deionized water (Table 4-2). The larger nanoplex size after re-hydration was likely caused by the irreversible aggregation of the nanoplex upon freeze drying as we reported earlier in Kiew et al. (2015) [129]. These results suggest that the DPI of CIP nanoplex was inferior to the DPI of CIP liposome in terms of its ability to undergo reconstitution back to the original nanoparticles upon its exposure to aqueous environment. Despite the larger size after re-hydration, the CIP payload of the CIP nanoplex was minimally affected by the drying process, unlike the CIP liposome, hence denoting the superior physical integrity of the CIP nanoplex in comparison to the CIP liposome.

Table 4-2. Physical characteristics of the CIP nanoplex after freeze drying and spray freeze drying (SFD)

CIP nanoplex	Size (nm)	Zeta potential (mV)	CIP payload (% w/w)
Before freeze drying	306 ± 38	-23 ± 2	80 ± 5
After freeze drying & re-hydration	420 ± 14	-20 ± 4	84 ± 6
After SFD & re-hydration	455 ± 30	-20 ± 2	76 ± 3

4.3.2 Aerosolization efficiency of DPI formulations

The DPI formulations of the CIP nanoplex and CIP liposome were prepared in the form of highly spherical porous nanocomposite particles as shown in their SEM images in Fig. 4-1A and B, respectively, in which the CIP nanoplex or the CIP liposome were physically dispersed in the porous D-Mannitol matrix. The DPIs of CIP nanoplex and CIP liposome possessed geometric size (d_G) in the range of 69 ± 19 and $58 \pm 18 \mu\text{m}$, respectively. The large size and high porosity were the typical morphology of powders prepared by SFD. This morphology was ideal for aerosolization as the large inertial force afforded by the large size minimized particle adhesion due to the surface forces, while the high porosity ensured that the particles remained light for aerosolization, despite their large size [23]. As a result, in the present approach, the addition of coarse carrier particles to facilitate the aerosolization of the CIP nanoplex or CIP liposome powders was not necessary as the powders could be effectively aerosolized off the inhaler on their own as demonstrated in the NGI results below.

Owing to their large porous morphology, both the DPIs of CIP nanoplex and CIP liposome were found to exhibit relatively high ED values at 79 ± 9 and $75 \pm 11\%$ (w/w), respectively (Fig. 4-1C). The ED values were comparable to the ED values of commercial DPI products [130]. The DPI of CIP nanoplex, however, exhibited far superior FPF for the emitted dose at $31 \pm 3\%$ (w/w) compared to only $5 \pm 1\%$ exhibited by the DPI of CIP liposome (Fig. 4-1C), indicating the would-be lower bronchial deposition of the CIP liposome. The low FPF despite the high ED exhibited by the DPI of CIP liposome signified the prevalence of in-flight agglomeration of the emitted particles, resulting in a significant portion of them to

be deposited in the PS sections of the NGI prior to Stage 1, which would represent the mouth and throat regions *in vivo*.

The high degree of in-flight agglomeration in the DPI of CIP liposome was likely caused by the significant presence of lipids in its formulation ($\approx 46\%$ w/w with the rests were made up of D-Mannitol and CIP) due to its low CIP payload. The high lipid content resulted in a high degree of particle cohesiveness in the DPI formulation due to the highly hygroscopic nature of phospholipids powders prepared by lyophilization [131]. The FPF of the DPI of CIP liposome could likely be improved by having an increased presence of D-Mannitol ($\gg 50\%$ w/w) to mask the hygroscopicity of the lipids as pursued in Sweeney et al. (2005) [132], who successfully delivered DPI of CIP liposome also prepared by SFD at high FPF by having adjuvant composition (i.e. lactose) in the excess of 75%. This approach, however, would lead to an even lower CIP payload in the formulation, hence it was not pursued here.

Figure 4-1: (A, B) SEM images of the DPIs of CIP nanoplex and CIP liposome prepared by SFD depicting large spherical porous morphology; (C) aerosolization efficiency of the DPIs of CIP nanoplex and CIP liposome as characterized by the ED, FPF, and MMAD.

Despite the low FPF, for the CIP liposome particles that successfully reached stages 1–7 of the NGI, the MMAD was equal to $3.3 \pm 0.2 \mu\text{m}$, which was comparable to that of the DPI of CIP nanoplex at $2.5 \pm 0.2 \mu\text{m}$, denoting their suitability to reach the upper respiratory tract (Fig. 4-1C).

It was noted that even though the ED of the DPI of CIP liposome was high and the MMAD suited for inhalation, the FPF was low. These results were not unexpected because in our studies the ED was determined from the mass ratio of powders recovered in the IP, PS, and stages 1–7 of the NGI to the initial mass placed in the inhaler (i.e. 10 mg). It was found that the large amount of the DPI of CIP liposome mainly deposited in the IP and PS sections of the NGI but not in the stages 1–7, so it still resulted in the high ED. This could be resulted in the in-flight agglomeration of the emitted particles because there was the significant presence of lipid contents (i.e. ~50% w/w) in the DPI of CIP liposome, leading to a high cohesiveness of particles during inhalation. On the other hand, MMAD was defined as the median d_A of the inhaled powders recovered in stages 1–7. And, FPF was defined as the mass percentage of the ED that possessed $d_A \leq 5.0 \mu\text{m}$ in which was only determined between stage 1 ($d_A = 6.7 \mu\text{m}$) and stage 2 ($d_A = 3.7 \mu\text{m}$). Therefore, the recovered FPF ($d_A \leq 5.0 \mu\text{m}$) was low for the DPI of CIP liposome.

In short, the low FPF of the DPI of CIP liposome coupled with its low CIP payload meant that a very large dosage would be needed to achieve the desired antibiotic exposure in the infected airways. The large dosage not only increased the burden to the patients, but also potentially increased the risks of adverse side effects. In the subsequent sections, we examined if the anticipated low antibiotic exposure delivered by the DPI of CIP liposome due to its inferior aerosolization

efficiency and CIP payload could be made up by its superior characteristics in terms of the mucus permeability and antimicrobial activity upon reaching the airways.

4.3.3 Dissolution characteristics in ABSM

The dissolutions of the CIP nanoplex and CIP liposome were characterized in both their aqueous suspension and DPI forms to evaluate the effect of the dry powder transformation on their dissolution (if any). In their aqueous suspension form, which was equivalent to the form used in nebulization delivery, the CIP nanoplex exhibited a faster release than the CIP liposome with $77 \pm 10\%$ (w/w) versus $30 \pm 4\%$ (w/w) dissolutions after 1 h, respectively (Fig. 4-2A). The CIP nanoplex exhibited nearly 100% dissolution after 2 h, whereas the CIP liposome took more than 8 h to reach 100% dissolution (data not shown). The faster CIP dissolution from the CIP nanoplex was not unexpected as CIP in the nanoplex was not encapsulated by any carrier matrix, unlike it was in the liposome. Instead, the CIP in the nanoplex was held by electrostatic binding with DXT, where the CIP-DXT complex readily dissociated in the presence of a high ionic concentration of the ABSM due to the ion exchange, resulting in the fast dissolution.

The same trend was observed in the DPI formulations, where the DPI of CIP nanoplex was found to exhibit a faster dissolution than its CIP liposome counterpart with $58 \pm 7\%$ versus $33 \pm 1\%$ dissolutions after 1 h, respectively (Fig. 4-2B). It was notable that the dissolution rate of the CIP nanoplex was slowed down after the dry-powder transformation, where nearly 100% dissolution of the DPI of CIP nanoplex was observed only after 4 h compared to 2 h observed previously for the CIP nanoplex suspension. The aforementioned increase in the CIP nanoplex size after

freeze drying and re-hydration (Section 4.3.1) led to reduced surface areas for dissolution, resulting in the slower dissolution of the CIP nanoplex after its dry-powder transformation. In contrast, the dissolution of the CIP liposome was minimally affected by the dry-powder transformation as the CIP liposome powder was shown earlier to be effectively re-constituted to its original size following re-hydration.

Figure 4-2: Dissolution time-profiles of the (A) aqueous suspensions and (B) DPI formulations of the CIP nanoplex and CIP liposome in the ABSM.

In addition to its fast dissolution, as reported earlier by Cheow and Hadinoto (2012) [22], the CIP nanoplex was also capable of producing high apparent solubility of CIP in aqueous environment owed to the supersaturated CIP concentration generated by its metastable amorphous state. The high apparent solubility of the CIP nanoplex was also evident in the ABSM, where the solubility of the CIP nanoplex suspension was multifold higher than the solubility of the native CIP at $(8.8 \pm 0.3) \times C_{\text{Sat}}$ ($\approx 2.3 \text{ mg/mL}$) (Fig. 4-3). The solubility of the CIP nanoplex was slightly lower at $(6.7 \pm 0.2) \times C_{\text{Sat}}$ ($\approx 1.8 \text{ mg/mL}$) in its DPI form, which was likely caused by the nanoplex fusion upon freeze drying, resulting in reduced surface areas for dissolution.

Figure 4-3: Apparent solubility of the CIP nanoplex (aqueous suspension) and DPI of CIP nanoplex in the ABSM.

The high solubility of the CIP nanoplex was maintained for 48 h in the ABSM before it started to decrease to eventually settle at the thermodynamically stable value of $\approx 1 \times C_{\text{Sat}}$ after 96 h (data not shown). Moreover, the fast dissolution of the CIP nanoplex signified that the high apparent solubility was achieved in a short period of time. The high solubility of the CIP nanoplex was beneficial for the antimicrobial activity because it enabled us to achieve a significantly higher antibiotic exposure than that produced by the native CIP or the CIP liposome as the maximum antibiotic exposures of the native CIP or the CIP liposome were limited to the thermodynamic value C_{Sat} .

To summarize, the CIP liposome offered a sustained CIP release, which in turn produced antibiotic concentration above the MIC over a prolonged period, whereas the CIP nanoplex offered a fast CIP release with multifold higher maximum achievable concentration. The effects of the contrasting dissolution characteristics exhibited by the DPIs of CIP nanoplex and CIP liposome on their antimicrobial activities were examined in Section 4.3.5.

4.3.4 *Ex vivo* mucus permeability

Besides the dissolution rates, another factor that influenced the degree of antibiotic exposure was the ability of the CIP nanoplex and CIP liposome to overcome the mucus barrier. In their aqueous suspension form, which was representative of the nebulization delivery mode, the permeabilities of the CIP nanoplex and the CIP liposome in the sputum from healthy donors ($n = 3$) were found to be similar in which the numerical variations were determined to be not statistically significant (Fig. 4-4A). Respectively, $48 \pm 12\%$ and $41 \pm 11\%$ (w/w) of

the CIP nanoplex and the CIP liposome that were initially placed at the sputum surface successfully permeated through the mucus barrier after 1 h. For comparison, the control run without sputum exhibited nearly 100% permeation after 1 h (data not shown). The permeated fraction increased to $84 \pm 3\%$ and $79 \pm 5\%$ after 6 h for the CIP nanoplex and CIP liposome, respectively.

In this regard, the similar mucus permeability between the aqueous suspension forms of the CIP nanoplex and CIP liposome was not unexpected as both of them had been purposely designed with mucus-inert surface properties. It was worth pointing out, however, that while the CIP nanoplex and CIP liposome permeated through the mucus, they simultaneously underwent dissolution. As a result of its faster dissolution rate and supersaturation generation, a larger fraction of the CIP nanoplex would have dissolved compared to the CIP liposome during the mucus permeation. The reported mucus permeability therefore included the contributions from the passive diffusion of the CIP nanoplex or CIP liposome, as well as the CIP molecules already dissolved in the mucus.

On the other hand, in their dry-powder form, the DPI of CIP liposome was found to exhibit slightly superior mucus permeability than the DPI of CIP nanoplex, particularly after 1 h (Fig. 4-4B). Specifically, $49 \pm 5\%$ of the DPI of CIP liposome successfully penetrated the mucus barrier after 6 h versus $32 \pm 6\%$ for the DPI of CIP nanoplex after the same period. Notably, the DPI formulations were found to exhibit slower mucus permeation rates than their aqueous suspension counterpart. This was not unexpected as the CIP nanoplex and the CIP liposome in the DPI formulations could only permeate the mucus upon dissolution of the D-Mannitol matrix, resulting in a time lag for the mucus permeation. After the D-Mannitol

matrix dissolved, the CIP nanoplex and the CIP liposome in the DPI formulations were liberated and transformed back to their individual nanoparticle states.

Figure 4-4: Mucus permeability of the (A) aqueous suspensions and (B) DPI formulations of the CIP nanoplex and CIP liposome in normal sputum ($n = 3$) (NS = not statistically significant, $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$).

The superior mucus permeability of the DPI of CIP liposome was attributed to the CIP liposome being liberated more effectively to its original nanoparticle state than the CIP nanoplex. The effective liberation of the CIP liposome from the D-Mannitol matrix was evident from the effective aqueous reconstitution of the DPI of CIP liposome to the original nanoscale liposome after re-hydration as discussed earlier in Section 4.3.1. The lower aqueous re-constitution of the DPI of CIP nanoplex resulted in a fraction of the nanoplex population exhibiting sizes that were too large for permeation through the aqueous mucus pores, hence the lower mucus permeability.

4.3.5 Antimicrobial activity in the presence of mucus barrier

In the presence of normal sputum ($n = 3$), both DPIs of CIP nanoplex and CIP liposome exhibited bactericidal activities against planktonic *P. aeruginosa* (Fig. 4-5A). Herein the bactericidal activity was defined as $> 3 \log_{10}$ CFU/mL reduction in the viable cells relative to the non-treated control. Similar bactericidal activities were observed against the planktonic PAO1 and clinical strains at $\approx 5 \log_{10}$ CFU/mL reduction in the viable cells. Against biofilm, the DPIs of CIP nanoplex and CIP liposome also exhibited bactericidal activities against *P. aeruginosa* biofilm in the presence of normal sputum ($n = 3$), albeit at slightly lower reductions in the viable cells ($\approx 4.5 \log_{10}$ CFU/mL) due to the higher antibiotic tolerance of biofilm cells compared to the planktonic ones (Fig. 4-5B). The bactericidal activities against the biofilm were also found to be independent of the bacterial strains.

Figure 4-5: Antimicrobial activities of the DPIs of CIP nanoplex and CIP liposome against the laboratory and clinical strains of *P. aeruginosa* in the presence of mucus barrier: (A) planktonic *P. aeruginosa* in normal sputum (n = 3); (B) biofilm *P. aeruginosa* in normal sputum (n = 3), and (C) biofilm *P. aeruginosa* in NCBF sputum (n = 2) (NS = not statistically significant).

Similar antibiofilm activities were observed when the study was repeated in the presence of NCFB sputum ($n = 2$) (Fig. 4-5C). On this note, the numerical variations between the antimicrobial activities of the DPI of CIP nanoplex and DPI of CIP liposome were determined to be statistically insignificant for both the planktonic and biofilm cells. Importantly, the similar antimicrobial activities exhibited by the DPIs of CIP nanoplex and CIP liposomes were observed despite the difference in their dissolution characteristics. This suggested that both types of dissolution (i.e. fast release at highly supersaturated concentration for the CIP nanoplex and sustained release above the MIC for the CIP liposome) were both appropriate for the bacterial killing in the presence of mucus barrier.

4.3.6 Cytotoxicity towards A549 and 16HBE14o-cells

4.3.6.1 A549 cells

In the lower range of CIP concentration investigated (i.e. $12.5 \leq [\text{CIP}] \leq 50$ $\mu\text{g/mL}$), both DPIs of CIP nanoplex and CIP liposome exhibited minimal cytotoxicity *in vitro* towards the A549 cells with cell survival higher than 85% (Fig. 4-6A). The DPI of CIP nanoplex remained to exhibit fairly low cytotoxicity with $\geq 75\%$ cell survivals as the CIP concentrations were increased to $100 \leq [\text{CIP}] \leq 250$ $\mu\text{g/mL}$. In contrast, the cell survivals upon treatment by the DPI of CIP liposome dropped to as low as 50% at the highest CIP concentration investigated (i.e. 250 $\mu\text{g/mL}$). In this regard, our previous study had shown that the native CIP exhibited minimal cytotoxicity ($\approx 85\%$ cell survival) towards the A549 cells at $[\text{CIP}] = 250$ $\mu\text{g/mL}$ [71]. Therefore, the cytotoxicity of the DPI of CIP liposome at higher CIP concentrations could only be caused by either (1) the D-Mannitol present in the DPI

formulation, (2) the residual solvents remaining after the CIP liposome preparation, or (3) the liposome itself. For this reason, the cytotoxicities of the spray-freeze-dried D-Mannitol, the residual solvents, and the DPI of blank liposome (without CIP) were investigated next.

Figure 4-6: Cytotoxicity of the (A) DPIs of CIP nanoplex and CIP liposome, and the (B) DPIs of blank nanoplex and blank liposome (without CIP) towards the A549 cells (NS =not statistically significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

The cytotoxicity of the spray-freeze-dried D-Mannitol was found to be negligible with nearly 100% cell survivals even at the highest concentration investigated (Fig. 4-7). The amount of the residual solvents were determined to be approximately equal to $1.83 \pm 1.30\%$ (w/w) of the total dry mass of the DPI of CIP liposome. For DPI of CIP liposome with dosage of $[CIP] = 12.5\text{--}250 \mu\text{g/mL}$, the amounts of the residual solvents were calculated to be in the range of $9\text{--}187 \mu\text{g/mL}$. The cytotoxicity tests of ethanol and tert-butanol performed in this concentration range revealed that the A549 cell viability was not reduced in the presence of the residual solvents with 100% cell survivals (Fig. 4-8). These results hence ruled out the D-Mannitol and the residual solvents as the causes for the cytotoxicity of the DPI of CIP liposome.

The cytotoxicity test of the DPI of blank liposome showed that the cell survivals gradually decreased from $91 \pm 3\%$ at $[CIP]_{eq} = 12.5 \mu\text{g/mL}$ to $70 \pm 2\%$ at $[CIP]_{eq} = 250 \mu\text{g/mL}$ indicating the adverse effects of the increased presence of liposomes on the cell viability (Fig. 4-6B). In contrast, the cell survivals upon treatment with the DPI of blank nanoplex remained at almost 100% at the highest $[CIP]_{eq}$ investigated (Fig. 4-6B). These results suggested that the liposome as the major reason for the higher cytotoxicity of the DPI of CIP liposome at higher CIP concentrations.

In this regard, studies by Knudsen et al. (2015) [133] and Roursgaard et al., 2016 [134] had indeed shown that liposome exhibited cytotoxicity towards the A549 cells at high concentrations ($> 100 \mu\text{g/mL}$) caused by the oxidative damage and inflammation of the cells induced by the high exposure to liposome. In the present work, as a consequence of its low CIP payload, a large amount of CIP

liposome must be delivered to achieve the targeted CIP dosage. At targeted CIP concentration of 100 µg/mL, the amount of liposome present in the DPI of CIP liposome formulation was very high at approximately 3 mg/mL, resulting in its cytotoxicity.

4.3.6.1 16HBE14o-cells

In the lower range of CIP concentration investigated (i.e. $3.125 \leq [\text{CIP}] \leq 25$ µg/mL), the DPI of CIP nanoplex exhibited lower cytotoxicity towards the 16HBE14o-cells than the DPI of CIP liposome. Specifically, the cell survivals upon treatment by the DPI of CIP nanoplex were equal to $94 \pm 5\%$ and $67 \pm 2\%$ at $[\text{CIP}] = 3.125$ and 25 µg/mL, respectively, compared to cell survivals of $76 \pm 3\%$ and $54 \pm 1\%$ upon treatment by the DPI of CIP liposome at the same CIP concentrations (Fig. 4-9A). A similar trend was observed in the cytotoxicity tests of the blank DPIs. The cell survivals upon treatment by the DPI of blank liposome at $3.125 \leq [\text{CIP}]_{\text{eq}} \leq 25$ µg/mL were about 50–60%, which were significantly lower than the $> 90\%$ cell survivals upon treatment by the DPI of blank nanoplex (Fig. 4-9B).

The cytotoxicity tests of the spray-freeze-dried D-Mannitol and the residual solvents (at 2–75 µg/mL) revealed their non-cytotoxicity towards the 16HBE14o-cells (Figs. 4-7 & 4-8, respectively). Therefore, similar to the finding for the A549 cells, the high cytotoxicity of the DPI of CIP liposome towards the 16HBE14o-cells was concluded to be caused by the excessive presence of liposome in its formulation.

Figure 4-7: Cytotoxicity of the SFD D-Mannitol used in the DPI formulations of CIP nanoplex and CIP liposomes towards (A) A549 cells and (B) 16HBE14o-cells. The cytotoxicity of the D-Mannitol was evaluated at the concentration that would be present in the DPI formulations at CIP dosage of 12.5-250 µg/mL and 3.125-100 µg/mL for the A549 cells and 16HBE14o- cells, respectively. The D-Mannitol concentrations investigated were thus expressed in terms of the equivalent CIP concentrations.

Figure 4-8: Cytotoxicity of the residual solvents remaining from the CIP liposome preparation towards (A) A549 cells and (B) 16HBE14o- cells. The cytotoxicity of the residual solvents was evaluated at the concentrations that would be present in the DPI of CIP liposome at CIP dosage of 12.5-250 μg/mL and 3.125-100 μg/mL for the A549 cells and 16HBE14o- cells, respectively. The residual solvents' concentrations investigated were thus expressed in terms of the equivalent CIP concentrations.

In comparison to the A549 cells, both DPIs of CIP nanoplex and CIP liposome exhibited higher cytotoxicity towards the 16HBE14o-cells due to the inherently low tolerance of the 16HBE14o-cells towards CIP as reported earlier by Tran et al. (2018) [71]. Consequently, at the two highest CIP concentration investigated (i.e. 50 and 100 $\mu\text{g/mL}$), the cell survivals dropped to below 50% for both the DPIs of CIP nanoplex and CIP liposome (Fig. 4-9A), where the similar cytotoxicity between the two DPI formulations at these high CIP concentration could be attributed to the fact that the cells were already dead due to the CIP exposure, thereby the influence of the adjuvants was minimal.

Figure 4-9: Cytotoxicity of the (A) DPIs of CIP nanoplex and CIP liposome, and the (B) DPIs of blank nanoplex and blank liposome (without CIP) towards the 16HBE14o-cells (NS= not statistically significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

4.4 Conclusions

The key findings from our comprehensive evaluations of the DPIs of CIP nanoplex and CIP liposome both prepared by SFD were as follows: First, both the CIP nanoplex and CIP liposome possessed size and mucus-inert surface that were ideal for overcoming the mucus barrier upon reaching the airways. This was reflected in their effective permeation of the *ex vivo* sputum with roughly 50% of the particles successfully diffused through the sputum after just 1 h. The DPI of CIP liposome exhibited slightly superior mucus permeability to the DPI of CIP nanoplex attributed to the more effective liberation of the CIP liposome from the D-Mannitol matrix after lyophilization.

Second, the CIP nanoplex exhibited a fast dissolution generating highly supersaturated CIP concentration in the ABSM, which was highly distinct from the sustained release below the saturation solubility exhibited by the CIP liposome. The distinct dissolution characteristics and mucus permeability between the DPIs of CIP nanoplex and CIP liposome, however, did not lead to variations in their antimicrobial activities in the presence of mucus barrier (both normal and NCFB sputum). Both the CIP nanoplex and CIP liposome exhibited bactericidal activities against planktonic and biofilm *P. aeruginosa*.

Third, while they exhibit similar mucus permeability and antipseudomonal activity, the DPI of CIP liposome had a significantly lower CIP payload than the DPI of CIP nanoplex ($\approx 84\%$ versus 3.5%) due to the inevitable loss of drug across the lipid membranes during lyophilization. Consequently, a much larger dosage was needed for the DPI of CIP liposome to achieve the same antibiotic exposure as the DPI of CIP nanoplex. The large dosage, which was made up mostly of the

liposome, led to higher cytotoxicity of the DPI of CIP liposome towards the human lung epithelium cells.

Fourth, while both DPIs of CIP nanoplex and CIP liposome could be effectively emitted off the inhaler ($ED \approx 80\%$) attributed to their large porous morphology, the DPI of CIP nanoplex exhibited far superior *in vitro* bronchial deposition efficiency (FPF $\approx 30\%$ versus $\approx 5\%$ for the DPI of CIP liposome), where the low FPF of the DPI of CIP liposome was believed to be caused by its high lipid content resulting in high degree of particle cohesiveness. In summary, our study showed that the DPI of CIP nanoplex represented a more viable antibiotic nanoparticle formulation strategy for NCFB therapy than the DPI of CIP liposome attributed to its (1) much simpler preparation, (2) superior aerosolization efficiency, (3) higher CIP payload, hence lower dosage requirement, and lastly (4) minimal cytotoxicity towards the human lung epithelial cells.

CHAPTER 5. IMPROVING THE MUCUS PERMEABILITY OF THE CIP NANOPLEX BY INCORPORATION WITH THE MUCOLYTIC PAPAIN PROTEASE

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5.1 Introduction

Bronchiectasis is a chronic lung disease characterized by irreversible dilatation and mucus infestation of the airways due to a vicious cycle of infection and inflammation, leading to the loss of lung function and eventually mortality [108]. Inhaled antibiotics aimed at lowering the airway bacterial load have been the mainstay in the management of cystic fibrosis (CF) and non-CF bronchiectasis for both acute exacerbations and long-term therapies [9, 33]. Recently, mucus-inert antibiotic nanoparticles were demonstrated in several *in vitro* and *in vivo* studies as an effective antibiotic formulation strategy for bronchiectasis by virtue of their superior antibacterial efficacy to the native form of the antibiotics (e.g. tobramycin [136-138], colistin [139], levofloxacin [140], ciprofloxacin [42, 71, 118, 119]). The superior antibacterial efficacy of the antibiotic nanoparticles was attributed to

controlled antibiotic release and improved mucus permeability that led to enhanced antibiotic exposure in the infected sites [21].

Among the antibiotics, nanoparticles of ciprofloxacin (CIP) have been one of the most actively investigated owed to strong clinical evidences of high bactericidal activity of CIP against *Pseudomonas aeruginosa* [104], which represents the predominant pathogen in bronchiectasis lungs [11, 106]. In fact, nanoscale CIP-encapsulated liposomes exhibiting sustained antibiotic release (ORBIT formulations) have reached Phase 3 clinical trials for non-CF bronchiectasis [141]. Nevertheless, the inconsistent results between trials for the ORBIT formulations suggest that the development of an alternative, and ideally superior, CIP nanoparticle formulation remains needed.

For this purpose, we previously developed mucus-penetrating CIP nanoparticle complex (or nanoplex in short) capable of rapidly generating a highly supersaturated CIP concentration owed to its metastable amorphous form, as opposed to the slow antibiotic release in the CIP liposome [71]. Its high mucus permeability and supersaturation generation capability enabled the CIP nanoplex to produce high and localized antibiotic exposures in the vicinity of the bacterial colonies. As illustrated in Fig. 5-1, the CIP nanoplex was prepared by electrostatically driven complexation between ionized CIP molecules and oppositely charged polyelectrolyte (i.e. sodium dextran sulfate, DXT). Soluble CIP-DXT complexes were formed upon mixing of the CIP and DXT solutions by virtue of their electrostatic binding. The soluble CIP-DXT complex subsequently aggregated among themselves due to the inter-drug hydrophobic interactions among

the bound drug molecules. Upon reaching a critical mass, the CIP-DXT complex aggregates precipitate out of the solution to form the CIP-DXT nanoplex [22].

Figure 5-1: Schematic of the CIP-(DXT-PAP) nanoplex formation.

In comparison to the native CIP, our previous study showed that the CIP-DXT nanoplex exhibited superior *ex vivo* mucus permeability in bronchiectasis sputum, which in turn led to its superior antibacterial efficacy in the presence of mucus barrier against clinical strains of *P. aeruginosa* isolated from bronchiectasis patients [71]. Moreover, when compared to CIP liposome, which shared similar formulation as the ORBIT formulations, our subsequent study showed that the CIP-DXT nanoplex exhibited comparable *ex vivo* mucus permeability and antipseudomonal activity [72]. The advantage that the CIP-DXT nanoplex had over the CIP liposome

was in its higher antibiotic payload that translated to its lower dosage requirement, thus decreased its risk of cytotoxicity towards human lung epithelium cells [72].

The high mucus permeability of the CIP-DXT nanoplex reported in these studies, however, was achieved upon its co-delivery with a well-established mucolytic agent (i.e. D-mannitol) [93] in the form of physical dispersion of the CIP-DXT nanoplex in the D-mannitol matrix. This formulation approach, while effective, inevitably reduced the overall antibiotic payload, and consequently increased the dosage requirement to achieve the desired antibacterial effect. The present work aimed to address this issue by incorporating a mucolytic enzyme into the CIP-DXT nanoplex at the nanoplex formation step, instead of in the later stage of the solid dosage formulation of the nanoplex, to equip the CIP nanoplex with a built-in mucolytic capability.

In this regard, mucolytic enzyme-decorated nanoparticles have been previously developed by ionic or covalent immobilization of the enzyme onto either polymer nanoparticles, or nanoscale self-emulsifying drug delivery systems [142-145]. These nanoparticles were intended as oral drug delivery systems to overcome the intestinal mucus barrier [26]. To the best of our knowledge, mucolytic enzyme-decorated nanoparticles for pulmonary drug delivery applications have not been developed before. In the present work, papain - a proteolytic enzyme extracted from papaya fruit - was used as the mucolytic enzyme. The strong mucolytic activity of papain (PAP) was attributed to its ability to cleave the peptide bonds of the mucus glycoproteins [146]. The tendency of PAP at below its isoelectric point to form electrostatic complex with polyanions has been reported by Izumi et al. (1994) [147]. Therefore, as depicted in Fig. 5-1, we aimed to develop PAP-decorated CIP-

DXT nanoplex by ionically binding PAP to DXT prior to the latter's complexation with CIP to produce ternary CIP-(DXT-PAP) nanoplex.

The objectives of the present work were to first determine the optimal preparation condition of the CIP-(DXT-PAP) nanoplex based on the resultant (i) size, zeta potential, (ii) preparation efficiency (i.e. CIP, PAP utilization rates, yield), and (iii) CIP and PAP payloads. The mucolytic activity and amorphous form stability of the optimal CIP-(DXT-PAP) nanoplex formulation were subsequently examined. The present work then evaluated and compared the CIP-(DXT-PAP) and CIP-DXT nanoplexes in terms of their (i) *in vitro* mucus permeability in synthetic mucus, (ii) dissolution characteristics in artificial bronchiectasis sputum medium, (iii) antibacterial efficacy against *P. aeruginosa* biofilm colonies that were embedded in mucus, and lastly (iv) cytotoxicity towards human lung epithelium cells.

5.2 Materials and Methods

5.2.1 Materials

Ciprofloxacin (CIP) ($\geq 98\%$ purity), papain (PAP) (23 kDa) from papaya latex with activity > 16 units/mg, and glacial acetic acid were purchased from Sigma-Aldrich (Singapore). Sodium dextran sulfate (DXT) (5 kDa) was purchased from Wako Pure Chemical (Japan). Pierce^{TM} bicinchoninic acid (BCA) protein assay kit was purchased from ThermoFisher Scientific (Singapore).

Materials for artificial bronchiectasis sputum medium (ABSM) and synthetic mucus preparations: mucin from porcine stomach, salmon sperm DNA, lecithin, bovine serum albumin (BSA), hydrogen chloride (HCl), Tris base, sodium chloride

(NaCl), potassium chloride (KCl), disodium ethylenediaminetetraacetate (Na₂EDTA), sodium phosphate dibasic heptahydrate (Na₂HPO₄·7H₂O), L-cysteine, and hydroxyethyl piperazineethanesulfonic acid (HEPES) were purchased from Sigma Aldrich (Singapore). Acetonitrile, methanol, and potassium dihydrogen phosphate (KH₂PO₄) for the dissolution experiment in ABSM were purchased from Merk Millipore (Singapore).

Materials for bacterial cell culture: clinical strain of *P. aeruginosa* was kindly provided by the clinical microbiology laboratory at National University Hospital (Singapore). Mueller Hinton broth (MHB), tryptic soy agar, and phosphate buffered saline (PBS, pH 7.4) were purchased from BD Diagnostics (Singapore), Gibco (USA), and Sigma Aldrich (Singapore), respectively.

Materials for cytotoxicity test: A549 human carcinoma lung epithelial cells were purchased from ATCC (USA). 16HBE14o- human bronchial epithelial cells were kindly provided by California Pacific Medical Center Research Institute (USA). Penicillin-streptomycin and CellTiter-Blue were purchased from PAA Laboratories (Austria) and Promega (Singapore), respectively. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were purchased from GE Healthcare Life Sciences (USA). Minimum Essential Medium with Eagle's salt (MEM), L-glutamin (200 mM), and 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) solution were purchased from Gibco (USA).

5.2.2 Methods

5.2.2.1 Preparation of CIP-DXT-PAP and CIP-DXT nanoplexes

DXT having pK_a of ≤ 2.0 [22] and PAP having an isoelectric point pI of ≈ 9.5 [143] existed at neutral pH as anionic DXT and cationic PAP molecules with charge densities of 4.8×10^{-6} and 4.7×10^{-7} mol charge/mg, respectively. DXT and PAP were co-dissolved in deionized (DI) water (pH 7) at a fixed mass ratio of DXT to PAP equal to 1.22, which corresponded to charge ratio of PAP to DXT ($R_{PAP/DXT}$) equal to ≈ 0.08 . Separately, CIP having pK_a of 6.1 and 8.6 [148] was dissolved at a fixed concentration of 5 mg/mL in 0.3% (v/v) aqueous acetic acid solution (pH ≈ 3) to produce cationic CIP molecules having charge density of 3.0×10^{-6} mol charge/mg.

Next, equal volumes of the CIP and the (PAP-DXT) solutions were mixed under gentle stirring. Three DXT concentrations (i.e. 3.14, 1.57, and 1.12 mg/mL) were investigated at a fixed mass ratio of DXT to PAP (i.e. 1.22) to examine the impacts of varying the charge ratio of the cations (i.e. CIP + PAP) to the anion (i.e. DXT), i.e. $R_{(CIP+PAP)/DXT}$. The resultant CIP-(DXT-PAP) nanoplex suspension underwent two cycles of centrifugation at 14,000 x g for 5 min and washing with DI water to remove excess CIP, DXT, and PAP that did not form the nanoplex. The washed nanoplex suspension was re-dispersed in DI water for characterizations. Two types of control run, i.e. (1) CIP solution was mixed with an equal volume of PAP solution without DXT, and (2) (PAP-DXT) solution was mixed with an equal volume of 0.3% (v/v) acetic acid solution without CIP, were performed. For comparison, the CIP-DXT nanoplex was prepared following the same procedures at CIP and DXT concentrations of 5 and 3.14 mg/mL, respectively, corresponding to $R_{CIP/DXT} = 1.0$.

5.2.2.2 Physical characterizations

The size, polydispersity index (PDI), and zeta potential of the CIP-(DXT-PAP) nanoplex were measured in triplicates after 100 times dilution by dynamic light scattering (DLS) using Brookhaven 90 Plus Nanoparticle Size Analyzer (Brookhaven Instruments Corporation, USA). The nanoplex size was verified by field emission scanning electron microscope (FESEM) (JSM 6700 F, JEOL, USA) using air-dried nanoplex as the representative sample.

The payloads of CIP and PAP, which were defined as the amounts of CIP (or PAP) per unit mass of the nanoplex, were determined in triplicates by dissolving a known mass of freeze-dried nanoplex in PBS. The amount of CIP dissolved in PBS was determined by UV-Vis spectrophotometry at wavelength of 324 nm (UV Mini 1240, Shimadzu, Japan), whereas the amount of PAP dissolved in PBS was determined by BCA protein assay kit. Briefly, 100 μ L of the PAP sample in PBS was mixed with 2 mL of BCA working reagent, followed by incubation of the resultant solution at 37 °C for 30 min. Afterwards, the solution was cooled down for 10 min at room temperature and the amount of PAP was determined by the UV–Vis spectrophotometry at absorbance wavelength of 562 nm. The existence of CIP and PAP in the CIP-(DXT-PAP) nanoplex was analyzed by Fourier transform infrared spectroscopy (FTIR) from 450 to 4000 cm^{-1} at 4 cm^{-1} spectral resolution using Spectrum One FTIR Spectrometer (Perkin-Elmer, USA).

The amorphous form of the CIP-(DXT-PAP) nanoplex was examined by powder x-ray diffraction (PXRD) performed between 5° and 70° (2 θ) with a step size of 0.02°/s using D8 Advance X-ray Diffractometer (Bruker, Germany). The PXRD analysis was performed thrice, (1) immediately after preparation, (2) after

eleven-month storage at 25 °C and 55% relative humidity (RH), and (3) two-week accelerated storage at 40 °C and 75% RH. The FTIR and PXRD analysis were also performed for the native CIP, free PAP, free DXT, CIP-DXT nanoplex, and physical mixture of DXT and PAP (only for FTIR).

The preparation efficiency of the CIP-(DXT-PAP) nanoplex was characterized from six replicates in terms of (1) the complexation efficiency (CE) of CIP and PAP and (2) the overall production yield. The complexation efficiency of CIP and PAP were defined in Eqs. 1 and 2, respectively, as the ratio of the CIP (or PAP) mass successfully transformed to the CIP-(DXT-PAP) nanoplex to the initial CIP (or PAP) mass added. The CIP (or PAP) mass transformed to the nanoplex was determined by the difference between the initial mass of CIP (or PAP) added and the mass of CIP (or PAP) recovered in the supernatant after the first centrifugation of the nanoplex suspension. The amounts of CIP and PAP in the supernatant were determined by UV-Vis spectrophotometry and BCA protein assay kit, respectively, as previously described. The overall yield defined in Eq. 3 was determined by the ratio of the dry mass of the CIP-(DXT-PAP) nanoplex recovered after 48-h freeze drying to the initial mass of CIP, PAP and DXT added.

$$\text{CE of CIP} \left(\% \frac{w}{w} \right) = \frac{\text{Mass of CIP that was transformed to CIP-(DXT-PAP)nanoplex}}{\text{Initial mass of CIP added}} \times 100$$

(1)

$$\text{CE of PAP} \left(\% \frac{w}{w} \right) = \frac{\text{Mass of PAP that was transformed to CIP-(DXT-PAP)nanoplex}}{\text{Initial mass of PAP added}} \times 100$$

(2)

$$\text{Yield } (\% \frac{w}{w}) = \frac{\text{Mass of CIP-(DXT-PAP)nanoplex formed}}{\text{Initial masses of CIP,DXT,and PAP added}} \times 100 \quad (3)$$

5.2.2.3 Mucolytic activity and *in vitro* mucus permeability

The mucolytic activity of PAP was investigated with the mucin solution. Briefly, 15 mg/mL mucin solution, which represented the typical mucin concentration present in the bronchiectasis sputum [149], was prepared by dissolving 1.5 g mucin, 2.68 g Na₂HPO₄·7H₂O, 0.242 g L-cysteine, and 0.186 g Na₂EDTA in 90 mL DI water after which the pH was adjusted to 6.0 by addition of 1.0 M HCl. Next, 2 mL of the nanoplex suspension containing 5 mg/mL PAP was added to 8 mL mucin solution maintained at 37 °C in a shaking incubator. At specific time points, the viscosity of the treated mucin solution was measured by Ubbelohde viscometer (USA). The reduced viscosity (η_{Reduced}) was then calculated as the ratio of the relative velocity increment (η_{Relative}) to the mass concentration of the mucin solution ([C] in g/mL) as defined in [150]. Control runs in which the mucin solution was not treated or treated with the free PAP and the CIP-DXT nanoplex were performed.

$$\eta_{\text{Reduced}} \text{ (mL/g)} = \frac{\eta_{\text{Relative}}}{[C]} = \frac{(\eta_{\text{Mucin}} - \eta_{\text{DI water}}) / \eta_{\text{DI water}}}{[C]}$$

The mucolytic activity of the CIP-(DXT-PAP) nanoplex was characterized from a minimum of six replicates by the change in the reduced viscosity (mL/g) of a mucin solution upon treatment with the nanoplex. The mucus permeability of the CIP-(DXT-PAP) and the CIP-DXT nanoplexes was investigated using synthetic mucus by the diffusion-tube method following Mueller et al. (2013) [143]. The

synthetic mucus was prepared following McGill et al. (2010) [151] by dissolving 60 mg/mL mucin, 3.2 mg/mL lecithin, and 32 mg/mL BSA in a buffer containing 85 mM Na⁺, 75 mM Cl⁻, 20 mM HEPES (pH 7.4).

The synthetic mucus (150 µL) was then added to a vertical 30-mm long silicon tube (diameter = 3.5 mm) covered with silicon caps on both ends. As illustrated in Fig. 5-2, aqueous suspension of the nanoplex was then added in six replicates to the top of the mucus layer and the tube was incubated at 37 °C for 6 h. At specific time points, the tube was flash frozen by liquid nitrogen after which the tube was cut into six 0.5 cm-long segments, where segments #1 and #6 represented the top and bottom parts of the mucus layer, respectively.

Figure 5-2: Diffusion-tube setup to characterize mucus permeability of the nanoplexes.

The CIP concentration contained in each segment was then determined by high performance liquid chromatography (HPLC) after dispersing the mucus segment in PBS. The HPLC analysis was performed in ZORBAX Eclipse Plus C18 column (250 × 4.6 mm, 5 µm particle size) at the optimal CIP detection wavelength of 324 nm. The mobile phase made up of acetonitrile: methanol: 0.1 M KH₂PO₄ prepared

at the ratio of 15: 25: 60 (v/v/v) was used at flow rate of 1.0 mL/min, resulting in the retention time of CIP at approximately 3.6 min.

The mucus permeability was characterized in terms of the mass percentage of CIP recovered in each segment calculated with respect to the initial mass of CIP added, where high percentages of CIP recovered in the segments nearer to the bottom (i.e. segments #5-6) signified good mucus permeability of the nanoplex in question. A control run in which the synthetic mucus was replaced with DI water of the same volume was performed.

5.2.2.4 Dissolution characteristics in ABSM

The CIP dissolutions from the CIP-(DXT-PAP) and CIP-DXT nanoplexes were investigated in triplicates in artificial bronchiectasis sputum medium (ABSM), which was formulated specifically to mimic the clinical bronchiectasis lung environment [152]. The ABSM was prepared following Yang et al. (2011) [153] as follows: 1 L of ABSM was prepared by dissolving 5 g mucin, 4 g salmon sperm DNA, 5.9 mg DPTA, 5 g NaCl, 2.2 g KCl, and 1.81 g Tris-base in 900 mL DI water adjusted to pH 7.0 by addition of 1.0 M HCl. Next, the medium was autoclaved at 110 °C for 15 min after which 5 mL egg yolk emulsion and 5 g/L sterile casamino acid solution were added. The CIP dissolution was investigated under sink condition at $\frac{1}{4}$ of C_{Sat} , where C_{Sat} denoted the thermodynamic saturation solubility of the native CIP in the ABSM.

The C_{Sat} was determined by adding native CIP in excess (≈ 20 mg) to 20 mL ABSM maintained at 37 °C in a shaking incubator. After 96 h incubation, 2 mL aliquot was withdrawn and subsequently centrifuged ($14,000 \times g$ for 5 min) and

syringe filtered (0.2- μ m pore size) to remove undissolved CIP. The CIP concentration in the filtered solution was then determined by HPLC as previously described. The CIP dissolution test was performed by adding aqueous suspension of the CIP-(DXT-PAP) nanoplex at $\frac{1}{4}$ C_{Sat} to a dialysis bag immersed in 50 mL ABSM maintained at 37 °C in a shaking incubator. At specific time points, 1 mL aliquot was withdrawn from the dissolution vessel, and replaced by the same volume of fresh ABSM. Next, the aliquot was centrifuged after which the CIP concentration in the supernatant was determined by HPLC as previously described.

5.2.2.5 Antibacterial efficacy in the presence of mucus barrier

The antibacterial efficacy of the CIP-(DXT-PAP) and CIP-DXT nanoplexes against biofilm colonies of clinical strain of *P. aeruginosa* was investigated using the aforementioned diffusion-tube setup with slight modifications. Briefly, 20 μ L overnight *P. aeruginosa* inoculums (10^6 CFU/mL) was deposited at the bottom of the silicon tube after which they were incubated for 24 h at 37 °C, resulting in the formation of biofilm colonies at the bottom. Subsequently, 150 μ L of the synthetic mucus was placed on top of the biofilm layer. Next, 15 μ L aqueous suspension of the nanoplex containing approximately 2 mg/mL of CIP, which represented more than ten-fold higher than the minimum inhibitory concentration (i.e. 0.125 μ g/mL) [71], was deposited on top of the mucus layer. After 24 h incubation at 37 °C, the tube was cut out 0.5 cm from the bottom. The cut-out segment was put in PBS and vortexed for 1 min to disperse the biofilm cells. The viable cells were then enumerated on MHB agar plates. The antibacterial efficacy was characterized from six independent replicates by the residual bacterial cell count ($\log_{10}$ CFU/mL). Two

control runs, i.e. (i) untreated biofilm cells and (ii) biofilm cells treated with the nanoplex but without the mucus barrier, were performed.

5.2.2.6 Cytotoxicity

The A549 cells were cultivated at the density of 10^4 cells/well in a 96-well tissue culture microplate containing DMEM supplemented with 10% (w/v) fetal bovine serum and 1% (w/v) penicillin-streptomycin solutions. The 16HBE14o- cells were grown in MEM supplemented with 10% (w/v) fetal bovine serum, 1% (w/v) L-glutamin (200 mM), and 1% (w/v) penicillin-streptomycin solution. After 24 h incubation at 37 °C in 5% CO₂ incubator, aqueous suspension of the CIP-(DXT-PAP) and CIP-DXT nanoplexes were added to the A549 and 16HBE14o- cells at CIP concentrations in the range of 12.5-200 µg/mL and 3.125-100 µg/mL, respectively. These CIP concentrations were within the typical range of CIP concentration recovered in the lungs (i.e. 33-237 µg/mL) upon inhaled delivery [100]. The treated cells were then incubated at 37 °C for 24 h after which 20 µL Celltiter-Blue® reagent was added to the cells and further incubated for 4 h. The viable cells were quantified from the concentration of resorufin determined by UV-Vis spectrophotometry at 570 nm and 600 nm for the background [154]. The cytotoxicity was determined from three replicates as the percentage of cell survival calculated from the ratio of OD₅₇₀₋₆₀₀ of the treated cells to OD₅₇₀₋₆₀₀ of the untreated cells. The cytotoxicity test was also performed for the free PAP at concentrations corresponding to the amount of PAP that would be present in the nanoplex at [CIP] = 12.5-200 µg/mL and 3.125-100 µg/mL for the A549 and

16HBE14o- cells, respectively. The PAP concentration used in the cytotoxicity test was therefore expressed as the equivalent CIP concentration, i.e. $[CIP]_{equiv}$.

5.2.2.7 Statistical analysis

All experiments were performed with a minimum of three replicates and the results were presented as mean $\pm$ standard deviation. The statistical significance was analyzed using Student t-test in GraphPad Prism software (USA). All p-values were two-sided and considered significant at $p \leq 0.05$, unless stated otherwise.

5.3 Results and Discussion

5.3.1 Optimal preparation condition for the CIP-(DXT-PAP) nanoplex

The effects of $R_{(CIP+PAP)/DXT}$ on the physical characteristics and preparation efficiency of the CIP-(DXT-PAP) nanoplex were investigated at $R_{(CIP+PAP)/DXT} = 1.1, 2.1, \text{ and } 2.9$, while keeping $R_{PAP/DXT}$ constant at 0.08 to ensure there were excess DXT charges to form electrostatic binding with PAP. As the study was performed at a fixed CIP concentration (i.e. 5 mg/mL), a higher $R_{(CIP+PAP)/DXT}$ value denoted a lower amount of anionic charges (i.e. DXT) available in the feed solution for complexation with the cationic charges (i.e. CIP and PAP).

The DLS results at $R_{(CIP+PAP)/DXT} = 1.1$ showed that the CIP-(DXT-PAP) nanoplex exhibited size and zeta potential of $223 \pm 99 \text{ nm}$ ($PDI = 0.51 \pm 0.05$) and $-51.0 \pm 1.9 \text{ mV}$, respectively (Fig. 5-3A). The negative zeta potential indicated the predominant presence of anionic DXT on the nanoplex surface. The nanoplex size was verified by the FESEM analysis, which showed roughly spherical nanoparticles in the size range of 100 to 200 nm (Fig. 5-3B), hence they were ideal for penetrating through the aqueous pores of the mucus, whose size was estimated around 60-300

nm [20]. On this note, the two control runs, i.e. (1) CIP solution mixed with PAP solution without DXT, and (2) (PAP-DXT) solution mixed with acetic acid solution without CIP, did not lead to any precipitated products, thus confirming that the nanoplex was made up of ternary complex of CIP and (PAP-DXT) as we verified later by FTIR.

Figure 5-3: (A) Effects of $R_{(CIP+PAP)/DXT}$ on the size and zeta potential of the CIP–(DXT–PAP) nanoplex; (B) FESEM image of the CIP–(DXT–PAP) nanoplex prepared at $R_{(CIP+PAP)/DXT} = 1.1$.

The results showed that increasing $R_{(CIP+PAP)/DXT}$ to 2.1 and 2.9 led to larger nanoplexes exhibiting lower zeta potentials. Specifically, at $R_{(CIP+PAP)/DXT} = 2.1$ and 2.9, the sizes were equal to 627 ± 77 nm (PDI = 0.46 ± 0.10) and 665 ± 57 nm (PDI = 0.33 ± 0.09), respectively, with zeta potentials of -43.0 ± 2.4 mV and -47.2 ± 1.4 mV, respectively (Fig. 5-3A). The FESEM analysis of the nanoplex prepared at $R_{(CIP+PAP)/DXT} = 2.9$, however, did not show larger nanoparticles in > 600 nm range, thus the larger size reported by DLS was likely attributed to agglomeration of the individual nanoplex.

The effect of $R_{(CIP+PAP)/DXT}$ on the CIP payload, on the other hand, was minimal, where the CIP payload remained at $60 \pm 5\%$ (w/w) independent of $R_{(CIP+PAP)/DXT}$ (Fig. 5-4A). In contrast, the PAP payload was found to decrease with increasing $R_{(CIP+PAP)/DXT}$ from $32 \pm 2\%$ (w/w) at $R_{(CIP+PAP)/DXT} = 1.1$ to $14 \pm 1\%$ (w/w) at $R_{(CIP+PAP)/DXT} = 2.9$ (Fig. 5-4A). The lower PAP payload at higher $R_{(CIP+PAP)/DXT}$ was not unexpected because, as mentioned earlier, the study was performed at fixed $R_{PAP/DXT} = 0.08$, thus there was less PAP in the feed solution when the amount of DXT was reduced at higher $R_{(CIP+PAP)/DXT}$. As a result of the decrease in the PAP payload, the total (CIP+PAP) payload decreased from $92 \pm 5\%$ (w/w) at $R_{(CIP+PAP)/DXT} = 1.1$ to $73 \pm 3\%$ (w/w) at $R_{(CIP+PAP)/DXT} = 2.9$ with the remaining mass was made up of DXT (Fig. 5-4A). The increased presence of DXT in the nanoplexes prepared at higher $R_{(CIP+PAP)/DXT}$ was believed to contribute to the aforementioned nanoplex agglomeration via the entanglement of the bound DXT chains.

With regard to the preparation efficiency, increasing $R_{(CIP+PAP)/DXT}$ resulted in lower CE values for both CIP and PAP denoting their lower utilization rates.

Specifically, the CE of CIP decreased from $88 \pm 2\%$ (w/w) at $R_{(CIP+PAP)/DXT} = 1.1$ to $42 \pm 1\%$ (w/w) at $R_{(CIP+PAP)/DXT} = 2.9$, respectively, while the CE of PAP decreased from $77 \pm 1\%$ (w/w) at $R_{(CIP+PAP)/DXT} = 1.1$ to $51 \pm 5\%$ (w/w) at $R_{(CIP+PAP)/DXT} = 2.9$, respectively (Fig. 5-4B). Consequently, the overall production yield also decreased from $56 \pm 2\%$ (w/w) at $R_{(CIP+PAP)/DXT} = 1.1$ to $40 \pm 4\%$ (w/w) at $R_{(CIP+PAP)/DXT} = 2.9$, despite the smaller initial amount of DXT in the feed solution at higher $R_{(CIP+PAP)/DXT}$ (Fig. 5-4B). In this regard, the lower CE of CIP observed at higher $R_{(CIP+PAP)/DXT}$ was caused by the smaller amount of DXT available for complexation at higher $R_{(CIP+PAP)/DXT}$. These results suggested that at least equal numbers of CIP and DXT charges were needed in the feed solution to maximize the CIP utilization rate, which was similar to the trend observed in the CIP-DXT nanoplex preparation reported earlier in Cheow and Hadinoto (2012) [22].

The lower CE of PAP at higher $R_{(CIP+PAP)/DXT}$, on the other hand, was believed to be caused by the competition between CIP and PAP, which both were positively charged, to form electrostatic binding with the negatively charged DXT. While the amount of DXT in the feed solution was lowered as $R_{(CIP+PAP)/DXT}$ was raised, the amount of CIP and $R_{PAP/DXT}$ were held constant. As a result, the same number of CIP molecules were competing with fewer PAP molecules to form electrostatic binding with fewer DXT molecules. This essentially made it more difficult for PAP to remain bound to DXT as DXT would preferentially bind with CIP because the charge density of CIP was almost ten times higher than that of PAP.

Figure 5-4: Effects of $R_{(CIP+PAP)/DXT}$ on (A) CIP and PAP payloads and (B) CEs of CIP and PAP and overall yield.

The above results led us to conclude that the optimal $R_{(CIP+PAP)/DXT}$ was found to be at 1.1 at which the size, zeta potential, PAP payload, and preparation efficiency of the CIP-(DXT-PAP) nanoplex were optimal. In comparison to the CIP-DXT nanoplex prepared at $R_{CIP/DXT} = 1.0$, the optimal CIP-(DXT-PAP) nanoplex showed a similar size (223 ± 99 nm versus 180 ± 55 nm), but with a higher PDI (0.51 ± 0.05 versus 0.36 ± 0.03) denoting the latter's higher polydispersity. Both CIP-DXT and CIP-(DXT-PAP) nanoplexes, nevertheless, possessed comparable zeta potentials of -57.0 ± 5.0 mV and -51.0 ± 1.9 mV, respectively. As expected, the CIP-(DXT-PAP) nanoplex exhibited a lower CIP payload ($60 \pm 5\%$ versus $70 \pm 5\%$) due to the inclusion of PAP. The CIP-(DXT-PAP) nanoplex exhibited a higher CIP utilization rate ($CE = 88 \pm 2\%$ versus $75 \pm 2\%$), but with a lower overall production yield ($56 \pm 2\%$ versus $66 \pm 5\%$) than the CIP-DXT nanoplex. From this comparison, it was concluded that the inclusion of PAP did not adversely affect the CIP-DXT nanoplex formation and with minimal impact on the nanoplex's physical characteristics.

5.3.2 FTIR and PXRD

The presence of CIP in the CIP-(DXT-PAP) nanoplex was verified by the appearance of the peak at 1628 cm^{-1} in the FTIR spectrum of the CIP-(DXT-PAP) nanoplex corresponding to the C=O stretching of the pyridone functional group that was unique to CIP [155] (Fig. 5-5A). The pyridone peak was slightly shifted from 1621 cm^{-1} observed in the native CIP's spectrum due to the protonation of CIP in the nanoplex. The pyridone peak expectedly also appeared in the FTIR spectrum of the CIP-DXT nanoplex. In this regard, the peak at 1732 cm^{-1} that appeared in the FTIR spectra of the CIP-(DXT-PAP) and CIP-DXT nanoplexes was attributed to

the C=O stretching of the uncharged COOH group of CIP in the nanoplex. This peak, nevertheless, did not appear in the FTIR spectrum of the native CIP because the COOH group of the native drug was charged due to its zwitterionic characteristics [156].

The characteristic peaks of PAP were evident in the FTIR spectrum of the free PAP at 1647 and 1530 cm^{-1} corresponding to the amide I (i.e. C=O stretching) and amide II (i.e. NH bending and C-N stretching) of the -CONH group of PAP [157], respectively. The amide I and II peaks, however, were not visible in the CIP-(DXT-PAP) nanoplex's spectrum due to the electrostatic complexation between the protonated amide groups of PAP and the SO_3^- group of DXT, which was represented by the peak at 1261 cm^{-1} in the free DXT's spectrum. In the absence of DXT-PAP complexation, the amide I and II peaks were visible as shown in the FTIR spectrum of the physical mixture of the free forms of DXT and PAP ("DXT-PAP mixture" in Fig. 5-5A).

Lastly, the electrostatic complexation between CIP and (DXT-PAP) complex was evident in the CIP-(DXT-PAP) nanoplex's spectrum from the disappearance of the peak at 1578 cm^{-1} corresponding to the NH_2 bending of the piperazine group of CIP. The disappearance of the NH_2 peak was attributed to the electrostatic binding between the protonated NH_2^+ group of CIP and the SO_3^- group of DXT. As expected, the disappearance of the NH_2 peak was also observed in the FTIR spectrum of the CIP-DXT nanoplex.

Figure 5-5: (A) FTIR spectra and (B) PXRD patterns of the CIP-(DXT-PAP) and CIP-DXT nanoplexes and their native/free constituents.

Similar to the CIP-DXT nanoplex, the PXRD analysis performed immediately after the nanoplex preparation showed that the CIP-(DXT-PAP) nanoplex existed in its amorphous forms, where the PXRD patterns did not show the strong intensity crystalline peaks observed in the PXRD pattern of the native CIP (Fig. 5-5B). Significantly, the amorphous form enabled the nanoplex to generate highly supersaturated CIP concentration upon dissolution, resulting in the high antibiotic exposure. Therefore, it was crucial for the nanoplex to maintain its amorphous form during its shelf-life. The results showed that the amorphous form of the CIP-(DXT-PAP) nanoplex remained stable after the eleven-month storage as evidenced by the absence of crystalline peaks in its PXRD pattern after storage (Fig. 5-5B). A similar observation was made after the two-week accelerated storage, which was approximately equal to two months storage under ambient condition [158]. The amorphous form stability could be attributed to the electrostatic binding between CIP and DXT that suppressed the molecular mobility of the amorphous CIP. In addition, the CIP and PAP payloads remained relatively stable after the eleven-month storage at $57 \pm 4\%$ and $27 \pm 2\%$, respectively, compared to $60 \pm 5\%$ and $32 \pm 2\%$ before the storage.

5.3.3 Mucolytic activity

The mucolytic activity of the CIP-(DXT-PAP) nanoplex was evident from the gradual decrease in $\eta_{Reduced}$ of the mucin solution as a function of time upon treatment with the CIP-(DXT-PAP) nanoplex (Fig. 5-6A). On this note, $\eta_{Reduced}$ of the untreated control was observed to decrease with time from 91 ± 4 mL/g at 5-min after preparation (0.08 h) to 80 ± 3 and 65 ± 2 mL/g after 1 h and 6 h, respectively,

due to the constant shearing in the shaking incubator. Compared to the untreated control, the rate of decrease in $\eta_{Reduced}$ was significantly faster for the mucin solution treated with the free PAP owed to the well-established mucolytic activity of PAP. Specifically, $\eta_{Reduced}$ of the mucin solution treated with the free PAP decreased to 54 ± 1 and 39 ± 2 mL/g after 1 h and 6 h, respectively.

In comparison to the free PAP, $\eta_{Reduced}$ of the mucin solution treated with the CIP-(DXT-PAP) nanoplex decreased at a slower rate to 73 ± 3 and 53 ± 2 mL/g after 1 h and 6 h, respectively. The slower mucolytic activity rate of the CIP-(DXT-PAP) nanoplex was not unexpected as PAP needed to be liberated from the nanoplex before it could perform its mucolytic function. Indeed, the difference between $\eta_{Reduced}$ of the mucin solution treated with the free PAP and the CIP-(DXT-PAP) nanoplex became smaller after 24 h, i.e. 36 ± 2 and 46 ± 1 mL/g, respectively (data not plotted). In contrast, $\eta_{Reduced}$ of the mucin solution treated with the CIP-DXT nanoplex decreased at the same rate as the untreated control due to the absence of mucolytic PAP in its formulation.

Figure 5-6: (A) Mucolytic activity of the CIP-(DXT-PAP) and CIP-DXT nanoplexes and free PAP; (B) mucus permeability of the nanoplexes in synthetic mucus after 3 h incubation and (C) after 24 h incubation (NS = not statistically significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

5.3.4 *In vitro* mucus permeability

Next, we investigated whether the established mucolytic activity of the CIP-(DXT-PAP) nanoplex did lead to its superior mucus permeability compared to the non-mucolytic CIP-DXT nanoplex. The results after 3 h incubation showed that $32 \pm 4\%$ (w/w) of the CIP-(DXT-PAP) nanoplex was able to permeate through the mucus layer to reach segments #5-6 near the bottom (Fig. 5-6B). In contrast, less than 3% (w/w) of the CIP-DXT nanoplex reached segments #5-6 because nearly all of it ($\approx 95\%$) remained near the top of the mucus layer (i.e. segments #1-2), as opposed to only $\approx 65\%$ for the CIP-(DXT-PAP) nanoplex. This result supported our hypothesis that the CIP-(DXT-PAP) nanoplex would exhibit superior mucus permeability to the CIP-DXT nanoplex owed to the mucolytic activity afforded by the PAP. For the control runs without mucus, the CIP-(DXT-PAP) and CIP-DXT nanoplexes exhibited comparable high permeation rates after 3 h with $93 \pm 5\%$ (w/w) and $95 \pm 8\%$ (w/w) of the nanoplexes were recovered in segments #5-6, respectively (data not plotted).

On this note, the high concentration of CIP was detected at the last section of the diffusion tube for the CIP-(DXT-PAP) nanoplex because of the diffusion-tube setup, which was covered with a cap at the bottom of the tube. And, at the collection time point (i.e. 3 h) to measure the CIP concentration, it was suggested that the PAP has already cleaved the mucin matrix in the synthetic mucus into small components, and the CIP-(DXT-PAP) nanoplex has penetrated through the mucus to reach the bottom of the tube. In future studies, if the collection time point is shorter (i.e. 1 h), the concentration of CIP may be high at the shorter segments (i.e.

segments #3-4), and may be low at the last segment. This needs to be further investigated for the future applications.

After 24 h incubation, a larger portion of the nanoplex permeated through the mucus layer as evidenced by the decrease in the proportions of the nanoplexes that remained in segments #1-2 of the mucus layer, i.e. $\approx 64\%$ and 43% , respectively, for the CIP-DXT and CIP-(DXT-PAP) nanoplexes (Fig. 5-6C). This decrease was accompanied by a visible increase in the proportion of the nanoplex recovered in segments #3-4. On this note, the proportion of the CIP-(DXT-PAP) nanoplex recovered in segment #6 after 24 h was found to be lower than that observed after 3 h likely due to the upward diffusion of the nanoplex upon reaching the bottom.

5.3.5 Dissolution characteristics

The CIP-(DXT-PAP) and CIP-DXT nanoplexes exhibited highly comparable CIP dissolution rates in the ABSM suggesting that the inclusion of PAP had minimal impact on the CIP dissolution from the nanoplex (Fig. 5-7A). Specifically, $62 \pm 1\%$ (w/w) of the CIP payload was dissolved from the CIP-(DXT-PAP) nanoplex after 60 min, compared to $68 \pm 10\%$ (w/w) dissolution for the CIP-DXT nanoplex over the same period. Both nanoplexes needed about 180 min to reach 100% dissolution. The CIP dissolution from the nanoplex was driven by the weakened electrostatic binding between CIP and DXT in the presence of high salt ion concentration in the ABSM.

5.3.6 Antibacterial efficacy in the presence of mucus barrier

Despite the presence of mucus barrier protecting the *P. aeruginosa* biofilm colonies, the CIP-(DXT-PAP) nanoplex was able to produce bactericidal activity against the biofilm as evidenced by $5.31 \pm 0.25 \log_{10}$ CFU/mL reduction in the viable bacterial cell counts (Fig. 5-7B). The bactericidal activity was herein defined as $> 3 \log_{10}$ CFU/mL reduction in the viable cells compared to the untreated control. For comparison, the reduction in the viable cell counts upon treatment with the CIP-DXT nanoplex was smaller at $4.09 \pm 0.58 \log_{10}$ CFU/mL. For the control run treated with the nanoplex in the absence of mucus barrier, the two nanoplexes produced identical reductions in the viable cell counts in which they completely eradicated the biofilm colonies (data not shown). Therefore, the superior antibacterial efficacy of the CIP-(DXT-PAP) nanoplex in the presence of the mucus barrier was attributed to its superior mucus permeability owed to the mucolytic action of PAP, resulting in higher antibiotic exposure in the vicinity of the biofilm colonies.

Figure 5-7: (A) CIP dissolution from the CIP-(DXT-PAP) and CIP-DXT nanoplexes; (B) antibacterial efficacy of the nanoplexes against *P. aeruginosa* biofilm in the presence of mucus barrier (** $p \leq 0.01$).

5.3.7 Cytotoxicity towards A549 and 16HBE14o-cells

5.3.7.1 A549 cells

The CIP-(DXT-PAP) and CIP-DXT nanoplexes exhibited comparable cytotoxicity profiles towards the A549 cells, where both nanoplexes exhibited minimal cytotoxicity with more than 90% cell survivals up to CIP concentration of 100 $\mu\text{g/mL}$ (Fig. 5-8A). At a higher CIP concentration of 200 $\mu\text{g/mL}$, however, the CIP-(DXT-PAP) nanoplex became more cytotoxic as evidenced by a significantly lower % cell survival at $53 \pm 1\%$, whereas the CIP-DXT nanoplex remained to exhibit minimal cytotoxicity with $\geq 88\%$ cell survival. These results indicated that the presence of PAP in the CIP-(DXT-PAP) nanoplex above a certain concentration threshold could be harmful to the A549 cells. The PAP concentration contained in the nanoplex at CIP concentration of 200 $\mu\text{g/mL}$ was calculated from the CIP and PAP payloads to be equal to approximately 107 $\mu\text{g/mL}$.

To confirm that the cytotoxicity of the CIP-(DXT-PAP) nanoplex was due to the presence of PAP, the cytotoxicity test of the free PAP was performed. The results showed that at $[\text{CIP}]_{\text{equiv}} = 12.5$ to 200 $\mu\text{g/mL}$ the free PAP exhibited a highly comparable cytotoxicity profile towards the A549 cells as the CIP-(DXT-PAP) nanoplex (Fig. 5-9A). The cytotoxicity result of the free PAP thus reaffirmed PAP as the main reason for the high cytotoxicity of the CIP-(DXT-PAP) nanoplex, which was not unexpected as PAP could cleave the components of the extracellular matrix surrounding the cells [159].

Figure 5-8: Cytotoxicity of the CIP-(DXT-PAP) and CIP-DXT nanoplexes towards (A) A549 and (B) 16HBE14o- cells (NS = not statistically significant, *** $p \leq 0.001$).

Figure 5-9: Cytotoxicity of the free PAP towards (A) A549 cells and (B) 16HBE14o- cells.

5.3.7.2 16HBE14o-cells

Compared to the A549 cells, both the CIP-DXT and CIP-(DXT-PAP) nanoplexes exhibited higher cytotoxicity towards the 16HBE14o- cells, where both nanoplexes exhibited increasing toxicity towards the 16HBE14o- cells with increasing CIP concentrations. This trend was not unexpected as our previous study had demonstrated the lower tolerance of the 16HBE14o- cells (compared to the

A549 cells) upon exposure to the native CIP [72]. Specifically, the % cell survivals upon treatment by the CIP-DXT and CIP-(DXT-PAP) nanoplexes were comparable at $\approx 90\%$ and 65% for $[\text{CIP}] = 6.25$ and $50 \mu\text{g/mL}$, respectively (Fig. 5-8B). At higher CIP concentration of $100 \mu\text{g/mL}$, however, the % cell survival upon treatment with the CIP-(DXT-PAP) nanoplex was equal to $30 \pm 3\%$, which was considerably lower than $51 \pm 1\%$ cell survival upon treatment with the CIP-DXT nanoplex.

Similar to the trend observed with the A549 cells, these results indicated that the presence of PAP in the CIP-(DXT-PAP) nanoplex above a certain concentration threshold was harmful to the 16HBE14o- cells. This was supported by the cytotoxicity results of the free PAP towards the 16HBE14o- cells, which showed nearly 100% cell survivals up to $[\text{CIP}]_{\text{equiv}} = 50 \mu\text{g/mL}$, but only 89% cell survival at $[\text{CIP}]_{\text{equiv}} = 100 \mu\text{g/mL}$ (Fig. 5-9B). On this note, at CIP concentration of $100 \mu\text{g/mL}$, the PAP concentration contained in the nanoplex was calculated from the CIP and PAP payloads to be equal to approximately $53 \mu\text{g/mL}$.

While the increased presence of PAP in the nanoplex would lead to higher mucolytic activity, thus improved mucus permeability, the cytotoxicity results showed that the increased presence of PAP could lead to harmful effect on the lung epithelium cells. Therefore, a trade-off existed between antibacterial efficacy and cytotoxicity upon the incorporation of PAP into the CIP-DXT nanoplex. A balancing effort in the dosage formulation and determination would therefore be needed to fully exploit the therapeutic benefits of the CIP-(DXT-PAP) nanoplex.

5.4 Conclusions

Mucolytic enzyme PAP was successfully incorporated into CIP-DXT nanoplex to produce CIP-(DXT-PAP) nanoplex. At the optimal preparation condition, the inclusion of PAP had minimal impact on the physical characteristics, preparation efficiency, and dissolution rate of the CIP nanoplex. The optimal CIP-(DXT-PAP) nanoplex exhibited size of ≈ 200 nm and zeta potential of ≈ -50 mV with CIP and PAP payloads of $\approx 60\%$ and 32% (w/w), respectively. The preparation of the CIP-(DXT-PAP) nanoplex was highly efficient in which 88% and 77% (w/w) of CIP and PAP were successfully transformed to the nanoplex. Compared to the CIP-DXT nanoplex, the CIP-(DXT-PAP) nanoplex exhibited a significantly higher mucus permeability (tenfold higher) attributed to the mucolytic actions of PAP. Its superior mucus permeability led to its higher bactericidal activity than the CIP-DXT nanoplex in the presence of mucus barrier. The CIP-(DXT-PAP) nanoplex, however, exhibited higher cytotoxicity towards the human lung epithelium cells than the CIP-DXT nanoplex above a certain PAP concentration threshold. Therefore, it is recommended that optimal toxicity profiles of PAP should be examined and evaluated before conducting antibacterial efficacy tests in biofilms in future studies. Lastly, the amorphous form and payloads of the nanoplex were demonstrated to be stable after long-term storage of nearly a year at room temperature.

CHAPTER 6. ANTI-BIOFILM AND ANTI-QUORUM SENSING OF *PSEUDOMONAS AERUGINOSA* BY THE QUERCETIN NANOPARTICLE COMPLEX

6.1 Introduction

Pseudomonas aeruginosa is an opportunistic pathogen causing high morbidity and mortality in bronchiectasis patients [160]. It can form biofilms in the mucus layer of the lung in which is very difficult for antibiotic penetration to eradicate all bacteria. The low dose exposure of antibiotics to the *P. aeruginosa* biofilm causes the resistance of many currently available antibiotics (i.e. aminoglycosides, quinolones, β -lactams and so on) [161]. And, overdose of antibiotics during treatment raised a risk of emergence for multidrug-resistant *P. aeruginosa* strains, leading to the ineffectiveness of the antibiotic therapy against this pathogen [162]. Therefore, the development of alternative non-antibiotic therapeutic strategies to eradicate *P. aeruginosa* is much increasingly paid attention [163].

Quorum sensing (QS) inhibition of *P. aeruginosa* is considered as one of novel promising strategies for treatment of bronchiectasis patients [74]. *P. aeruginosa* utilizes QS systems to communicate cell-cell interaction, regulate gene expression and secrete virulence factors [164]. The use of QS inhibitors to inhibit of *P. aeruginosa* QS is attractive due to the reduction of biofilm formation and virulence factor production, and preventing from the development of bacterial drug resistance [165]. The QS inhibitors can be from either synthetic or natural sources.

Flavonoids, prominent components of medicinal herbs, have attracted much interest as their unique antibacterial properties for inhibition of *P. aeruginosa* QS

[32]. Quercetin (QUE), a natural flavonoid mainly found in vegetables and fruits, has recently shown a wide range of biological activities in antioxidation [166], anticancer [167], and the effective anti-biofilm and anti-quorum sensing activity against *P. aeruginosa* strains [31, 32, 168]. Nevertheless, it was found that the aqueous solubility of QUE is very low, leading to a low bioavailability [169]. In addition, the native QUE form was easily degraded by enzymes to other metabolites, reducing its activity at the target sites [167]. In this regards, nanotechnology has developed the QUE nanoparticles to solve these issues. QUE has been encapsulated into PLA, PLGA nanoparticles [169, 170], liposome [171] and chitosan nanoparticles [83, 172]. These studies showed that entrapment of QUE into polymers enhanced its solubility, biological effects and bioavailability [83, 169].

On the other hand, despite various strategies to enhance QUE solubility, the nanoencapsulation of QUE has major limitations for its implementation on a wider scale. First, costs for QUE nanocapsule preparation are expensive due to costly materials of the carriers and complicated preparation steps. The carrier materials are required to be biocompatible and biodegradable, which hindered for options of polymeric carriers (i.e. the costly PLGA). Polysaccharides (i.e. chitosan) were considerably used as low-cost carriers to encapsulate QUE, however, those QUE nanocapsules exhibited a low payload $\leq 5\%$ (w/w) [83, 173], leading to the requirement of high dose for applications. As a result, it is required to develop a cost-effective formulation strategy for QUE nanoparticles which can exhibit a high payload and simple preparation process.

In our previous studies, a cost-effective bioavailability enhancement strategy has been developed in the amorphous form of nanoparticle complex (in short nanoplex), which was produced by the self-assembly drug–polysaccharide complexation, and exhibited the supersaturation solubility and high payload [52, 174, 175]. The cost-effective formulation strategy in our studies is attributed to (i) the simple but highly efficient preparation process, which was performed by only mixing of drugs and polymer solutions under ambient condition, and (ii) the use of naturally biodegradable polymers as the carrier (i.e. chitosan). With benefits of this preparation strategy, we developed the amorphous formulation of QUE-CHI nanoplex to enhance solubility and bioavailability of QUE.

In the present work, the aims were to investigate the formulation of QUE-CHI nanoplex for physical characteristics (i.e. size, complexation efficiency, production yield and payload). From this investigation, the optimal preparation condition of the QUE-CHI nanoplex was determined and subsequently used to investigate the dissolution, supersaturation generation, efficacies of anti-biofilm and anti-quorum sensing against *P. aeruginosa*. In addition, cytotoxicity of the QUE nanoplex toward lung epithelial cells was *in vitro* investigated.

6.2 Materials and Methods

6.2.1 Materials

Materials for QUE nanoplex preparation and characterization: quercetin (QUE) (purity $\geq 95\%$), low molecular weight chitosan (CHI) (50–190 kDa), glacial acetic acid (AA), potassium hydroxide (KOH), acetonitrile, formic acid, potassium bromide (KBr) were purchased from Sigma-Aldrich (Singapore).

Materials for bacterial cell culture: *P. aeruginosa* PAO1 laboratory strain was purchased from ATCC (USA). Mueller Hinton Broth (MHB), tryptone, and phosphate buffered saline (PBS, pH 7.4) for the bacterial cell culture were purchased from BD Diagnostics (Singapore), Gibco (USA), and Sigma-Aldrich (Singapore), respectively.

Materials for cytotoxicity tests: A549 human carcinoma lung epithelial cells were purchased from ATCC (USA). Penicillin-streptomycin and CellTiter-Blue were purchased from PAA Laboratories (Austria) and Promega (Singapore), respectively. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were purchased from GE Healthcare Life Sciences (USA). 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) solution was purchased from Gibco (USA).

6.2.2 Methods

6.2.2.1 Preparation of QUE-CHI nanoplex

QUE having pKa of 5.9 and 8.5 [176] was fully deprotonated at pH 13 to form the negatively charged QUE with a charge density of 6.62×10^{-6} mol-charge/mg. CHI having pKa of 6.5 [177] was protonated in aqueous acetic acid (AA) solution to form the positively charged CHI with a charge density of 5.58×10^{-6} mol-charge/mg. Briefly, 2.5 mg/mL of QUE was dissolved in 0.1 M KOH and 2.96 mg/mL of CHI calculated at the charge ratio of 1 ($R_{\text{CHI/QUE}}$) was dissolved in aqueous AA solution with a range of different concentrations (i.e. 0.4–1.0% v/v). The QUE solution upon its preparation was immediately mixed with an equal volume of CHI solution under stirring for 1 min. Next, the resultant QUE-CHI

nanoplex solution was centrifuged (14,000 x g for 30 min) and washed with DI water for two cycles to remove excess QUE and CHI that did not form the QUE nanoplex. Subsequently, the pellets of QUE nanoplex was re-dispersed in DI water for characterizations. The QUE solution mixed with 0.5% (v/v) AA solution at an equal volume ratio was examined as the control.

6.2.2.2 Physical characterizations

The size, polydispersity index (PDI), and zeta potential of the QUE nanoplex were characterized in triplicates by dynamic light scattering (DLS) after 100 × dilution using Brookhaven 90 Plus Nanoparticle Size Analyzer (Brookhaven Instruments Corporation, USA). The size and morphology of the optimal QUE-CHI nanoplex were examined by field emission scanning electron microscope (FESEM) using JSM-6700F (JEOL, USA).

The preparation efficiency of QUE nanoplex was characterized in terms of the complexation efficiency (CE), production yield and payload. The CE was defined in Eq. (1) as utilization rate of QUE that formed the QUE-CHI nanoplex, which was determined by the difference between the initial amount of QUE added and the amount of QUE remaining in the supernatant after the first centrifugation. The amount of QUE in the supernatant was determined by High Performance Liquid Chromatography (HPLC) using Agilent 1100 (Agilent Technologies, USA) after 20 times dilution of the supernatant in acetonitrile. The HPLC analysis was performed in ZORBAX Eclipse Plus C18 column (250 × 4.6 mm, 5 μm particle size) at 294 nm representing the optimal detection wavelength for QUE. The mixture of

acetonitrile : 0.1% formic acid (85 : 15 v/v) was used as the mobile phase at 0.8 mL/min, resulting in the retention time of QUE at approximately 2.8 min.

The production yield was defined in Eq. (2) as utilization rates of QUE and CHI to produce QUE-CHI nanoplex, which was determined by the ratio of the dry mass of the QUE nanoplex recovered after 48 h freeze-drying to the initial mass of QUE and CHI added. The payload of QUE in the nanoplex was determined as Eq. (3) in triplicates by dissolving a known mass of the freeze-dried QUE-CHI nanoplex in acetonitrile, and the amount of the dissolved QUE was determined by HPLC as described above.

$$\text{CE of QUE } \left(\% \frac{w}{w} \right) = \frac{\text{Mass of QUE that was transformed to QUE nanoplex}}{\text{Initial mass of QUE added}} \times 100 \quad (1)$$

$$\text{Yield } \left(\% \frac{w}{w} \right) = \frac{\text{Mass of QUE nanoplex formed}}{\text{Initial masses of QUE and CHI added}} \times 100 \quad (2)$$

$$\text{Payload } \left(\% \frac{w}{w} \right) = \frac{\text{Mass of QUE in nanoplex}}{\text{Mass of QUE nanoplex}} \times 100 \quad (3)$$

The presence of QUE in the nanoplex was examined by Fourier transform infrared spectroscopy (FTIR) from 450 and 4000 cm^{-1} at 4 cm^{-1} spectral resolution in Spectrum One (Perkin–Elmer, USA). The FTIR was analyzed for the native QUE, native CHI, QUE-CHI nanoplex, and physical mixture of the two natives. The amorphous form of the optimal QUE–CHI nanoplex was examined by powder X-ray diffraction (PXRD). The PXRD analysis was performed using D8 Advance

X-ray diffractometer equipped with Cu K α radiation (Bruker, Germany) from 5° to 70° (2 θ) with a step size of 0.02°/s.

6.2.2.3 Dissolution characteristics

The thermodynamic saturation solubility of the native QUE (i.e. C_{Sat}) in the PBS was determined by adding excess native QUE (i.e. 20 mg) to 20 mL PBS in a shaking incubator at 37 °C. After 48 h incubation, 2 mL aliquot was withdrawn and subsequently centrifuged (14,000 $\times$ g for 5 min) and filtered (0.2- μ m pore size) to remove the undissolved QUE. The QUE concentration in the filtered solution was then determined by HPLC as previously described.

The apparent solubility of the QUE nanoplex was determined by adding the QUE nanoplex suspension in excess (i.e. $\approx 10 \times C_{\text{Sat}}$) to 10 mL PBS placed in a shaking incubator at 37 °C and 150 rpm. At specific time points over 24 h, 400 μ L aliquot was withdrawn and filtered (0.2 μ m pore size), followed by 50 times dilution with fresh PBS to suppress QUE precipitation from the supersaturated solution. The QUE concentration in the diluted solution was then quantified by HPLC. The apparent solubility was reported as the ratio of the supersaturated QUE concentration generated by the QUE nanoplex to the thermodynamic saturation solubility of the native QUE in PBS ($C_{\text{Sat}} = 145 \pm 10 \mu\text{g/mL}$). The apparent solubility was obtained from three independent replicates using three different batches of the optimal QUE-CHI nanoplex.

The dissolution characteristics of the QUE-CHI nanoplex and native QUE in the PBS were determined in triplicates under sink condition (1/5 of C_{Sat}) by adding 0.5 mL of the QUE-CHI nanoplex suspension (or 1.5 mg of the native QUE) to a

dialysis bag immersed in an opaque bottle containing 50 mL PBS maintained at 37 °C in a shaking incubator. At specific time points over 24 h, 1 mL aliquot was withdrawn and the same volume of fresh PBS was added back to the dissolution vessel. The aliquot was centrifuged and filtered, and the QUE concentration in the supernatant was determined by HPLC.

6.2.2.4 Antimicrobial activity

Antimicrobial activities of the QUE-CHI nanoplex and the native QUE against clinically derived isolate of *P. aeruginosa* PAO1 were examined by the minimum inhibitory concentration (MIC). The MIC was performed in triplicates by microbroth dilution method according to the guidelines of Clinical and Laboratory Standards Institute (CLSI) [178]. Briefly, overnight inoculum of *P. aeruginosa* PAO1 was adjusted to 0.5 McFarland standards and diluted in MHB for 1.0×10^6 CFU/mL. Next, 200 µL the diluted cell suspension was added to a 96-well microplate. Afterwards, 200 µL the QUE-CHI nanoplex suspension in the range of 50-500 µg/mL QUE concentration was added to the cell suspension. The plate was then incubated at 37°C for 24 h. Wells without the nanoplex or without cells were prepared as the positive and negative controls, respectively. The bacterial cell growth was evaluated at OD₆₀₀ by microplate reader (Synergy HT, Biotek, USA), where OD₆₀₀ < 0.1 characterized no visible growth. The same protocol was used to determine the MIC of native QUE.

6.2.2.5 Anti-biofilm formation activity

The effects of the QUE nanoplex and the native QUE on biofilm formation were evaluated in triplicates as the method previously described [31]. Briefly, *P.*

aeruginosa PAO1 was inoculated in LB broth overnight, and then diluted to OD₆₀₀ of 0.05. Next, the planktonic bacterial suspension was incubated with the native QUE or the QUE nanoplex at a range of QUE concentrations (10 – 200 µg/mL) in a 24-well microplate. After 24 h incubation at 37 °C, biofilms were washed three times with PBS to remove non-adherent cells. Subsequently, biofilms were stained with 0.1% (w/v) crystal violet solution for 15 min. The cells were then washed three times with PBS to remove excess stain, followed by air-drying for 15 min. The crystal violets bound to biofilm were extracted with 30% (v/v) acetic acid solution. The amount of crystal violets in wells was determined by a microplate reader at OD₅₉₀. The biofilm formation was determined in triplicates by the ratio of OD₅₉₀ of the treated cells to OD₅₉₀ of the untreated cells. Wells without QUE or without cells were prepared as the positive and negative controls, respectively.

6.2.2.6 Anti-quorum sensing efficacy

6.2.2.6.1 Swimming assay

The swimming motility of *P. aeruginosa* was carried out as previously described [179]. Briefly, 2 µL of overnight inoculum *P. aeruginosa* was spotted on the soft agar plate containing 10 mg/mL tryptone, 5 mg/mL NaCl, 0.3% (w/v) agar and 200 µg/mL QUE for the native QUE or the QUE nanoplex. The plates were incubated in upright position at 37 °C for 24 h. The agar medium plates without QUE or without cells were used as the positive and negative controls, respectively.

6.2.2.6.2 Pyocyanin quantification assay

Production of pyocyanin in *P. aeruginosa* PAO1 treated by native QUE and QUE nanoplex were investigated in triplicates as the method previously described [180]. Briefly, the agar plate containing *P. aeruginosa* PAO1 treated with native QUE or QUE nanoplex was cut into small squares, followed by addition of chloroform to extract the pyocyanin. Next, the pyocyanin in the chloroform solution was re-extracted by adding 4 mL of 0.2N HCl. The amount of pyocyanin in a pink solution was determined by UV-Vis spectrophotometer at OD₅₂₀. The agar medium plates without QUE and without the bacteria were used as the positive and negative controls, respectively.

6.2.2.7 Cytotoxicity

The A549 cells cultivated in DMEM supplemented with 10% (w/v) fetal bovine serum and 1% (w/v) penicillin-streptomycin solution at 10⁵ cells/mL in a 96-well tissue culture microplate were incubated in 5% CO₂ incubator for 24 h at 37 °C. Afterwards, the QUE nanoplex suspension was added to the cells in the range of 10–100 µM of QUE concentration. This concentration range represented the typical range of QUE concentration towards A549 cells [181, 182].

The treated cells were incubated at 37°C for 24 h after which 20 µL Celltiter-Blue® reagent was added to the cells and further incubated for 4 h. The viable cells were quantified from the concentration of resorufin determined by UV-Vis spectrophotometry at 570 nm and 600 nm for the background. The cytotoxicity was determined from three replicates as the percentage of cell survival calculated from

the ratio of OD₅₇₀₋₆₀₀ of the treated cells to OD₅₇₀₋₆₀₀ of the untreated cells. The cytotoxicity tests were also performed for the native QUE.

6.3 Results and Discussion

6.3.1 Physical characteristics of QUE-CHI nanoplex

The formulation of the QUE-CHI nanoplex was investigated in the range of AA concentration (i.e. 0.4 – 1.0 % v/v). DLS results at the charge ratio $R_{\text{CHI/QUE}} = 1$ showed that the particle sizes of the QUE nanoplex were in the range of 143 – 600 nm (PDI = 0.2 - 0.4) corresponding to the AA concentration used (Fig. 6-1A). And, the QUE nanoplexes exhibited positive charges of 35–41 mV zeta potentials resulted from the presence of CHI. Furthermore, the size of the QUE nanoplex was reaffirmed by FESEM in which nanoplexes prepared at different AA concentrations were similarly observed in the identical and round shapes with the size range of 35 ± 8 nm (Fig. 6-1B).

The different sizes between FESEM and DLS measurements showed that there was an agglomeration of the QUE nanoplex. It was suggested that a lower magnification of FESEM images should be investigated to check further agglomerations. On the other hand, it was noted that the sizes of the QUE nanoplex prepared at 0.4 and 0.5% AA (v/v) and measured by the DLS were about 150 nm, indicating that the aggregation of the nanoplex at these two preparation conditions were quite small. Taken this into account, the effect of the QUE nanoplex's aggregation on future studies should be investigated (i.e. DPI formulation).

In terms of the preparation efficiency, it was found that the complexation efficiency (CE) was increased from $30 \pm 1\%$ (w/w) at 0.4% AA (v/v) to $57 \pm 1\%$

(w/w) at 0.5% AA (w/w), and then reduced to $8 \pm 2\%$ (w/w) at 1.0% AA (v/v) (Fig. 6-2A). The same trend was found for production yield, where the QUE nanoplex was obtained with $30 \pm 13\%$ (w/w) at 0.4% AA (v/v), increased to $40 \pm 2\%$ (w/w) at 0.5% AA (v/v) and then decreased to $6 \pm 3\%$ (w/w) at 1.0% AA (v/v). On the other hand, it was found that the payload of QUE nanoplex was not significantly different with about 25% (w/w) as it was prepared at 0.5 – 0.8% (v/v) of AA concentration (Fig. 6-2B). From these results, the optimal preparation condition for the QUE nanoplex at $R_{\text{CHI/QUE}} = 1$ was determined to be at 0.5% (v/v) AA.

Figure 6-1: (A) Effects of the AA concentration on the size and zeta potential of the QUE-CHI nanoplex prepared at $R_{\text{CHI/QUE}} = 1$; (B) FESEM image of the QUE-CHI nanoplex prepared at 0.5% (v/v) AA and $R_{\text{CHI/QUE}} = 1$.

A)

B)

Figure 6-2: Effects of the AA concentration on (A) the complexation efficiency and production yield, and (B) payload of the QUE-CHI nanoplex prepared at $R_{\text{CHI/QUE}} = 1$.

6.3.2 FTIR and PXRD

The presence of QUE in the QUE-CHI nanoplex was verified by the FTIR as shown in the Fig. 6-3A. The peak at 1620 cm^{-1} in the FTIR spectrum of the QUE-CHI nanoplex was corresponding to the C=C aromatic ring stretch bands that was unique to QUE [183]. The spectral peak slightly shifted from 1610 cm^{-1} observed in the native QUE's spectrum due to the deprotonation of QUE in the nanoplex. In addition, the appearance of peaks at 1379 cm^{-1} and 1165 cm^{-1} in the FTIR spectrum of the QUE-CHI nanoplex corresponding to the OH bending of the phenols and the C-CO-C stretch and bending in the ketone, respectively, was also observed in the native QUE [183]. The results of FTIR spectra revealed that the functional groups of QUE were maintained in the QUE-CHI nanoplex.

The PXRD patterns showed that the QUE-CHI nanoplex existed in the amorphous state as it only exhibited the broad halo peaks, which were opposite with the PXRD pattern of the sharp crystalline peaks observed native QUE (Fig. 6-3B). The existence of amorphous form was important as the nanoplex could generate highly supersaturated concentration upon dissolution, resulting in the high drug exposure. Therefore, the stability of the amorphous form for nanoplex was important for its shelf-life. Significantly, the QUE-CHI nanoplex stored for the six-month period still exhibited the amorphous form, which was evidenced by the absence of sharp crystalline peaks in its PXRD pattern (Fig. 6-3B).

Figure 6-3: (A) FTIR spectra and (B) PXRD patterns of the QUE-CHI nanoplex and the native QUE.

6.3.3 Dissolution characteristics

As shown in the Fig. 6-4A, the QUE nanoplex exhibited the “spring and parachute” supersaturation time-profiles, where the QUE nanoplex upon dissolution in PBS exhibited the supersaturated QUE level highly above the saturation solubility of QUE (“spring”) after which the supersaturated level gradually reduced to the saturation solubility value (“parachute”) due to crystallization of the supersaturation solution [184]. The QUE nanoplex exhibited a maximum supersaturation level of 4.7 ± 0.9 after 20 min, followed by its gradual decrease to 2.4 ± 0.2 after 24h (Fig. 6-4A).

The supersaturated solubility generation of the QUE nanoplex could be resulted from its fast dissolution rate in PBS, which was attributed to their amorphous nanosize and the weakened electrostatic binding between QUE and CHI in the presence of high salt ion concentrations of the PBS. Indeed, it was found that the QUE nanoplex exhibited a faster dissolution rate than the native QUE. Specifically, the QUE nanoplex exhibited $25 \pm 2\%$ and $45 \pm 3\%$ dissolution after 1h and 4h, respectively, compared to $16 \pm 2\%$ and $31 \pm 3\%$ dissolution of the native QUE in the same period (Fig. 6-4B).

A)

B)

Figure 6-4: (A) Supersaturation solubility of the QUE nanoplex in the PBS solution expressed as a multiple of the saturation solubility of the native QUE (C_{Sat}); (B) dissolution rates of the QUE nanoplex and the native QUE in the PBS solution.

6.3.4 Antimicrobial activity

The MIC of the QUE-CHI nanoplex against the clinical strain of *P. aeruginosa* was determined to be greater than 500 $\mu\text{g/mL}$ with $\text{OD}_{600} = 0.048 \pm 0.067$, which was found to be similar to the MIC of the native QUE with $\text{OD}_{600} = 0.097 \pm 0.029$. This indicated that the antimicrobial activity of QUE was maintained after its complexation with CHI, which boded well for potential clinical applications of the QUE-CHI nanoplex. This MIC was similarly found with the study by Ouyang et al. which showed the MIC of QUE against the *P. aeruginosa* PAO1 was greater than 256 $\mu\text{g/mL}$ [31].

6.3.5 Anti-biofilm formation activity

The anti-biofilm activities of the QUE nanoplex and the native QUE were investigated in the concentration range of 10 – 200 $\mu\text{g/mL}$. And, it was found that the QUE nanoplex exhibited a superior inhibition of *P. aeruginosa* biofilm to its native counterpart. Specifically, the native QUE reduced $\approx 30 - 40\%$ biofilm formation, whereas QUE nanoplex significantly reduced biofilm formation of *P. aeruginosa* PAO1 to $\approx 60 - 70\%$ at 10 - 200 $\mu\text{g/mL}$ of QUE in comparison to the untreated control (Fig. 6-5). Especially, *P. aeruginosa* PAO1 biofilm was reduced up to $62 \pm 9\%$ as it was treated with the QUE nanoplex at 200 $\mu\text{g/mL}$ of QUE. Therefore, the QUE concentration of 200 $\mu\text{g/mL}$ was used in further experiments. Not unexpectedly, the higher biofilm inhibition of QUE nanoplex was attributed to its faster dissolution and supersaturation solubility generation, which was showed in the section 6.3.3.

Figure 6-5: Effects of the QUE nanoplex and native QUE on biofilm formation of *P. aeruginosa* PAO1 at different QUE concentrations after 24 h at 37 °C.

6.3.6 Anti-quorum sensing efficacy

6.3.6.1 Anti-swimming motility

The swimming motility and quorum sensing were coordinated during the biofilm formation of *P. aeruginosa* [185]. The swimming motility of *P. aeruginosa* was found to be essential in the first stage of invasion and growth for biofilm formation. It was reported that inhibition of swimming motility resulted in prevention of biofilm formation of *P. aeruginosa* [186]. Therefore, the anti-quorum sensing efficacy of the QUE nanoplex and the native QUE were first evaluated by the swimming motility of *P. aeruginosa* PAO1. As shown in the Fig. 6-6, the colony diameters of clinical strain *P. aeruginosa* PAO1 on the plates containing the native QUE (i.e. 5.3 ± 0.4 cm) and the QUE nanoplex (i.e. 4.2 ± 0.2 cm) exhibited poorer flagellum-driven motility compared to the control without QUE (i.e. 6.8 ± 0.4 cm). Furthermore, it was observed that the plate containing the QUE nanoplex

exhibited superior motility inhibition to its native counterpart, which was not unexpected as the nanoplex could generate the supersaturation solubility and faster QUE dissolution. This result revealed that the superior motility inhibition of the QUE nanoplex resulted in the superior reduction of biofilm formation of *P. aeruginosa* in comparison to its native counterpart.

Figure 6-6: Effects of the QUE nanoplex and native QUE on the swimming motility of *P. aeruginosa* PAO1. The plate supplemented with the QUE nanoplex had the orange color that was resulted from the agglomeration of the QUE nanoplex.

It was found that the agglomeration of the QUE nanoplex appeared when the QUE nanoplex was added to the soft-agar medium. And, it was noted that the agglomeration of the QUE nanoplex in the Fig. 6-6 was different with the one in Fig. 6-1. The agglomeration of the QUE nanoplex in Fig. 6-1 was resulted in the redispersion of the nanoplex in deionized water, whereas the agglomeration of the QUE nanoplex in the Fig. 6-6 was resulted in the disintegration of the QUE nanoplex to free QUE molecules in the high salt ion concentrations of the medium.

The disintegration of the nanoplex was not unexpected as the electrostatic binding between QUE and CHI was weakened in the high salt ion concentrations of the medium due to the ion exchange. Moreover, it was noted that the agglomeration of the QUE nanoplex was inevitably observed in the swimming motility test because the medium used to examine the motility of *P. aeruginosa* was an agar-solidified form, so the QUE nanoplex was not yet completely dissolve while the medium agar was solidified. This was expected because the QUE needed time to be liberated from the QUE-CHI complex.

On the other hand, the effect of the agglomeration on the motility of *P. aeruginosa* in the swimming motility test can be further investigated by a microscope to check the twitching and protrusions of *P. aeruginosa* colony edges [187]. If the agglomeration of the QUE nanoplex has an effect on the motility of *P. aeruginosa*, we will observe a different phenomenon of the colony edges compared to the native QUE.

6.3.6.2 Anti-virulence factor production

Quorum sensing regulates the expression of genes involved in production of virulence factors (i.e. pyocyanin, rhamnolipid, elastase) [188]. Pyocyanin, a redox-active phenazine, caused the persistent infection of *P. aeruginosa* [189], and the damage of host cells or tissues in patients [188]. To further evaluate the anti-quorum sensing efficacy of the QUE nanoplex and the native QUE against *P. aeruginosa* PAO1 biofilm, the amount of pyocyanin produced in the swimming motility assay was investigated. As shown in the Fig. 6-7, in comparison to the untreated control the pyocyanin production was reduced by $63 \pm 2\%$ for the plate

supplemented with the native QUE owed to the well-established anti-quorum sensing activity of native QUE [31]. Indeed, the cell growth of *P. aeruginosa* PAO1 between the untreated control and the QUE-treated cells were found to be similar (Fig. 6-7), indicating that the inhibition of pyocyanin production was from the anti-quorum sensing activity of QUE.

In comparison to the native QUE, the QUE nanoplex exhibited a lower reduction of pyocyanin production with about $29 \pm 4\%$ (Fig. 6-7). The lower reduction of pyocyanin production but higher inhibition of biofilm and swimming motility for the QUE nanoplex indicated that the nanoplex could already more inhibit for other virulence factors (i.e. pyoverdinin, rhamnolipid, elastase, etc.) which need to be further investigated.

Figure 6-7: Effects of the QUE nanoplex and native QUE on pyocyanin production and cell growth of *P. aeruginosa* PAO1.

6.3.7 Cytotoxicity

The QUE nanoplex and the native QUE exhibited comparable cytotoxicity profiles towards the A549 cells. It was found that both native QUE and nanoplex exhibited minimal cytotoxicity with more than 90% cell survival at 10 μM of QUE (Fig. 6-8). However, at higher concentration of QUE (50 – 100 μM) the native QUE and the QUE nanoplex became more toxicity as shown by a significantly lower % cell survival at $\approx 70\%$ and $\approx 50\%$, respectively. These results suggested that the QUE could be harmful for A549 cells at the certain concentration threshold, which was similarly found in previously reported studies [181, 182]. In addition, it indicated that the QUE nanoplex did not alter the cytotoxicity profile of QUE towards A549 cells.

Figure 6-8: Cytotoxicity of the QUE nanoplex and native QUE towards A549 cells.

6.4 Conclusions

First, the amorphous formulation of the QUE nanoplex was successfully prepared by a simple and cost-effective drug-polysaccharide complexation method. The QUE nanoplex exhibited a good payload (i.e. $\approx 25\%$) and generated supersaturation solubility at ≈ 5.0 times of its saturation solubility. Second, the amorphous state of the QUE nanoplex was demonstrated to be stable more than 6-months at the room temperature, indicating that its physical stability was preserved. Third, the QUE nanoplex exhibited comparable antimicrobial activity and minimal cytotoxicity to its native counterpart, indicating that QUE was not affected by its complexation with CHI. Fourth, even though the inhibition of pyocyanin production of the QUE nanoplex was lower than the native QUE, the efficacy of anti-biofilm and anti-swimming motility of the QUE nanoplex was superior than its native counterpart, indicating that the other virulence factors (i.e. pyoverdine, rhamnolipid, protease, elastase) could be more inhibited by the QUE nanoplex, which should be further investigated. On the other hand, the mechanism of QUE on anti-QS of *P. aeruginosa* should be further investigated. An evaluation of the gene expression involved in the QS pathways should be performed in future studies (i.e. quantitative real-time PCR). Indeed, in Ouyang's study the effect of QUE on the expression of genes (i.e. *lasI*, *lasR*, *rhlI*, *rhlR*) associated with the QS pathways were already examined, and it was found that QUE significantly reduced the expression levels of *lasI*, *lasR*, *rhlI*, and *rhlR* to 34%, 68%, 57%, and 50%, respectively [31]. In summary, the positive preliminary results achieved in the present study showed that the QUE nanoplex could be a promising formulation strategy for an effective inhibition of *P. aeruginosa* biofilm and quorum sensing.

CHAPTER 7. CONCLUSIONS AND RECOMMENDATIONS

7.1 CONCLUSIONS

Bronchiectasis characterized by permanent bronchial dilatation and recurrent infections has been clinically managed by long-term intermittent inhaled antibiotic therapy among other treatments. Inhaled antibiotic nanoparticles have emerged as an effective strategy to control infection in bronchiectasis lung owed to their mucus-penetrating ability. And, the effectiveness of the CIP nanoplex in the mucus environment of NCFB, its bactericidal activity against clinical NCFB *P. aeruginosa* strains, and its cytotoxicity towards the lung cells had not been previously investigated. Herein the objectives of this dissertation were to evaluate the effectiveness of DPI of CIP nanoplex against its native CIP counterpart (Chapter 3) and the well established drug delivery system represented by the CIP liposome (Chapter 4) in the NCFB environment in terms of (1) dissolution characteristics in artificial sputum bronchiectasis medium (ABSM), (2) *ex vivo* mucus permeability in the sputum expectorated by healthy individuals and NCFB patients, (3) antibacterial efficacy in the presence of sputum against clinical isolates of *P. aeruginosa* (both planktonic and biofilm), (4) cytotoxicity towards human lung epithelial cells, and (5) *in vitro* aerosolization efficiency.

First, the DPI of CIP nanoplex was evaluated against its native CIP counterpart in the NCFB environments. It was found that even though both DPI forms of CIP nanoplex and native CIP exhibited the similarly fast dissolution rates in sputum, the DPI of CIP nanoplex exhibited superior mucus permeability to the native CIP (5–7

times higher) attributed to its built-in ability to generate highly supersaturated CIP concentration in the sputum, leading to the CIP nanoplex's higher antibacterial efficacy ($> 3 \log_{10}$ CFU/mL). Especially, the DPI of CIP nanoplex exhibited similar cytotoxicity towards the lung epithelial cells as the native CIP indicating its low risk of toxicity.

Second, the DPI of CIP nanoplex was further evaluated against the DPI of CIP liposome in the NCFB environment. The results showed that the CIP nanoplex exhibited fast dissolution with CIP supersaturation generation, in contrast to the slower release of the liposome. Both nanoparticles exhibited the good mucus penetration (50% permeation after 1 h), leading to their similarly high antipseudomonal activity. Nevertheless, the CIP liposome possessed much lower CIP payload than the nanoplex (3.5% versus 84%), resulting in high lipid contents in its DPI formulation that led to higher cytotoxicity and lower aerosolization efficiency.

Third, to further establish the CIP nanoplex as a viable inhaled antibiotic delivery system for NCFB management, the mucus permeability of CIP–DXT nanoplex was improved by incorporation with mucolytic protease papain (PAP) to produce the ternary CIP–(DXT–PAP) nanoplex in Chapter 5 to enhance the higher antibacterial efficacy. The results showed that the physical characteristics and the dissolution of the CIP–(DXT–PAP) nanoplex was found to be similar to the CIP nanoplex, which exhibited size of 200 nm, zeta potential of -50 mV, and payloads of 60% (w/w) CIP and 32% (w/w) PAP. The CIP–(DXT–PAP) nanoplex exhibited ten-fold improvement in the mucus permeability compared to its CIP–DXT nanoplex counterpart, resulting in the CIP–(DXT–PAP) nanoplex's superior

bactericidal activity against clinical *P. aeruginosa* biofilm in the presence of mucus barrier. However, the incorporation of PAP into the CIP nanoplex caused cytotoxicity towards human lung epithelium cells at above a certain concentration threshold. Therefore, the optimal dosing of the CIP–(DXT–PAP) nanoplex must be investigated *in vivo*.

Fourth, to further enhance the antibacterial efficacy and prevent the antibiotic resistant *P. aeruginosa* biofilm, the amorphous QUE nanoplex was developed and evaluated in Chapter 6 in terms of (i) dissolution characteristics, (ii) antibacterial and anti-biofilm efficacy, (iii) anti-quorum sensing against *P. aeruginosa*, and (iv) cytotoxicity towards human lung epithelial cells. The results showed that the QUE nanoplex exhibited a supersaturation generation and a faster dissolution rate than the native QUE, resulting the QUE nanoplex 's higher anti-biofilm and anti-swimming motility efficacy. In addition, both QUE nanoplex and native QUE exhibited a minimal cytotoxicity towards A549 cells, indicating that the activity of QUE was maintained after its complexation with CHI. These results suggested that the QUE nanoplex can be a potential anti-biofilm and anti-quorum sensing formulation strategy against *P. aeruginosa* for the treatment of bronchiectasis patients.

7.2 RECOMMENDATIONS FOR FUTURE WORKS

Based on the obtained results in this dissertation, some further promising studies are recommended as the following:

- *In vivo* studies including evaluations of effectiveness of DPI formulations in the bronchiectasis environment should be investigated in the future.
- It is necessary to find out the mechanisms that how the nanoplex interacts with the components in the mucus and enhance the mucus permeability and antibacterial efficacy.
- The CIP-(DXT-PAP) nanoplex suspension can be formulated to the DPI form as the DPI inhalation showed some advantages over nebulizer. Next, the DPI of CIP-(DXT-PAP) nanoplex will be investigated for aerosolization efficacy, mucus permeability, antibacterial efficacy and cytotoxicity towards human lung epithelial cells. And, it is recommended that optimal toxicity profiles of PAP should be examined and evaluated before conducting antibacterial studies in biofilms.
- The QUE-CHI nanoplex suspension can be developed to the DPI forms. Next, both QUE-CHI nanoplex in suspension and DPI forms will be investigated for the mucus permeability, efficacy of anti-biofilm and anti-

quorum sensing against clinical strains of *P. aeruginosa* in the presence of mucus barrier.

- On the other hand, it was reported that combination therapy between conventional antibiotics and anti-drug resistant therapy has exhibited a great potential for the treatment of pathogen infections [190, 191]. As a result, it is recommended that co-delivery of the CIP nanoplex with the QUE nanoplex can be a promising therapy to completely eradicate *P. aeruginosa* biofilm for the management of bronchiectasis.

CHAPTER 8. LISTS OF PUBLICATIONS

Tran TT, Vidaillac C, Yu H, Yong VFL, Roizman D, Chandrasekaran R, Lim AYH, Low TB, Tan GL, Abisheganaden JA, Koh MS, Teo J, Chotirmall SH, Hadinoto K, “A new therapeutic avenue for bronchiectasis: Dry powder inhaler of ciprofloxacin nanoplex exhibits superior *ex vivo* mucus permeability and antibacterial efficacy to its native ciprofloxacin counterpart”, *Int J Pharm.*, 547(1-2), 368-376, 2018.

Tran TT, Yu H, Vidaillac C, Lim AYH, Abisheganaden JA, Chotirmall SH, Hadinoto K, “An evaluation of inhaled antibiotic liposome versus antibiotic nanoplex in controlling infection in bronchiectasis”, *Int J Pharm.*, 559: 382–392, 2019.

Tran TT, Hadinoto K, Ternary nanoparticle complex of antibiotic, polyelectrolyte, and mucolytic enzyme as a potential antibiotic delivery system in bronchiectasis therapy, *Colloids and Surfaces B: Biointerfaces*, 193: p. 111095, 2020.

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A new therapeutic avenue for bronchiectasis: Dry powder inhaler of ciprofloxacin nanoplex exhibits superior ex vivo mucus permeability and antibacterial efficacy to its native ciprofloxacin counterpart

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An evaluation of inhaled antibiotic liposome versus antibiotic nanoplex in controlling infection in bronchiectasis**Author:**

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Ternary nanoparticle complex of antibiotic, polyelectrolyte, and mucolytic enzyme as a potential antibiotic delivery system in bronchiectasis therapy

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