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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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Abstract

Non-cystic fibrosis bronchiectasis (NCFB) characterized by permanent bronchial dilatation and recurrent infections has been clinically managed by long-term intermittent inhaled antibiotic therapy among other treatments. Herein we investigated dry powder inhaler (DPI) formulation of ciprofloxacin (CIP) nanoplex with mannitol/lactose as the excipient for NCFB therapy. The DPI of CIP nanoplex was evaluated against DPI of native CIP in terms of their (1) dissolution characteristics in artificial sputum medium, (2) *ex vivo* mucus permeability in sputum from NCFB and healthy individuals, (3) antibacterial efficacy in the presence of sputum against clinical *Pseudomonas aeruginosa* strains (planktonic and biofilm), and (4) cytotoxicity towards human lung epithelial cells. Despite their 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. The superior mucus permeability led to the CIP nanoplex's higher antibacterial efficacy ($> 3 \log_{10}$ CFU/mL). The DPI of CIP nanoplex exhibited similar cytotoxicity towards the lung epithelial cells as the native CIP indicating its low risk of toxicity. These results established the promising potential of DPI of CIP nanoplex as a new therapeutic avenue for NCFB.

Keywords: *bronchiectasis; ciprofloxacin; dry powder inhaler; nanoparticles; Pseudomonas aeruginosa*

47 **List of abbreviations**

48	AA	acetic acid
49	ASM	artificial sputum medium
50	bc	basolateral compartment
51	C _{Sat}	saturation solubility of the native CIP
52	CF	cystic fibrosis
53	CFM	confocal fluorescence microscope
54	CFU	colony forming units
55	CIP	ciprofloxacin
56	[CIP]	ciprofloxacin concentration
57	DPI	dry powder inhaler
58	DMSO	dimethyl sulfoxide
59	DMEM	Dulbecco's modified Eagle's medium
60	DNA	deoxyribonucleic acid
61	DPTA	diethylenetriamine pentaacetic acid
62	DXT	dextran sulfate
63	EDTA	ethylenediaminetetraacetic acid
64	HPLC	high performance liquid chromatography
65	MEM	Minimum Essential Medium with Eagle's salt
66	MHB	Mueller Hinton broth
67	MIC	minimum inhibitory concentration
68	MLR _{Nanoplex}	Mannitol to lactose ratio for the CIP nanoplex
69	MLR _{Native}	Mannitol to lactose ratio for the native CIP
70	NCFB	non-cystic fibrosis bronchiectasis
71	OD ₆₀₀ , OD ₅₀₀	optical density at 600 and 500 nm
72	PBS	phosphate buffered saline
73	PR	permeability ratio
74	UV-Vis	ultraviolet-visible spectroscopy

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 (Weycker et al., 2005). Bronchiectasis is traditionally classified as either cystic fibrosis (CF) bronchiectasis or non-cystic fibrosis bronchiectasis (NCFB) (Bilton and Jones, 2011; Chalmers and Sethi, 2017). Historically, the true prevalence of NCFB has been underestimated and consequently has received less attention than its CF counterpart (Chang and Bilton, 2008; Grimwood et al., 2014). 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 (McShane et al., 2013; Weycker et al., 2017).

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 (Chalmers et al., 2015a). Antibiotics play an important role in the management of NCFB, both in acute exacerbations and in long-term intermittent therapy (Brodt et al., 2014; Chalmers et al., 2012). 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 (Finch et al., 2015; Wilson et al., 2016).

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 (Chalmers et al., 2015b). However, no inhaled antibiotics have been approved at present for NCFB due to a lack of long-term efficacy data (Sugianto and Chan, 2016). 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 (Justo et al., 2013).

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) (Aksamit et al., 2018; De Soya et al., 2018), and (2) dual-release liposomal CIP delivered by nebulization (ORBIT-3 and ORBIT-4) (Haworth et al., 2017). 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 (De Soyza and Aksamit, 2016). 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 (Weers, 2015). 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 (Brillault et al., 2017), 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 (Yang et al., 2011). 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 (Cipolla et al., 2016).

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) (Cheow and Hadinoto, 2012; Yu et al., 2016).

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 (Suk et al., 2009). 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) (Hadinoto and Cheow, 2014). 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 (Cheow and Hadinoto, 2012), 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 (Cheow and Hadinoto, 2012). We had also successfully developed DPI formulation of the CIP nanoplex (Yu et al., 2016) prepared by spray drying of the CIP nanoplex with mannitol - a mucolytic agent widely used in bronchiectasis therapy (Bilton et al., 2014; Yang et al., 2011). 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.

2. Materials and Methods

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 our previous work (Chotirmall et al., 2010). The patients' demographics and clinical information were provided Table S1 in the Supplementary Materials. 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-glutamin (200 mM), and 0.25% trypsin ethylenediaminetetraacetic acid (EDTA) solution were purchased from Gibco (USA).

2.2. Methods

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 and Hadinoto (2012). 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. (2016). 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 Yu et al. (2016) 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.

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. (2015) with a slight modification on the mucin concentration used. Briefly, DPI of CIP nanoplex was added at [CIP] = 50 µg/mL to 1 mL PBS containing 6.5 mg/mL mucin representing the typical maximum concentration of mucin observed in bronchiectasis mucus (Henderson et al., 2014). 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 × 4.6 mm, 5-µm particle size) at detection wavelength of 324 nm. Acetonitrile: methanol: 0.1 M KH₂PO₄ (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. A control experiment in which mucin was not added to the PBS was carried out to determine the mucin interaction.

2.2.3. Dissolution characteristics in ASM

The ASM was prepared according to the protocols described in Yang et al. (2011). 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 (Jayaraman et al., 2001).

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 μg/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.

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. S1 of the Supplementary Materials. 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°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.

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.

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

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

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

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 µL 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 µg 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 to 0.125 µg/mL.

Next, 50 µL aliquot was withdrawn after 1 h from the basolateral compartment and diluted in 450 µL MHB, followed by filtration using 0.2-µm 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 µg 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. (2014). 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.

2.2.6. Confocal fluorescence microscope

Overnight cultures of *P. aeruginosa* (CS#1-3 strains) adjusted to optical density OD₆₀₀ = 0.70 in MHB were diluted to reach a starting inoculum of 1×10^6 CFU/mL. Subsequently, 200 μ L of the inoculum 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 543nm, respectively. The images were analyzed using COMSTAT 2 software (Technical University of Denmark) to determine the cells' Live/Dead ratios.

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-glutamin (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 to 250 mg/L. This concentration range represented the typical range of CIP concentration recovered in the lungs (i.e. 33 to 237 mg/L) upon inhaled CIP delivery (Cipolla et al., 2016; Stass et al., 2013).

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).

2.2.8. Statistical analysis

All experiments were performed in at least three replicates and the results were presented as mean ± standard deviation. The statistical analysis was performed using GraphPad Prism software (USA). All p-values were two-sided and considered significant at $p \leq 0.05$, unless stated otherwise. The details of the statistical analysis were provided in the Supplementary Materials.

3. Results and discussion

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. S2 of the Supplementary Materials 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. (2015).

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.

3.2. 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. 1A). 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 (Konno and Taylor, 2006).

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 ($> 96 \text{ h}$) 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. 1A). 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. 1B). 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 (Yu and Hadinoto, 2015).

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. 1B). 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 (Rennard et al., 1986). 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. S3 of the Supplementary Materials). 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. Mucus permeability

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. 2A). 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.

As both DPIs of the CIP nanoplex and the native CIP were previously established in Section 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.

3.3.2. 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. 2B). 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. 2B).

A closer look at the data showed 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 (Fig. S3 of the Supplementary Materials). 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 (Fig. S4 of the Supplementary Materials).

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. 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. 3A). 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.

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. 3B). 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.

3.4. Antibacterial efficacy in the presence of sputum

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 (Brazas and Hancock, 2005) (Fig. 4A). 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. 4A). 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. 4B).

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. 5). 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.

3.4.2. 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. 6A). 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. 6A).

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. 6B). 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. 6C). The lower Live/Dead ratio signified the lower residual bacterial cell count resulted from the CIP nanoplex treatment ($p \leq 0.001$).

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. 7A). 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. S5 of the Supplementary Materials.

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. 7B), 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. S4 of the Supplementary Materials).

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

474 therapeutic advantages afforded by the DPI of CIP nanoplex must be evaluated in the future from *in vivo* studies and
475 subsequently in human clinical trials.

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568

569

Figure captions

- Fig. 1 (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) dissolution rates of the DPI of CIP nanoplex and DPI of native CIP prepared with mannitol as the excipient in the normal ASM
- Fig. 2 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)
- Fig. 3 MLR ratios of (A) DPI of CIP nanoplex and (B) DPI of native CIP in normal and NCFB sputum (NS = not statistically significant)
- Fig. 4 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 (* $p \leq 0.05$)
- Fig. 5 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 (* $p \leq 0.05$, ** $p \leq 0.01$)
- Fig. 6 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 (** $p \leq 0.01$, *** $p \leq 0.001$)
- Fig. 7 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)