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2019

Tran, T.-T., Yu, H., Vidaillac, C., Lim, A. Y. H., Abisheganaden, J. A., Chotirmall, S. H., & Hadinoto, K. (2019). An evaluation of inhaled antibiotic liposome versus antibiotic nanoplex in controlling infection in bronchiectasis. *International Journal of Pharmaceutics*, 559, 382–392. doi:10.1016/j.ijpharm.2019.01.062

<https://hdl.handle.net/10356/144358>

<https://doi.org/10.1016/j.ijpharm.2019.01.062>

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**An Evaluation of Inhaled Antibiotic Liposome versus Antibiotic Nanoplex
in Controlling Infection in Bronchiectasis**

The-Thien Tran¹, Hong Yu¹, Celine Vidaillac², Albert Y.H. Lim³, John A. Abisheganaden³, Sanjay H. Chotirmall²,
Kunn Hadinoto^{1*}

¹School of Chemical and Biomedical Engineering, Nanyang Technological University, Singapore 637459

²Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore

³Department of Respiratory and Critical Care Medicine, Tan Tock Seng Hospital, Singapore

*To whom correspondence should be addressed:

Tel.: (65) 6514 8381, Fax: (65) 6794 7553, E-mail: kunnong@ntu.edu.sg

Abstract

Inhaled antibiotic nanoparticles have emerged as an effective strategy to control infection in bronchiectasis lung owed to their mucus-penetrating ability. Using ciprofloxacin (CIP) as the model antibiotic, we evaluated dry powder inhaler (DPI) formulations of two classes of antibiotic nanoparticles (i.e. liposome and nanoplex) in their (1) physical characteristics (i.e. size, zeta potential, CIP payload, preparation efficiency), (2) dissolution in artificial sputum medium, (3) *ex vivo* mucus permeability, (4) antimicrobial activity against *Pseudomonas aeruginosa* in mucus, (5) cytotoxicity towards human lung epithelium cells, and (6) *in vitro* aerosolization efficiency. The results showed that the CIP nanoplex exhibited fast dissolution with CIP supersaturation generation, in contrast to the slower release of the liposome (80 versus 30% dissolution after 1h). Both nanoparticles readily overcame the mucus barrier attributed to their nanosize and mucus-inert surface (50% permeation after 1h), leading to their similarly high antipseudomonal activity. The CIP liposome, however, possessed much lower CIP payload than the nanoplex (84% versus 3.5%), resulting in high lipid contents in its DPI formulation that led to higher cytotoxicity and lower aerosolization efficiency. The CIP nanoplex thus represented a superior formulation owed to its simpler preparation, higher CIP payload hence lower dosage, better aerosolization, and lower cytotoxicity.

Keywords: ciprofloxacin; liposomes; nanoparticle complex; dry powder inhaler; bronchiectasis

45 **List of abbreviations***

46	AA	acetic acid
47	ABSM	artificial bronchiectasis sputum medium
48	C _{Sat}	saturation solubility of the native CIP
49	CE	complexation efficiency
50	CF	cystic fibrosis
51	CFU	colony forming units
52	CIP	ciprofloxacin
53	[CIP]	ciprofloxacin concentration
54	[CIP] _{eq}	equivalent ciprofloxacin concentration
55	d _A	aerodynamic diameter of the inhalable powders
56	d _G	geometric diameter of the inhalable powders
57	DLS	dynamic light scattering
58	DMSO	dimethyl sulfoxide
59	DMEM	Dulbecco's modified Eagle's medium
60	DNA	deoxyribonucleic acid
61	DPI	dry powder inhaler
62	DPTA	diethylenetriamine pentaacetic acid
63	DXT	sodium dextran sulfate
64	ED	emitted dose
65	EDTA	ethylenediaminetetraacetic acid
66	EE	encapsulation efficiency
67	FEV1	first forced expiratory volume
68	FPF	fine particle fraction
69	HPLC	high performance liquid chromatography
70	HRCT	high resolution computed tomography
71	IP	induction port
72	M _{CIP/liposome}	molar ratio of CIP to liposome
73	MEM	Minimum Essential Medium with Eagle's salt
74	MHB	Mueller Hinton broth
75	MIC	minimum inhibitory concentration
76	MMAD	mass median aerodynamic diameter
77	NCFB	non-cystic fibrosis bronchiectasis
78	NGI	next generation impactor
79	OD ₆₀₀ , OD ₅₀₀	optical density at 600 and 500 nm
80	PBS	phosphate buffered saline
81	PDI	polydispersity index
82	PET	powder entrainment tube
83	PS	pre-separator
84	SFD	spray freeze drying
85	UV-Vis	ultraviolet visible spectroscopy

86 *To appear as footnotes

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 (Chalmers and Sethi, 2017). 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 (Chotirmall and Chalmers, 2018a; Weycker et al., 2017). Therefore, the primary goal of bronchiectasis management therapy has been to reduce the frequency of exacerbations and slow down the disease progression (Chalmers et al., 2015; McShane et al., 2013).

Inhaled antibiotics has been proposed to have a role in bronchiectasis management as a long-term intermittent therapy to reduce the airway bacterial burden (Brodt et al., 2014; Smiths et al., 2018), particularly from *Pseudomonas aeruginosa*, where its colonization has been associated with increased frequency of exacerbations and mortality (Dhand, 2018; Finch et al., 2015; Wilson et al., 2016). 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 (Agent and Parrott, 2015).

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 (Chalmers and Chotirmall, 2018; Sugianto and Chan, 2016). 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 (Grimwood et al., 2014; Justo et al., 2013). At present, two inhaled CIP products, i.e. (1) nebulized liposome encapsulating CIP (ORBIT-3 and 4 trials) (Haworth et al., 2017) and (2) DPI of native CIP (RESPIRE 1 and 2 trials) (Aksamit et al., 2018; De Soyza et al., 2018) 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 (Chotirmall and Chalmers, 2018b). 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 (Weers, 2015). 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 (Bos et al., 2017). The problem is compounded by the rapid systemic absorption of those antibiotic molecules that have overcome the mucus barrier (Gontijo et al., 2014). 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 (Maselli et al., 2017).

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 (Cheow et al., 2010; Hadinoto and Cheow, 2014; Martin and Finlay, 2018; Schneider et al., 2017). 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) (Cipolla et al., 2016; Cipolla et al., 2015). 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 (Ong et al., 2014). Besides CIP liposome, another example of CIP nanoparticle delivery system intended for bronchiectasis therapy was in the form of CIP-encapsulated polymer nanoparticles (Tureli et al., 2017; Tureli et al., 2016).

Our group previously developed an alternative formulation of antibiotic nanoparticles in the form of CIP nanoparticle complex (or nanoplex in short) (Cheow and Hadinoto, 2012). 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 (Tran et al., 2018). Moreover, the CIP nanoplex was shown to exhibit similar a cytotoxicity profile towards human lung epithelium cells as the native CIP, hence denoting its low risk of toxicity (Tran et al., 2018).

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 (De Soya and Aksamit, 2016). 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 (Alhaddad et al., 2015). In place of spray drying used in Tran et al. (2018), 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 (Adi et al., 2010; Lee et al., 2009) - was used as the drying adjuvant.

2. Materials and Methods

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 (KH₂PO₄) 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 (Chotirmall et al., 2010). 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-glutamin (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.

2.2. Methods

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). 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 (14,000×g 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.

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). 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 (14,000×g 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 14,000×g 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 $\approx$ 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.

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). 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°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°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..

2.2.4. Aerosolization efficiency

The aerosolization efficiency of the DPI formulations was characterized following Yu et al. (2016) 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 (Buttini et al., 2016), 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 to 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 (Darquenne, 2012).

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 to 7, was taken from the 50% point of the cumulative d_A distribution curve.

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 (Kirchner et al., 2012). The ABSM was prepared by supplementing the artificial sputum medium's recipe presented in Yang et al. (2011) with NaCl and KCl at concentrations of 115mM and 130mM, respectively, which were representative of the salt concentrations present in bronchiectasis sputum (Jayaraman et al., 2001). 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 timepoints over 96 h, 400 μL aliquot was withdrawn, centrifuged (14,000 $\times g$ 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 ($C_{\text{Sat}} = 263 \pm 3 \mu\text{g/mL}$). 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.

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) and Tran et al. (2018). Briefly, 100 μ L fresh undiluted sputum from healthy donors was placed in the apical compartment of a Transwell[®] polycarbonate membrane inserts (12-mm diameter with 3- μ m 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 was 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.

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. S1 of the Supplementary Materials. 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 μ g/mL that was significantly higher than the minimum inhibitory concentrations (MIC) of the PAO1 and clinical strains (i.e. 0.03 to 0.125 μ g/mL).

After 1 h incubation, 50 μ L aliquot of the bacterial cell suspension was withdrawn from the basolateral compartment and diluted in 450 μ L MHB. The aliquot was subsequently filtered using 0.2- μ m 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). 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.

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 to 250 µg/mL and 3.125 to 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 to 237 mg/L) upon inhaled CIP delivery (Cipolla et al., 2016; Stass et al., 2013).

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 to 250 μ g/mL and 3.125 to 100 μ 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 amount of residual solvents was determined by gravimetric analysis as detailed in Section 2 of the Supplementary Materials.

2.2.9. 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.

3. Results and discussion

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 to 300 nm (Suk et al., 2009). 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 (Cipolla et al., 2016).

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 (Ingvarsson et al., 2011). 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%) (Cipolla et al., 2016).

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 S1A of the Supplementary Materials). 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 re-hydration 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.

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 S1B of the Supplementary Materials). 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). 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.

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 Figs. 1A and 1B, 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 ± 18 μm , 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 (Yu et al., 2016). 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. 1C). The ED values were comparable to the ED values of commercial DPI products (Abdelrahim, 2010). 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. 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*. On this note, the raw data of the amount of powders deposited in each stage of the NGI were presented in Table S2 of the Supplementary Materials.

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 (Franze et al., 2018). The FPF

of the DPI of CIP liposome could likely be improved by having an increased presence of D-Mannitol ($>> 50\%$ w/w) to mask the hygroscopicity of the lipids as pursued in Sweeney et al. (2005), 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.

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. 1C). 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.

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. 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. 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 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.

In addition to its fast dissolution, as reported earlier by Cheow and Hadinoto (2012), 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 $(8.8 \pm 0.3) \times C_{\text{Sat}}$ (≈ 2.3 mg/mL) (Fig. S2 of the Supplementary Materials). The solubility of the CIP nanoplex was slightly lower at $(6.7 \pm 0.2) \times C_{\text{Sat}}$ (≈ 1.8 mg/mL) in its DPI form, which was likely caused by the nanoplex fusion upon freeze drying, resulting in reduced surface areas for dissolution.

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 3.5.

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. 3A). 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. 3B). 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.

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 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.

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. 4A). 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. 4B). The bactericidal activities against the biofilm were also found to be independent of the bacterial strains.

Similar antibiofilm activities were observed when the study was repeated in the presence of NCFB sputum ($n = 2$) (Fig. 4C). 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.

3.6. Cytotoxicity towards A549 and 16HBE14o- cells

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. 5A). 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 $[\text{CIP}] = 250 \mu\text{g/mL}$ (Tran et al., 2018). 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.

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. S3 of the Supplementary Materials). 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 $[CIP] = 12.5$ to $250 \mu\text{g/mL}$, the amounts of the residual solvents were calculated to be in the range of 9 to $187 \mu\text{g/mL}$. 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. S4 of the Supplementary Materials). 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 $[CIP]_{\text{eq}} = 12.5 \mu\text{g/mL}$ to $70 \pm 2\%$ at $[CIP]_{\text{eq}} = 250 \mu\text{g/mL}$ indicating the adverse effects of the increased presence of liposomes on the cell viability (Fig. 5B). In contrast, the cell survivals upon treatment with the DPI of blank nanoplex remained at almost 100% at the highest $[CIP]_{\text{eq}}$ investigated (Fig. 5B). 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); (Roursgaard et al., 2016) 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 $100 \mu\text{g/mL}$, the amount of liposome present in the DPI of CIP liposome formulation was very high at approximately 3 mg/mL , resulting in its cytotoxicity.

3.6.2. 16HBE14o- cells

In the lower range of CIP concentration investigated (i.e. $3.125 \leq [CIP] \leq 25 \mu\text{g/mL}$), 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 $[CIP] = 3.125$ and $25 \mu\text{g/mL}$, 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. 6A). 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 $3.125 \leq [CIP]_{\text{eq}} \leq 25 \mu\text{g/mL}$ were about 50-60%, which were significantly lower than the $> 90\%$ cell survivals upon treatment by the DPI of blank nanoplex (Fig. 6B).

The cytotoxicity tests of the spray-freeze-dried D-Mannitol and the residual solvents (at 2 to $75 \mu\text{g/mL}$) revealed their non-cytotoxicity towards the 16HBE14o- cells (Figs. S3 and S4 of the Supplementary Materials, 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.

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). 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. 6A), 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.

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.

5. Acknowledgement

This research is supported by (1) LKCMedicine-SCBE Collaborative Grant (CG-04/16) (S.H.C and K.H.O), (2) LKCMedicine Start-Up Grant (S.H.C) both from Nanyang Technological University, and (3) the Singapore Ministry of Health's National Medical Research Council under its Transition Award (NMRC/TA/0048/2016) (S.H.C). The authors would like to acknowledge The Academic Respiratory Initiative for Pulmonary Health (TARIPH) for the collaboration supports.

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Figure captions

- Fig. 1 (A, B) SEM images of the DPIs of CIP nanoplex and CIP liposome prepared by SFD depicting large spherical porous morphology; (B) aerosolization efficiency of the DPIs of CIP nanoplex and CIP liposome as characterized by the ED, FPF, and MMAD
- Fig. 2 Dissolution time-profiles of the (A) aqueous suspensions and (B) DPI formulations of the CIP nanoplex and CIP liposome in the ABSM
- Fig. 3 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, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$)
- Fig. 4 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 NCFB sputum (n = 2) (NS = not statistically significant)
- Fig. 5 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, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$)
- Fig. 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 16HBE14o- cells (NS = not statistically significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$)

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Supplementary Material

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