Engineered antibiotic nanoparticles for inhaled anti-biofilm therapy

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**ENGINEERED ANTIBIOTIC NANOPARTICLES
FOR
INHALED ANTI-BIOFILM THERAPY**

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SCHOOL OF CHEMICAL AND BIOMEDICAL ENGINEERING

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**ENGINEERED ANTIBIOTIC NANOPARTICLES
FOR
INHALED ANTI-BIOFILM THERAPY**

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ABSTRACT

Biofilm, a sessile community of bacterial cells that is enclosed by a self-secreted matrix composed of an extracellular polymeric substance, is implicated in the chronic pulmonary infection of patients with impaired lung mucociliary clearance mechanism, such as cystic fibrosis and chronic obstructive pulmonary disease (COPD) patients. The higher tolerance of biofilms to antimicrobial agents (10–1,000 fold less susceptible to antimicrobial agents compared to its planktonic counterparts) necessitates a targeted and controlled delivery of antibiotics. To this end, engineered antibiotics nanoparticles for inhaled anti-biofilm therapy via the dry powder inhaler (DPI) platform are investigated in this dissertation.

First, *in vitro* investigations into the effects of antibiotic release profiles afforded by the encapsulation of antibiotics in nanoparticles on *E. coli* biofilm susceptibility shows that a biphasic extended release profile is necessary for maximum biofilm eradication. A high initial antibiotic concentration ensures that maximum biofilm cells were killed, while the subsequent slower extended release maintained an antibiotic concentration high enough to arrest biofilm proliferation. The initial maximum killing of biofilm cells is crucial as surviving biofilm cells are less susceptible to antibiotics.

Second, lipid–polymer hybrid nanoparticles which combine the advantages of (i) the biofilm targeting ability of liposomes and (ii) the structural integrity and stability of polymeric nanoparticles, has been shown to result in higher antibiotic encapsulation efficiency compared to polymeric nanoparticles alone, hence are able to provide a higher initial antibiotic concentration. Furthermore, hybrid nanoparticles have been found to demonstrate higher *in vitro* efficacy against biofilm of *P. aeruginosa* compared to non-hybrid nanoparticles. For these reasons, lipid–polymer hybrid nanoparticles can be utilized as a vehicle for inhaled anti-biofilm therapy. However,

the encapsulation of antibiotics in lipid–polymer hybrid nanoparticles must be tailored to the type of antibiotic and lipid used, as experiments performed show that ionic interactions between oppositely charged drug molecules and lipids prevent nanoparticle formation.

Third, a new drug-polyelectrolyte nanoplexation method developed in the present work has resulted in antibiotic nanoparticles with high drug loading (up to 80%). The simple method, which only involves the mixing of two aqueous solutions of oppositely charged antibiotic and polyelectrolyte, has also been applied in the production of amorphous nanoparticles of a sparingly soluble antibiotic. The resultant amorphous antibiotic nanoparticle exhibits (i) faster dissolution rate, (ii) enhanced saturation solubility, and (iii) higher achievable supersaturation level compared to those of the raw crystalline drug.

Finally, the engineered antibiotic nanoparticles can be transformed into inhalable dry powder nano-aggregates for pulmonary delivery by means of the spray drying or spray-freeze-drying (SFD) technique, both of which have been investigated in the present work. While the spray drying method to prepare dry powder nano-aggregates is more rapid and straightforward, the SFD is more suitable for the processing of thermosensitive nanoparticles due to the avoidance of a high operating temperature. Adjuvant selection in SFD is crucial as the adjuvant dictates the resulting nanoparticle-adjuvant structures, which in turn influence the aerodynamic diameter and aqueous re-dispersibility of the dry-powder aggregates.

Overall, this dissertation contributes to future works on successful inhaled anti-biofilm therapy, based on (i) antibiotic release profile tailored for optimal anti-biofilm therapy, (ii) antibiotic-loaded lipid–polymer hybrid nanoparticles with higher anti-biofilm efficacy, and (iii) the transformation of nanoparticles into inhalable dry powder nano-aggregates. Furthermore, a simple preparation method to produce antibiotic nanoparticles has been developed, for both water-soluble and sparingly soluble antibiotics, offering the possibility of further functionalization of the antibiotic nanoparticles.

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ABBREVIATIONS

AA	Acetic acid	Nanoplex	Drug-polyelectrolyte complex nanoparticle
API	Active pharmaceutical ingredient	NGI	Next generation pharmaceutical impactor
CE	Complexation efficiency	NP	Nanoparticles
CF	Cystic fibrosis	NPC	Nanoprecipitation
CFU	Colony forming unit	OD	Optical density
CI	Carr's compressibility index	OFX	Ofloxacin
CIP	Ciprofloxacin	PAH	Poly(allylamine hydrochloride)
COPD	Chronic obstructive pulmonary disease	PBS	Phosphate buffer saline
DCM	Dichloromethane	PC	Phosphatidylcholine
DESE	Double-emulsification-solvent-evaporation	PCL	Poly(caprolactone)
DI	Deionized	PCS	Photon correlation spectroscopy
DPI	Dry powder inhaler	PE	Polyelectrolyte
DSC	Differential scanning calorimetry	PEG	Poly(ethylene glycol)
DXT	Dextran sulfate	PLGA	Poly(DL-lactide-co-glycolide)
ED	Emitted dose	PSS	Poly(styrene sulfonate)
EPS	Extracellular polymeric substance	PSS	Pre-separator
ESE	Emulsification-solvent-evaporation	PVA	Poly(vinyl alcohol)
FE-SEM	Field emission scanning electron microscope	PXRD	Powder X-ray diffraction
FPF	Fine particle fraction	RHL	Rhamnolipid
HPMC	Hydroxypropylmethylcellulose	SA	Stearylamine
IP	Induction port	SEM	Scanning electron microscope
LEV	Levofloxacin	SFD	Spray-freeze-drying
MBIC	Minimum biofilm inhibitory concentration	SPET	Standardized powder entrainment tube
MHB	Mueller–Hinton broth	TGA	Thermogravimetric analysis
MIC	Minimum inhibitory concentration	TOB	Tobramycin
MMAD	Mass median aerodynamic diameter	TPGS	Vitamine E-PEG 1000

NOMENCLATURE

$C_{s,\infty}$	Solubility of drug particles having radius $r = \infty$
$C_{s,r}$	Solubility of drug particles having radius r
d_A	Aerodynamic diameter
d_G	Geometric diameter
K	Absolute temperature
K_B	Boltzmann constant
$\log P$	Octanol/water partition coefficient
N_A	Avogadro's number
N/M	Nanoparticle to mannitol concentration ratio
N/P	Nanoparticle to PVA concentration ratio
pK_a	Dissociation constant
R	Gas constant
r	Particle radius
$R_{CIP/DXT}$	Charge ratio of CIP to DXT
$R_{drug/PE}$	Charge ratio of drug to polyelectrolyte
$R_{LEV/DXT}$	Charge ratio of LEV to DXT
$R_{OFX/DXT}$	Charge ratio of OFX to DXT
S_f	Final nanoparticle size after re-dispersion
S_i	Initial nanoparticle size before SFD
S_o	Aqueous molar solubility
T	Temperature
T_g	Glass transition temperature
V_m	Drug molar volume
W_{PC}	Mass of PC
W_{PLGA}	Mass of PLGA
γ	Interfacial tension
ρ_e	Effective density
ρ_s	Standard density
ρ_{tap}	Tap density

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3. W.S. Cheow and K. Hadinoto. “Activity of levofloxacin loaded polymeric nanoparticles against biofilm of *E. coli*”, 3rd East Asian Pacific Student Workshop on Nano-Biomedical Engineering, Singapore. December 2009. (Oral)
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Chapter 1

Introduction

1.1 Biofilms and the need for inhaled antibiotics

Biofilm, a sessile community of bacterial cells that is enclosed by a self-secreted matrix composed of an extracellular polymeric substance (EPS), is the predominant mode of growth. Biofilm renders bacterial cells 10–1,000 fold less susceptible to antimicrobial agents compared to their planktonic cell counterparts¹. The main defensive barrier of the human lungs, i.e. the phagocytic defense mechanism, is incapable of removing the biofilm cells protected by the EPS matrix. Therefore, pulmonary biofilm colonization causes persistent infections and inflammations in the airways that eventually lead to the deterioration of the lung function if they are not properly treated.

Individuals with an impaired mucociliary clearance in their respiratory systems are vulnerable to chronic biofilm infections due to the consequential mucus build-up in their respiratory airways that provides a nutrient-rich environment ideal for biofilm growth. The pulmonary disease that is typically associated with biofilm infections is bronchiectasis, which is an abnormal irreversible dilatation of bronchi and bronchioles due to the recurring infections and inflammations.

Bronchiectasis includes congenital bronchiectasis (e.g. cystic fibrosis) whose occurrence is limited to a small population, and acquired bronchiectasis, which is prevalent among individuals suffering from chronic obstructive pulmonary disease (COPD). COPD is the fourth leading cause of death globally², where 50% of adults with moderate to severe COPD are found to exhibit bronchiectasis symptoms³. Unfortunately, the high biofilm tolerance towards antibiotics renders the current

antibiotic therapy for bronchiectasis ineffective in curing the disease completely. Antibiotic therapies for moderate to severe bronchiectasis are therefore primarily aimed to alleviate the symptoms and to minimize the exacerbations³.

The high tolerance of the biofilm cells towards antimicrobial agents, particularly antibiotics, has been attributed to various causes, such as (i) the non-growing state of a majority of the biofilm cells rendering them less susceptible to certain antibiotics (e.g. β -lactam), (ii) the shielding of the biofilm cells from antibiotics by the EPS matrix hence allowing the bacterial cells to release their adaptive stress responses in time, and (iii) the existence of subpopulation persister cells in biofilm colonies that remain invulnerable regardless of the antibiotic concentration and its duration of exposure⁴.

With the exception of fluoroquinolones, most antibiotics delivered by the oral route are ineffective against respiratory tract bacterial infections⁵⁻⁶, requiring a higher systemic bioavailability as provided by intravenous delivery of antibiotics. The potential of systemic toxicity as a result of high doses and prolonged concentration of antibiotics encouraged the development of antibiotic delivery through the pulmonary route.

1.2 Antibiotic nanoparticles

Currently, a majority of inhaled antibiotics for respiratory tract infections are delivered by nebulization. The readily available aqueous solutions of soluble antibiotics and suspensions of insoluble antibiotics intended for intravenous delivery are frequently nebulized using jet and ultrasonic nebulizers⁷. The nebulization of TOBI, a solution of the antibiotic tobramycin is the most successful application of inhaled antibiotics, and remains the only Federal Drug Administration-approved antibiotics for pulmonary delivery.

However, inhaling antibiotics in the solution form requires frequent dosing as the small antibiotic molecules are rapidly absorbed from the lungs, and results in the eradication of bacterial cells only at the periphery of the biofilm but not the cells embedded deeper in the biofilm^{1, 8}. Controlled formulations from antibiotic-loaded carriers, on the other hand, will provide a constant supply of antibiotic to ensure the antibiotic concentration in the local vicinity of the biofilm remains above the minimum biofilm inhibitory concentration (MBIC). Furthermore, encapsulation of antibiotic in

carriers offers the possibility of tailoring the antibiotic release profile to that required for optimum biofilm eradication. The optimum release profile however, is not universal but instead depends on the carrier type.

While encapsulation of drugs in micro-sized carriers facilitates sustained drug deliveries through the oral and intravenous routes, similar micron sized particles will simply be removed from the lungs by the alveolar macrophages in less than 12 h⁹. In contrast, the pulmonary phagocytic clearance mechanism is largely unable to efficiently remove nano-sized particles¹⁰, making sustained release formulations in the nano-scale an ideal candidate for maintaining therapeutic antibiotic concentrations in the lungs. Nevertheless, nanoparticles are ultimately removed from the lungs by phagocytosis, similarly by alveolar macrophages, albeit at a rate much slower than micron sized particles⁹.

The application of polymeric nanoparticles has been favorably demonstrated in protein, gene and drug encapsulation due to their potential for high encapsulation efficiency, adapted release, stability during long-term storage and structural integrity. Utilizing biodegradable and biocompatible polymers such as poly(lactic-*co*-glycolic acid) (PLGA) and poly(caprolactone) (PCL), nanoparticles can be prepared by various methods such as nanoprecipitation, emulsification-solvent-evaporation or emulsification-solvent displacement¹¹. Besides reducing the dosing frequency required, encapsulation of antibiotics in the polymeric nanoparticles will prevent the binding of antibiotics to the sputum before reaching the biofilms. The hindered penetration through sputum due to interaction between antibiotic and sputum content is well documented for various antibiotics commonly used in the treatment of lung biofilm infections¹²⁻¹⁵.

In addition to encapsulating antibiotics in nanoparticles, antibiotics can also be converted into nanoparticles, i.e. instead of the antibiotic being dispersed in the nano-carrier, the nanoparticle is predominantly made up of the antibiotic itself (see Fig. 1.1). Further to the possibility of surface functionalization of the antibiotic nanoparticle, the formulation of sparingly soluble antibiotic into antibiotic nanoparticle is particularly beneficial in terms of enhancing the bioavailability of these antibiotic with low aqueous solubilities.

The antibiotics, whether in the form of antibiotic nanoparticles or encapsulated in polymeric nano-carriers, are ideally delivered by the dry powder inhaler (DPI) platform attributed to the advantages of the DPI compared to nebulization, in terms of (i) higher delivery efficiency, (ii) shorter administration time, (iii) higher stability, and (iv) higher portability¹⁶.

Figure 1.1: Antibiotic-loaded nanoparticle versus antibiotic nanoparticle.

1.3 Aims and Objectives

This dissertation aims to engineer antibiotic-loaded nanoparticles and antibiotic nanoparticles for inhaled anti-biofilm therapy. Specifically, the objectives are to:

- (i) Determine the optimum antibiotic release profile for anti-biofilm therapy
- (ii) Investigate the physical characteristics and anti-biofilm efficacy of (a) antibiotic-loaded polymeric nanoparticles and (b) antibiotic-loaded polymeric nanoparticles modified with lipids (i.e. lipid-polymer hybrid nanoparticles)
- (iii) Develop an antibiotic nanoparticle preparation method that is simple and rapid for both water-soluble and sparingly water soluble antibiotics
- (iv) Examine the feasibility of transforming antibiotic-loaded nanoparticles into nano-aggregates for dry powder inhaler delivery

1.4 Thesis organization

The present Introduction chapter lays down the background information and leads to the Literature Review in **Chapter 2**, where current research status relevant to the thesis objectives stated are presented. **Chapter 3** details the Methods and Materials commonly applied in the later chapters.

Antibiotic-loaded polymeric nanoparticles are prepared and the effect of the antibiotic release profiles on the efficacy against biofilm of *E. coli* is presented in **Chapter 4**. In **Chapter 5**, the standard polymeric nanoparticle preparation methods are modified, resulting in a successful attempt to double the amount of antibiotic loaded in the polymeric nanoparticles (i.e. drug loading) used in Chapter 4. The optimal nanoparticle identified incorporates lipid as part of its formulation, resulting in lipid–polymer hybrid nanoparticles. Furthermore, the feasibility of transforming the hybrid nanoparticles into inhalable dry powder using the spray drying technique is demonstrated.

The anti-biofilm efficacy of the lipid–polymer hybrid nanoparticles is compared to that of non-hybrid nanoparticles in **Chapter 6**, for hybrid nanoparticles in both suspension and dry powder form. The next chapter, **Chapter 7**, expands upon the use of lipid in nanoparticle formulation, where three other antibiotics are similarly encapsulated in the lipid–polymer hybrid nanoparticles. The effect of lipid inclusion is discussed in terms of the physical properties of the antibiotics investigated.

While the encapsulation of antibiotic in both polymeric and hybrid nanoparticles is feasible, the relatively low drug loading encourages the development of a polymer-free antibiotic nanoparticle, the results of which is presented in **Chapter 8**. The technique developed, drug-polyelectrolyte nanoplexation, is further applied in **Chapter 9** specifically to a sparingly soluble antibiotic, where it is shown that the nanoplexation method results in stable amorphous antibiotic nanoparticle with enhanced dissolution rate and saturation solubility.

In **Chapter 10**, the feasibility of transforming thermosensitive antibiotic-loaded nanoparticles into inhalable dry powder nano-aggregates by the spray-freeze-drying technique is investigated. Finally, **Chapter 11** summarizes the important findings and presents a conclusion, as well as lays down recommendations for worthwhile future works.

Chapter 2

Literature Review

2.1 Importance of the antibiotic release profile

Biofilm susceptibility towards antibiotics is typically characterized by the minimum biofilm inhibitory concentration (MBIC), which is defined as the minimum antibiotic concentration needed to eradicate a majority of the non-persister cells and to inhibit the visible biofilm growth initiated by the surviving persister cells¹⁷. The conventional wisdom for an effective antibiotic therapy is to expose the biofilm cells to antibiotic concentrations that are equal or above the MBIC value. In this regard, bacterial cells exposed to a sub-inhibitory antibiotic concentration have been found to ramp up their biofilm mode of growth as a defensive response to the antibiotic¹⁸. Furthermore, non-persister cells have been found to self-transform into persister cells upon an exposure to antibiotics as part of their adaptive defense mechanism¹⁹.

The defensive response of the biofilm cells upon their exposure to antibiotics is therefore highly dynamic as the biofilm cells can adaptively react to promote their survival. Consequently, despite their similar overall dose, a constant antibiotic concentration over time above the MBIC value may not yield the same antibacterial efficacy as that obtained from a gradually decreasing or increasing antibiotic concentration that is also maintained above the MBIC value. Nevertheless, several studies have reported that a higher antibacterial efficacy is obtained from a sustained antibiotic release profile as a result of encapsulated antibiotics, compared to that obtained from a faster release profile in the free-drug formulations²⁰⁻²². The superiority of the sustained release profile observed in these studies, however, is attributed more to the immediate clearance of the antibiotic in the free-drug formulations rather than

the effect of having a sustained antibiotic concentration on the biofilm defensive response itself.

For antibiotic-loaded nanoparticle formulations, however, the role of the immediate antibiotic clearance becomes less significant as the antibiotic is released locally inside the biofilm matrix. The application of encapsulated antibiotics to the treatment of biofilm-associated diseases is a relatively new development, with reports of such studies available only in very recent years. The *in vivo* efficacy of liposomal amikacin was investigated by Meers *et al.*²¹, while Halwani *et al.* studied the antimicrobial efficacy of liposomal gentamicin and tobramycin against biofilm of *P. aeruginosa*²³⁻²⁴. However, the effects of antibiotics release profiles afforded by the encapsulation of antibiotics in nanoparticles on the biofilm susceptibility remains to be investigated.

2.2 Lipid–polymer hybrid nanoparticles

For anti-biofilm therapy, liposomes are beneficial as carriers because liposomes have been found to preferentially adsorb onto biofilm surfaces²⁵⁻²⁶ and can interact closely with bacterial cells by fusing into bacterial cell membranes²⁷. However, liposomes are not physically robust as they are built resembling biological membranes (i.e. composed of phospholipid bilayers), which renders their effective delivery to the lung in a clinical setting a highly challenging task, due to potential drug leakage, temperature sensitivity, and short shelf-life²⁸. On the other hand, solid lipid nanoparticles, although similarly possess a lipidic surface, are limited in their potential as antibiotic delivery vehicles because they are only applicable to drugs that are soluble in lipid²⁹.

In this regard, lipid–polymer hybrid nanoparticles (Fig. 2.1), where antibiotic-loaded polymeric nanoparticle core is coated with lipid layers represent a promising anti-biofilm formulation as the hybrid nanoparticles possess physical robustness afforded by the polymer matrix, while at the same time they possess the high biofilm compatibility characteristics of liposomes. Moreover, owing to their robustness, hybrid nanoparticles can withstand the physical transformation from their aqueous suspension state into the pharmaceutical solid dosage form, hence enabling their delivery by DPI.

In addition to the physical robustness afforded by the polymeric core, the presence of a lipid coat surrounding the polymeric nanoparticles limits outward transport of water-soluble drugs, hence potentially increasing the drug encapsulation efficiency, while the fragile lipid layer is conferred structural integrity. Thus, in a well-designed hybrid lipid–polymer nanoparticle, the polymeric core functions to encapsulate either water or oil-soluble drugs and to provide robust structures, whereas the external lipid coat functions as (i) a biocompatible shield, (ii) a template for surface modifications, and (iii) a barrier preventing the fast leakage of water-soluble drugs.

Figure 2.1: Lipid–polymer hybrid nanoparticles with lipid coat enveloping the polymeric nanoparticle core.

Currently, only three methods have been reported for the preparation of lipid–polymer hybrid nanoparticles. The first method is a two-step procedure involving co-incubation of separately pre-formed nanoparticles and lipid vesicles (or lipid films)³⁰⁻³¹. The method was initially developed for the purpose of preparing solid-supported lipid bilayers as an artificial cell membrane. In this method, polymeric nanoparticles and lipid vesicles interact electrostatically resulting in the formation of a lipid layer on the nanoparticle surface. However, this method of preparation inadvertently reduces the drug encapsulation efficiency due to the incubation step during which the encapsulated drug, especially the highly water-soluble ones, may have leaked out before a lipid coat is formed on the nanoparticle surface.

The second method is a single-step method, where a modified nanoprecipitation method is employed to prepare hybrid nanoparticles in a fast and reproducible manner³²⁻³⁵. In this method, the polymer and drug are first dissolved in a water-miscible solvent, and subsequently added to an aqueous phase containing lipids, while

under sonication. The diffusion of the solvent into the aqueous phase co-precipitates the drug and polymer into nanoparticles, the surface onto which the lipids self-assemble. While simultaneously elegant and rapid, the applicability of this method is limited, in that the method only allows for the encapsulation of water-insoluble drugs that are soluble in the water-miscible solvent (usually acetone or acetonitrile).

The third method is based on an emulsification method with lipids replacing traditional surfactants, such as PVA, as the stabilizer³⁶⁻³⁷. In this method, the lipid is first dissolved in a water-immiscible solvent (i.e. oil phase) together with the polymer and the drug. When the oil phase is emulsified in an aqueous phase, an oil-in-water emulsion is formed, where the hydrophobic tails of the lipids self-assemble and attach onto the polymeric core, whereas the hydrophilic heads extend outwards towards the aqueous phase, thus effectively forming lipid-coated polymeric core (i.e. hybrid nanoparticles). To date, only water-insoluble drug, Paclitaxel, has been encapsulated in hybrid nanoparticles prepared by the emulsification-based method³⁷. Consequently, the potential of employing the emulsification-based method for encapsulating different drugs has yet to be tested.

2.3 Antibiotic nanoparticle by nanoplexation

In addition to encapsulating antibiotics in polymeric or hybrid nanoparticles, antibiotics can also be converted into nanoparticles. The surface functionalization of the antibiotic nanoparticles, for example with a thin polymeric shell, provides a barrier through which antibiotic release can be controlled. However, the intricacies associated with the existing drug nanoparticles preparation methods are believed to play a role in the limited employment of drug nanoparticles in smart drug delivery systems, such as the one envisioned for the anti-biofilm therapy. Therefore, a simpler preparation method of antibiotic nanoparticles is desired.

Recognizing that a majority of antibiotics are amphiphilic and soluble in acid or basic solutions³⁸, antibiotic nanoparticles can be prepared via a self-assembly amphiphile-polyelectrolyte complexation process. The field of amphiphile-polyelectrolyte complexation is well established with a wide range of applications from waste-water treatment to personal care products and pharmaceuticals³⁹. A majority of studies on the complexation of amphiphilic drugs with polyelectrolyte (PE),

however, tend to focus on the extent of drug adsorption onto the PE chains without specific practical drug delivery applications⁴⁰⁻⁴¹.

Only recently, few studies began to utilize the amphiphile-PE complexation principle to produce drug nanoparticles⁴²⁻⁴³. The active pharmaceutical ingredient (API) used in Sun *et al.*⁴², however, was a polysaccharide (i.e. enoxaparin), not small-molecule drugs, like antibiotics. Ranjan *et al.*⁴³ did prepare nanoparticles of a small-molecule drug using synthetic block copolymers as the PE. The drug loading, however, is low at $\approx 20\%$ (w/w) hence it does not represent a significant improvement over the conventional lipid or polymer-based nano-carriers.

In the amphiphile-PE complexation process, nanoparticle complex (or nanoplex in short) of amphiphilic drug is prepared by promoting electrostatic adsorptions of the ionized amphiphiles onto oppositely charged flexible PE chains in the presence of salt. Upon reaching a critical concentration, the amphiphile-adsorbed-on-PE molecules (i.e. complex) form aggregates via hydrophobic interactions of the amphiphiles resulting in the nanoplex formation⁴⁴. Unlike the existing methods, drug nanoparticle preparation by complexation is simple, fast, energy-minimal, solvent-free, and importantly highly efficient.

The principles behind drug nanoplex formation by the self-assembly amphiphile-PE complexation are presented in Fig. 2.2 using cationic antibiotics as an example. The antibiotics, which are uncharged at neutral pH, are dissolved in acidic solution to bring them to their ionized state. The drug solution is then added into PE solution, where cationic drug molecules electrostatically adsorb onto anionic PE chains. Upon reaching a certain critical concentration, whose value depends on the drug hydrophobicity, there are enough drug-adsorbed-on-PE molecules in the vicinity of each other, such that they form aggregates via hydrophobic interactions of the drug molecules. The coupling of the hydrophobic interactions with charge neutralization of the drug, as a result of the electrostatic interactions, leads to drug precipitation into the nanoplex, where the PE in effect functions as electrostatic stabilizers of the nanoplex.

Figure 2.2: Drug nanoplex formation via self-assembly amphiphile-PE complexation in the presence of salt.

2.3.1 Amorphous nanoplex of sparingly-soluble antibiotic

When the drug-polyelectrolyte nanoplexation method is extended to sparingly-soluble antibiotic, the combination of rapid precipitation due to drug-drug interaction and strong drug-polyelectrolyte interactions prevents the drug molecules from assembling into ordered crystalline structures. As a result, drug-polyelectrolyte nanoparticle complex in the form of amorphous antibiotic nanoparticle is produced.

Owed to their small size, drug nanoparticles possess high surface area to volume ratios resulting in higher dissolution rates. Concurrently, the nano-size increases the drug saturation solubility as theoretically predicted by the Ostwald–Freundlich equation (Eq. 2.1).

$$\ln \frac{C_{s,r}}{C_{s,\infty}} = \frac{2\gamma V_m}{rRT} \quad (2.1)$$

where $C_{s,r}$ and $C_{s,\infty}$ (g/L) are solubilities of drug particles having radius r and $r = \infty$ (m), respectively, γ is the interfacial tension between the drug particles and solubilisation medium (N/m), V_m is the drug molar volume (m^3/mol), R is the gas constant (8.314 J/mol·K), and T is the absolute temperature (K).

The amorphous form is known to possess higher saturation solubility than their crystalline counterpart⁴⁵. In addition, the amorphous form is known for its ability to generate a supersaturated drug solution upon dissolution, which is multi-folds higher than that of its crystalline equivalent⁴⁶. A number of *in vitro-in vivo* studies have reported that the supersaturated solution generated by the amorphous form observed in the *in vitro* experiment does lead to *in vivo* bioavailability enhancement provided that the supersaturation is maintained for a time period sufficient for absorption⁴⁷⁻⁴⁹. Stable amorphous drug nanoparticles are therefore viewed as highly promising as they combine the bioavailability enhancement characteristics of the amorphous form and the nanoscale formulation.

Attributed to their bioavailability enhancing potential, the development of the stabilized amorphous form of drug nanoparticles have been investigated⁵⁰⁻⁵⁴, particularly for highly water-insoluble drugs. Amorphous drug nanoparticles can be prepared using techniques similar to those employed in the production of API nanocrystals, i.e. the top-down approaches by size reduction (e.g. wet milling) and the bottom-up approaches by controlled precipitation (e.g. nanocrystallization), or by less conventional techniques, such as spray drying⁵⁰ and sonoprecipitation⁵². Table 2.1 shows a summary of the non-conventional techniques of preparing amorphous drug nanoparticles. A majority of the techniques requires the use of specialized equipment and machines, such as reactors, vortex mixers, and microfluidic channels. Furthermore, most of these approaches face the challenge of inhibiting crystal growth after nucleation, such that intricate processes requiring drug-specific stabilizers or solvents are needed⁵⁵. Moreover, the bottom-up approach involves heavy use of organic solvents necessitating multiple purification steps and a large amount of toxic waste. The drug–polyelectrolyte nanoplexation technique hence represents a simpler method to prepare not only amorphous antibiotic nanoparticles, but also amorphous nanoparticles of other drugs.

Table 2.1: Non-conventional methods to prepare amorphous drug nanoparticles.

Method	API	Size (nm)	Reference
Multi-inlet vortex mixer flash nanoprecipitation	β -carotene	< 100	54
Tube-in-tube microchannel reactor flash nanoprecipitation	Ceferuxime axetil	420	56
Microchannel reactor flash nanoprecipitation	Ceferuxime axetil	350	57
Microchannel reactor flash nanoprecipitation	Atorvastatin	480	58
High gravity antisolvent precipitation	Ceferuxime axetil	100–400	53
High gravity antisolvent precipitation	Cephadrine	200–400	59
Microfluidic precipitation	Hydrocortisone	80–450	60
Evaporative precipitation into solution	Cyclosporin A	120–600	61
Evaporative precipitation into solution	Quercetin	250	62
Antisolvent precipitation with hydrophobins	Itraconazole	100	63
Antisolvent precipitation with hydrophobins	Beclomethasone dipropionate	100	64
Supercritical anti solvent	Rifampicin, amoxicilin, nitrotriazole, astermizole	60–270	65
Rapid expansion of supercritical solution into liquid solvent	Paclitaxel	40–200	66
Sonoprecipitation	Ceferuxime axetil	130	52
Emulsification solvent evaporation	Dexamethasone	130	67
Emulsion freeze drying	Indomethacin	140–290	68
Emulsion freeze drying	Simvastatin	50–60	69
Microfluidic spray dryer	Danazol	20–60	70
Aerosol flow reactor	Beclomethasone dipropionate	85–125	71
Aerosol flow reactor	Salbutamol sulfate	70–130	72
Nano spray dryer	Furosemide	1250	73
Ultra rapid freezing	Itraconazole	190	47
Acid-base neutralization	Itraconazole	260	74

2.4 Aerosol antibiotic delivery by dry powder inhaler

The dry powder inhaler as a platform for antibiotic delivery to the lungs is a relatively uncharted area compared to nebulization, with which solutions of different classes of antibiotics such as aminoglycosides, β -lactams and polymyxins have been delivered over the years, even though none are approved by the FDA except for TOBI⁷. In contrast, the dry powder ciprofloxacin (a fluoroquinolone antibiotic) currently in Phase II of clinical trial³⁹ and the dry powder colistimethate sodium (Phase III of clinical trial)⁷⁵ are the nearest DPI antibiotics to commercialization potential.

Several investigations into the applicability of DPI antibiotic using human subjects have also been carried out, notably the tobramycin Pulmosphere[®] formulation⁷⁶⁻⁷⁷, colistin sulfate⁷⁸⁻⁷⁹, tobramycin⁸⁰ and gentamicin⁸¹. All studies demonstrated the superiority of aerosol delivery using the DPI platform, in that DPI delivery is capable of delivering the same dose as nebulization within a much shorter time frame and with higher delivery efficiency. Another DPI antibiotic study implementing *in vivo* animal studies is the dry powder capreomycin, where results show higher therapeutic antibiotic concentration in the lungs compared to intravenous delivery⁸².

DPI antibiotics are typically prepared by spray drying due to its efficiency, simplicity and low cost. Antibiotics solutions and suspensions converted into dry powder include azithromycin⁸³, tobramycin⁸⁴, ciprofloxacin suspension⁸⁵, a combination of doxycycline and ciprofloxacin⁸⁶, lipid-coated tobramycin powder⁸⁷, tobramycin co-spray dried with sodium stearate⁸⁸, ceftazidime and ciprofloxacin co-spray dried with dipalmitoylphosphatidylcholine (DPPC), albumin and lactose⁸⁹⁻⁹⁰. However, as these DPI formulations are prepared from solutions or suspensions of antibiotics, improved delivery efficiencies by the DPI platform to the intrapulmonary region does not guarantee a sustained therapeutic concentration, necessitating the encapsulation of antibiotics in nanoparticles.

In this regard, antibiotics have been encapsulated in nano-carriers in attempts to prolong the release of antibiotics from the DPI formulations⁹¹⁻⁹³. Example of an *in vivo* study include the dry powder microparticles encapsulating isozianid and rifampicin produced by a solvent extraction and evaporation method⁹⁴. In the study, the poly(lactic acid) (PLA) microparticles produced were intended for macrophage targeting in anti-tuberculosis therapy. The results following administration to rats via an inhalation chamber indicated that inhalable microparticles has the potential for aerosol delivery with the advantages of reduced dosing frequency, direct targeting and minimized of toxicity⁹⁴.

2.4.1 Spray drying of nanoparticles into inhalable aggregates

The conversion of nanoparticles into dry powder nano-aggregates for inhalation is non-trivial because nanoparticles have a strong tendency to agglomerate resulting in difficult physical handling. Nanoparticles, with the exception of particles < 50 nm in

size, are predominantly exhaled from the lung without depositing due to their low aerodynamic diameters (d_A)⁹, while large particles with $d_A > 5\mu\text{m}$ has been shown to deposit due to inertial impaction in the throat and mouth⁹⁵. In this regard, inhaled particles should possess d_A defined in Eq. 2.2 between 1–5 μm to effectively deposit in the lungs.

$$d_A = d_G \sqrt{\frac{\rho_e}{\rho_s}} \quad (2.2)$$

where d_G is the particle geometric diameter, $\rho_s = 1 \text{ g/cm}^3$, and ρ_e is the particle effective density defined as the mass of particles divided by their total volume including the open and closed pores.

To facilitate their delivery by inhalation, nanoparticles are typically transformed by spray drying into low-density micro-scale nanoparticle aggregates (i.e. nano-aggregates) with large d_G ($> 5 \mu\text{m}$) and low ρ_e ($\ll 1 \text{ g/cm}^3$) using various pharmaceutical excipients as drying adjuvants^{93, 96-98}. The large d_G of the nano-aggregates alleviates the problem of particle agglomerations resulting in effective aerosolization off the inhaler, without the need for coarse carrier particle inclusion, and improved physical handling. Their low ρ_e , which is owed to either hollow or porous morphologies, ensures their d_A to fall within the range suitable for effective lung depositions despite the large d_G . Another advantage of this carrier-free DPI formulation is in eliminating the concern of poor liberations of drug particles from carrier particles observed in the conventional carrier-based DPI formulation.

The spray dryer is typically operated at inlet temperatures $\geq 100^\circ\text{C}$ to achieve a fast convective drying rate needed to obtain the low ρ_e . Exposure to high temperatures in the spray dryer, where the outlet spray dryer temperatures vary in the range of $60\text{--}70^\circ\text{C}$, however, is prohibitive when low melting-point or thermally sensitive biodegradable polymers, such as PCL and poly(propylene succinate), are employed as therapeutic carriers. The high temperature can jeopardize the structural integrity of the nanoparticles, even in the presence of drying adjuvants, leading to nanoparticle degradations or coalescences that diminish their therapeutic functions.

2.4.2 Spray-freeze-drying of thermally-sensitive nanoparticles

Owed to its non-elevated temperature operation, spray freeze drying (SFD) represents a viable alternative to spray drying to produce dry powder nano-aggregates. In terms of its applications, SFD has primarily been employed to produce dry powder proteins as the sub-zero temperature, unlike the high temperature, does not adversely affect protein structures and functions⁹⁹⁻¹⁰⁰. SFD has also been used to produce solid dosage forms of poorly water-soluble drugs¹⁰¹⁻¹⁰² and to transform aqueous suspensions of drug-loaded liposomes into their dry powder forms using lactose, sucrose, and mannitol as freeze drying adjuvants¹⁰³⁻¹⁰⁴.

Figure 2.3: Physical mechanisms of the spray-freeze-drying (SFD) process.

The two-step SFD process (i.e. atomization into a cryogen followed by lyophilization) to produce nano-aggregates is illustrated in Fig. 2.3. The first step involves channeling of the nanoparticle suspension through a nozzle to be atomized into tiny droplets. As the nanoparticle suspension is atomized into droplets, the cold temperature provided by the cryogen (i.e. liquid nitrogen) is sufficient to freeze water molecules present within the air-borne droplets. The droplets are completely frozen when they are immersed in the liquid nitrogen. The rapid freezing allows the water

molecules to be frozen into ice crystals that are interspersed among the dissolved or suspended materials consisting of nanoparticles and freeze drying adjuvants. Excess liquid nitrogen is subsequently removed from the slurry containing the frozen droplets by evaporation or decanting. The evaporation of the liquid nitrogen is carried out by holding the slurry at a temperature between the boiling point of the liquid nitrogen and the melting point of ice (i.e. between -196°C and 0°C) to ensure the droplets remain frozen while the liquid nitrogen is being evaporated.

To obtain dry powders, the ice crystals are removed from the frozen droplets via sublimation in the lyophilization stage. The sublimation is carried out below the triple point of water (6 mbar, 0.01°C) to prevent the melting of ice crystals into liquid water that can destroy the solid structure of the nano-aggregates. As the ice crystals in the frozen droplets are sublimed interstitially, the frozen droplets retain their size and do not shrink upon sublimation. Therefore, for identical atomizing conditions, SFD produces particles having larger d_G compared to spray drying in which the atomized droplets shrink due to convective evaporation of the aqueous medium. Furthermore, due to the interstitial sublimation, particles produced by SFD exhibit highly porous structures attributed to the presence of voids previously occupied by the sublimed ice crystals. As a result, nano-aggregates having large d_G and low ρ_e are straightforwardly produced by SFD.

In SFD of nanoparticle suspension, freeze drying adjuvants need to be included in the formulation (i) to function as cryoprotective agents protecting the nanoparticles from the sub-zero temperature, and (ii) to facilitate re-dispersions of the nano-aggregates back into primary nanoparticles, so that the nanoparticles can perform their intended therapeutic functions. In inhaled drug delivery, aqueous re-dispersibility is an important characteristic of the micro-scale nano-aggregates as they must readily disassociate into primary nanoparticles upon exposure to the lung interstitial fluid in order to avoid lung phagocytosis that is least effective for particles smaller than $1\text{--}2\text{ }\mu\text{m}$ ¹⁰⁵. In this regard, the adjuvant inclusion prevents the nanoparticles from forming irreversible coalescences upon freezing caused by mechanical stresses exerted by the ice crystal formation. The presence of adjuvants leads to the formation of “adjuvant bridges” that interconnect the nanoparticles hence preventing direct inter-nanoparticle contacts upon freezing. As a result, the nano-aggregate aqueous re-dispersibility is

governed by the dissolution rate of the “adjuvant bridges” instead of the intrinsic attractive forces (e.g. van der Waals).

2.5 Chapter Summary

To summarize, this chapter reviews the current literature regarding (i) the development and application of antibiotic-loaded nanoparticles for inhaled anti-biofilm therapy, (ii) drug–polyelectrolyte nanoplexation to prepare antibiotic nanoparticles, and (iii) the transformation of nanoparticles suspension into inhalable dry powder. Significantly, there remains significant gap between the research and application fronts in these areas, and hence presenting an opportunity for further research to be conducted.

In particular, the development of antibiotic-loaded nanoparticles specifically for inhaled anti-biofilm therapy requires the careful tailoring of the antibiotic release profile to yield maximum anti-biofilm efficacy, necessitating the study of said optimum antibiotic release profile as well as the design of nanoparticles capable of the same. On the other hand, antibiotic nanoparticles for inhaled anti-biofilm therapy can also be prepared using the drug–polyelectrolyte nanoplexation method. To further narrow the gap between research and application of nanoparticles for inhalation, the transformation of the nanoparticles in suspension form into inhalable dry powder form is necessary. These research directions form the objectives of this dissertation (as outlined in Section 1.3), and are presented in the chapters that follow.

Chapter 3

Methodology

The Materials and Methods that are commonly applied throughout the course of the experimental work involved in this thesis are presented here. Methods that are specific to certain chapters, however, are presented in the individual chapters, with a goal of clarity and brevity.

3.1 Materials

3.1.1 Active Pharmaceutical Ingredients

The antibiotics used in the experiments, i.e. levofloxacin (LEV), ciprofloxacin (CIP), ofloxacin (OFX), and tobramycin (TOB) were purchased from Sigma–Aldrich (USA).

3.1.2 Antibiotic-loaded nanoparticles

The polymers used to prepare the antibiotic-loaded nanoparticles i.e. poly(caprolactone) (PCL, MW = 80,000) was purchased from Sigma–Aldrich (USA) and poly(DL-lactide-*co*-glycolide) (PLGA, Purasorb 5004A) was received as a gift from PURAC Biomaterials (Netherlands).

Analytical/HPLC grade chemicals used in the nanoparticle synthesis, in particular (i) solvents such as dichloromethane (DCM), acetone, and ethyl acetate, (ii) surfactants such as poly (vinyl alcohol) (PVA, MW = 23,000), Pluronic F-68, d- α -tocopheryl polyethylene glycol 1000 succinate (TPGS, Vitamin E-PEG 1000), polyethylene glycol (PEG, MW = 4000), and tyloxapol, and (iii) lipids such as phosphatidylcholine (PC) and stearylamine (SA), as well as chitosan were purchased from Sigma–Aldrich (USA).

3.1.3 Other chemicals and materials

Sodium chloride (NaCl), potassium hydroxide (KOH), glacial acetic acid, hydroxypropylmethylcellulose (HPMC), and the spray-freeze-drying adjuvants (i.e. L-Leucine, α -lactose monohydrates, and D-mannitol) were purchased from Sigma–Aldrich (USA). The polyelectrolytes used, i.e. poly(allylamine hydrochloride), (PAH, MW 120–200,000), was purchased from Alfa Aesar (USA) while dextran sulfate, (DXT, MW 5,000 Da) was purchased from Wako Pure Chemical (Japan). Rhamnolipid (RHL) (JBR–515, Jeneil Biosurfactant, US) produced by *P. aeruginosa* biofilm cells was donated by Teesside University (UK) in the form of 50% (w/v) mixtures of mono-RHL and di-RHL at 15% (w/v) concentration.

The phosphate buffer saline solution (PBS, pH = 7.4) and the dialysis membrane with a molecular weight cut-off size of 12,400 g/mol used in the *in vitro* drug release study were purchased from 1st Base (Singapore) and Sigma–Aldrich (USA), respectively.

Bacterial growth medium (i.e. Mueller Hinton Broth (MHB) and Luria–Bertani (LB) broth) and agar were purchased from BD Biosciences (USA).

3.1.4 Bacterial strains

Bacterial strains used in the antimicrobial testing experiments, i.e. *E. coli* K-12 (W3110) strain, was obtained from Coli Genetic Stock Center, Yale University (USA) while the *P. aeruginosa* PAO1 strain was obtained from American Type Culture Collection (BAA-47, ATCC, USA).

3.2 Methods

3.2.1 Preparation of antibiotic-loaded nanoparticles

3.2.1.1 Nanoprecipitation (NPC) to prepare antibiotic-loaded nanoparticles

The nanoprecipitation (NPC) method of Govender *et al.*¹⁰⁶ was one of the methods employed to obtain antibiotic-loaded nanoparticles. The polymer and the antibiotic were dissolved in water-miscible acetone. Upon addition of the polymer/antibiotic solution into water, the acetone rapidly diffused into the aqueous phase resulting in the formation of polymeric nanoparticles in which the antibiotic is

predominantly adsorbed on the nanoparticle surface resulting in the burst release profile.

Briefly, 8 mg of antibiotic and 100 mg of PLGA were dissolved in 5 mL of acetone after which the solution was poured into 10 mL aqueous solution of 0.1% (w/v) Pluronic F-68. The resulting nanoparticulate suspension was stirred overnight at room temperature to evaporate off the acetone present in the aqueous phase, which was followed by two centrifugations at 13,000×g to remove the non-encapsulated antibiotic from the nanoparticulate suspension. Antibiotic-loaded PCL nanoparticles were prepared following the same procedures but using 75 mg of PCL instead. Blank nanoparticles were prepared using the same procedures but without incorporating any antibiotic.

3.2.1.2 Preparation of antibiotic-loaded nanoparticles by emulsification-solvent-evaporation (ESE)

The emulsification-solvent-evaporation (ESE) method of Sung *et al.*⁹³ was another method employed to prepare antibiotic-loaded nanoparticles. The polymer and antibiotic were dissolved in a volatile and water-immiscible solvent (i.e. DCM), where upon addition of the polymer/antibiotic solution into water, an oil-in-water nano-emulsion was formed by ultrasonication. Next, the DCM was slowly evaporated to transform the nano-emulsion into a nanoparticulate suspension in which antibiotic was encapsulated inside the polymer matrix resulting in an extended release profile compared to the NPC method.

Briefly, 8 mg of antibiotic and 80 mg of PLGA were dissolved in 2 mL of DCM after which the solution was poured into 6 mL aqueous solution of 1.0% (w/v) PVA. The resulting solution was emulsified for 60 s using a Vibra-Cell probe sonicator (VC 5040, Sonics and Materials, USA). The nano-emulsion was next added to 10 mL aqueous solution of 0.1% (w/v) PVA and was stirred overnight at room temperature to evaporate off the DCM resulting in the nanoparticle production. The resulting nanoparticulate suspension was then centrifuged twice at 11,000×g to remove the non-encapsulated antibiotic. The same procedures were used to prepare the blank nanoparticles and the antibiotic-loaded PCL nanoparticles.

3.2.1.3 Antibiotic-loaded nanoparticles by double-emulsification-solvent-evaporation (DESE)

PLGA and antibiotic were dissolved separately in a water-immiscible solvent (i.e. DCM) and deionized (DI) water, respectively. The two solutions were emulsified under ultrasonication to form w/o nano-emulsion, which was subsequently added to a second aqueous phase to form w/o/w nano-emulsion. The antibiotic was encapsulated in both the aqueous core as well as the polymer matrix, producing an extended release profile where the antibiotic was first released from the polymer matrix, followed by antibiotic release from the aqueous core.

Specifically, 9 mg of antibiotic was dissolved in 300 μ L of DI water and 90 mg of PLGA was dissolved in 3 mL of DCM. The aqueous antibiotic solution was emulsified in the PLGA organic solution by sonication for 60 s. The resulting nano-emulsion was poured into 12 mL aqueous solution of 2.0% (w/v) PVA and was sonicated for 60 s. The nano-emulsion was stirred overnight at room temperature to evaporate off the DCM. The resulting nanoparticle suspension was centrifuged and washed following similar procedures described in the NPC and ESE method.

3.2.2 Physical characterizations of antibiotic-loaded nanoparticles

The nanoparticle size and zeta potential were measured by photon correlation spectroscopy (PCS) using a Brookhaven 90Plus Nanoparticle Size Analyzer (Brookhaven Instruments Corporation, USA) in DI water at neutral pH. The drug encapsulation efficiency (i.e. % encapsulation) was determined from the ratio of the amount of the encapsulated antibiotic to the amount of antibiotic initially added. The amount of the encapsulated antibiotic was determined by subtracting the antibiotic amount present in the supernatant after the first centrifugation from the amount of antibiotic initially added. The antibiotic concentration in the supernatant was measured using UV-Vis spectrophotometer (UV Mini-1240, Shimadzu, Japan) absorbance wavelengths of 227, 254, and 291 nm for OFX, LEV, and CIP, respectively. The drug loading was determined from the ratio of the amount of the encapsulated antibiotic to the total amount of the nanoparticles produced.

To obtain the nanoparticle morphology, the nanoparticle suspension was freeze dried for 24 h in a Alpha 1-2 LD Plus freeze dryer (Martin Christ, Germany) at -52°C

and 0.05 mbar. The dried nanoparticles were sputter coated with gold and imaged using a Scanning Electron Microscope (SEM) model JSM-6700F (JEOL, USA).

3.2.3 *In vitro* drug release from antibiotic-loaded nanoparticles

The antibiotic release rate from the nanoparticles was determined using the dialysis bag method under a sink condition. The dialysis bag containing 2 mL of the antibiotic-loaded nanoparticulate suspension was placed in an opaque bottle filled with 6 mL of PBS as the release medium. The dissolution test was conducted at 37°C in a water bath under a gentle stirring for a period of time. The opaque bottle was used as fluoroquinolone antibiotics (LEV, CIP, and OFX) in their aqueous solution form are known to be photodegradable¹⁰⁷. At fixed time intervals, 4 mL of the release medium was withdrawn and the amount of antibiotic released was immediately measured by the UV-Vis spectrophotometer. The withdrawn sample was replaced with 4 mL of fresh PBS.

3.2.4 Antibiotic susceptibility testing

3.2.4.1 Minimum Inhibitory Concentration (MIC) determination

The minimum inhibitory concentration (MIC) is defined as the lowest antibiotic concentration that inhibits a visible planktonic bacterial growth after an overnight incubation at 37°C. An optical density measurement at 600 nm (OD_{600}) is typically used to examine the visible bacterial growth in which $OD_{600} < 0.1$ indicates a zero bacterial growth. Briefly, the MIC was determined by broth micro-dilution using a 96-well microplate (F96 MicroWell, Nunc, Denmark). A bacterial suspension to be used as the inoculum was adjusted to a 0.5 McFarland standard in MHB after which it was further diluted by 100-fold to produce a bacterial cell suspension having 1.0×10^6 colony forming units (CFU)/mL. Next, 100 μ L of antibiotic solution in MHB was added to 100 μ L of the bacterial cell suspension in each well of the microplate to yield a final cell concentration of 5.0×10^5 CFU/mL. After 24 h incubation at 37°C, OD_{600} of the cell suspension in each well was measured using a microplate reader (Synergy HT, Biotek, USA). The lowest antibiotic concentration that yields $OD_{600} < 0.1$ was determined as the MIC.

3.2.4.2 Minimum Biofilm Inhibitory Concentration (MBIC) determination

The minimum biofilm inhibitory concentration (MBIC) is defined as the lowest antibiotic concentration that inhibits a visible biofilm cell growth. The biofilm cultivation method and MBIC determination follow the methods of Harrison *et al.*¹⁰⁸ and Ceri *et al.*¹⁰⁹, respectively. Briefly, a bacterial cell suspension to be used as the inoculum was adjusted to 1.0 McFarland standard and was diluted by thirty-fold in MBH to produce 1.0×10^7 CFU/mL. Next, 150 μ L of the inoculum was transferred to each well of a 96-well microplate and a 96-peg lid was fitted on top of the microplate to provide a surface for the biofilm growth (see Fig. 3.1). After 24-hour-incubation in a shaking incubator at 150 RPM and 37°C, the peg lid was lifted and the biofilm formed on the pegs was rinsed with PBS to remove loosely attached planktonic cells.

Figure 3.1: Peg-plate system used for biofilm growth.

Next, two pegs were broken off used to enumerate the biofilm cells formed. The biofilm formed in the remaining 94 pegs were exposed in a new microplate to 200 μ L of antibiotic solution in MHB. After a 24-hour-incubation at 37°C, the peg lid was lifted and the pegs were rinsed with PBS to remove the dead biofilm cells. Next, the non-eradicated biofilm cells were detached from the pegs by 5 min sonication and transferred to a recovery plate containing 200 μ L of fresh MHB. After a 24-hour-incubation at 37°C, OD₆₀₀ in each well of the recovery plate was measured using the microplate reader. The lowest antibiotic concentration that results OD₆₀₀ < 0.1 was determined as the MBIC. Both the MIC and MBIC determinations were repeated three times on three different days using three replicates on each day.

3.2.5 Antibiotic nanoplex preparation

The antibiotic was dissolved in 1 mL aqueous acetic acid (AA) solution after which it was added to an equal volume of aqueous solution of DXT and NaCl salt. DXT is anionic at pH above its pK_a ($\approx 1-2$). The role of salt is to provide a charge screening effect to minimize intramolecular and intermolecular repulsions of the like-charged PE chains, which can inhibit the complex aggregate formation. Significantly, the nanoplex was not formed in the absence of salt despite the drug-PE electrostatic interactions. The resulting nanoplex suspension was left for 3 h at ambient conditions for the complexation to reach equilibrium. The suspension was centrifuged to remove the salt, AA, and excess drug and DXT (i.e. those that are not complexed) followed by three-cycle washing with DI water.

3.2.6 Physical characterizations of antibiotic nanoplex

The complexation efficiency (CE) was characterized by the mass percentage of the drug that forms nanoplex per the amount of drug initially added. The amount of drug that forms nanoplex was determined from the amount of non-complexed drug recovered in the supernatant after centrifugation of the unwashed nanoplex suspension. Drug concentrations in the supernatant were measured by UV-Vis spectrophotometer at absorbance wavelengths specified in Section 3.2.2. The yield was characterized by the total mass of nanoplex produced per the total mass of drug and PE initially added. The drug loading was determined by measuring the drug concentration after complete dissolution of a known mass of nanoplex in PBS. Characterizations of CE, yield, and drug loading were performed with a minimum of two replicates.

3.2.7 Nanoplex dissolution rate and saturation solubility

The drug dissolution profile was determined by a dialysis bag method under a sink condition. Briefly, a dialysis bag containing 2 mL nanoplex suspension was placed in 48 mL PBS at 37°C in a water bath under gentle stirring. The volume of PBS used was much higher than that used in the *in vitro* antibiotic release experiments (in Section 3.2.3) as one of the antibiotics (CIP) used in the nanoplex preparation have lower aqueous solubility. At fixed intervals, 2 mL of the dissolution medium was withdrawn to measure the amount of drug released after which the withdrawn solution was replaced with 2 mL fresh PBS.

To determine the saturation solubility, nanoplex in the suspension and dry powder forms were added in excess to 20 mL PBS. After incubation for 24–48 h in a shaking incubator at 37°C and 100 RPM, 2 mL aliquot of the incubated sample was centrifuged and filtered through a 0.02 µm filter (Minisart, Sartorius, Germany) and the dissolved drug concentration was measured.

To examine drug nanoplex dissolution behavior under supersaturated conditions, 2 mL of nanoplex suspensions at concentrations equal to 10 and 25 times of the drug's saturation solubility were added to 48 mL PBS at 37°C under constant stirring. At specified time points, 2 mL PBS was withdrawn and filtered and the dissolved drug concentration was measured. More specifically, 200 µL of the 2 mL filtered drug solution was immediately diluted 10-fold with PBS to prevent drug precipitation under supersaturated conditions during UV-Vis measurement. The withdrawn sample was replaced with 2 mL fresh PBS. The experiments for the dissolution rate, saturation solubility, and dissolution under supersaturated conditions were performed using at least two replicates and they were repeated for the raw drug crystals as comparisons.

3.2.8 Nanoplex physical stability

To examine their physical stability, the nanoplex powders were stored for one month at 25°C and 55% relative humidity. Afterwards, powder X-ray diffraction (PXRD) patterns of the nanoplex powders were examined using D8 Advance X-Ray diffractometer equipped with Cu K α radiation (Bruker, Germany) from 10° to 60° (2 θ) with a step size of 0.02°/s. Thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) analysis were also carried out using SDT Q600 (TA Instruments, USA), where 5 mg nanoplex powders were filled into an alumina pan and heated from 30°C to 400°C at 10°C/min.

Chapter 4

Effect of Antibiotic Release Profile on Anti-Biofilm Efficacy of Nanoparticles

4.1 Introduction

The primary objective of the present chapter is to examine the effect of the antibiotic release profile on the antibacterial efficacy of antibiotic-loaded nanoparticles (the importance of which was detailed in Section 2.1) in a testing condition where the immediate antibiotic clearance is not a major determining factor. Owing to the highly dynamic nature of the biofilm defensive response, the antibacterial efficacy of the antibiotic-loaded nanoparticles is examined in a time-dependent biofilm susceptibility testing in which the influences of the antibiotic exposure history, the biofilm age, and the *in vivo* drug removal on the results are taken into account.

In the present chapter, *Escherichia coli* (*E. coli*) biofilm, which is present in 7% of patients hospitalized for lower respiratory tract infections¹¹⁰, is used as the biofilm model. Levofloxacin (LEV), a fluoroquinolone antibiotic, is used as the antibiotic model as it is known to be effective against a wide range of dormant respiratory bacterial pathogens¹¹¹, and, unlike aminoglycoside antibiotics, it can diffuse through the EPS matrix to reach mature biofilm cells embedded deeply in the biofilm matrix¹¹².

The antibiotic is encapsulated into biodegradable polymeric nanoparticles that are specifically engineered to produce different antibiotic release profiles (i.e. burst, intermediate, and slow). The biofilm susceptibility testing is conducted over a six-day period with daily dosing of different antibiotic concentrations that correspond to the antibiotic release profile. In addition, the antibiotic susceptibility testing is also conducted for floating planktonic cells, which are steadily shed from the biofilm

matrix as part of the biofilm mode of growth. These biofilm-derived planktonic cells are of significant importance as they have been found to be the main cause of exacerbations by forming new biofilm colonies elsewhere¹¹³.

4.2 Methodology

LEV-loaded PLGA and PCL nanoparticles were prepared by using the NPC and ESE methods previously detailed in Section 3.2.1. The (i) physical characteristic of the nanoparticles prepared, (ii) *in vitro* release of LEV from the nanoparticles, and (iii) antimicrobial susceptibility (MIC and MBIC) of the nanoparticles were determined using the methods detailed in Sections 3.2.2–3.2.4. The LEV solutions collected from the six days of *in vitro* release with at 24 h intervals (i.e. LEV1, LEV2, and ...) were stored in opaque bottles to be subsequently used, after a ten-fold dilution, in the time-dependent antibiotic susceptibility testing (see Section 4.2.2). The *in vitro* release study was also conducted using 6 mL of MHB as the release medium for comparison. In addition, the antimicrobial activity of the blank nanoparticles and the time-dependent anti-biofilm of the LEV-loaded nanoparticles were characterized.

4.2.1 Blank nanoparticle susceptibility testing

To investigate whether the PLGA and PCL nanoparticles exhibit any antibacterial activities, biofilm cells were cultivated on a 96-peg lid following the same procedures used to determine the MBIC. Afterwards, the biofilm formed on the pegs were exposed in a microplate to a suspension containing 40 μ L of the blank nanoparticulate suspension (1.5 % w/v) and 160 μ L of MHB. Biofilm cells exposed to 40 μ L of PBS and 160 μ L of MHB were used as the experimental controls. After 6, 12, and 24 h of incubation at 37°C, the biofilm formed on the pegs was rinsed with PBS and was later detached from the pegs into a new microplate by sonication.

The recovered biofilm cells were serially diluted tenfold and were plated onto agar plates and incubated overnight at 37°C to determine the viable CFU. The biofilm-derived planktonic cells, which were present in the wells of the microplate after the blank nanoparticle exposure, were enumerated after 24 h. The experiment was conducted in two replicates. The viable CFU counts of the biofilm-derived planktonic cells and the biofilm cells, which have been exposed to the blank nanoparticles, were compared with those of the experimental controls.

4.2.2 Time-dependent biofilm susceptibility testing

For the six-day time-dependent biofilm susceptibility testing (i.e. time-kill), the biofilm was cultivated on six 96-peg lids following the same procedures used to determine the MBIC. On the 1st Day of the time-kill, the biofilm cells from the six peg lids were exposed to an antibiotic solution in a microplate, which contains 20 μ L of the aforementioned LEV1 solution obtained from the *in vitro* drug release study and 180 μ L of MHB. A solution containing 20 μ L of PBS and 180 μ L of MHB was used as the positive control. The fresh MHB must be supplied daily to sustain the biofilm existence over the six-day time-kill period. After a 24-hour-incubation at 37°C (i.e. 2nd Day of the time-kill), one of the six peg lids was rinsed with PBS and is sonicated to recover the non-eradicated biofilm cells.

Figure 4.1: An illustration of the time-dependent biofilm susceptibility testing using the LEV solution from the *in vitro* drug release study (LEV-NP: LEV-loaded nanoparticles).

The recovered biofilm cells were transferred to a new microplate containing 200 μ L of PBS. Next, the viable CFU was determined using two replicates following the same procedures used in the blank nanoparticle susceptibility testing. In addition, OD₆₀₀ of the biofilm-derived planktonic cell suspension present in the wells of the first

microplate was measured. The remaining five peg lids were exposed to an antibiotic solution containing 20 μL of the LEV2 solution and 180 μL of MHB. These procedures were repeated daily for five consecutive days using LEV3, LEV4, LEV5, and LEV6 antibiotic solutions in sequence as illustrated in Fig. 4.1. The biofilm susceptibility testing was repeated in a pseudo-time-kill study using one-day-old biofilm cells to examine the effect of the antibiotic exposure history on the biofilm susceptibility.

4.3 Results

4.3.1 LEV-loaded nanoparticles

A summary of the LEV-loaded nanoparticles produced is presented in Table 4.1. The experimental uncertainties in the % encapsulation and the drug loading measurements based on three replicates are between 4–7% and 5%, respectively. The results indicate that the NPC method produces smaller nanoparticles (≈ 100 nm) compared to those produced by the ESE method (≈ 200 nm) for both the PCL and PLGA. Importantly, nanoparticles in this size range have been found to be ideal for the biofilm penetration¹¹⁴. The high aqueous solubility of LEV results in a majority of LEV to diffuse out into the aqueous phase during the preparation steps resulting in low % encapsulation ($\leq 16\%$) and consequently relatively low drug loadings (0.30–1.10% w/w). Significantly, PLGA nanoparticles exhibit higher % encapsulation and drug loadings than their PCL nanoparticle counterparts.

Table 4.1: Physical characteristics of the nanoparticles produced.

Nanoparticle	Preparation method	Size (nm)	% Encapsulation	Drug loading (%)
PLGA	NPC	80 ± 30	14.7	0.65
	ESE	190 ± 50	15.8	1.10
PCL	NPC	110 ± 40	5.0	0.40
	ESE	230 ± 80	4.0	0.30

4.3.2 *In vitro* LEV release profiles

The *in vitro* LEV release profiles of the different nanoparticle formulations in PBS examined over a six-day period are presented in Fig. 4.2. The experimental uncertainties in the *in vitro* LEV release profiles based on three replicates are equal to

approximately 5%. The effect of changing the release medium to MHB is found to be negligible. Both LEV-loaded PCL and PLGA nanoparticles prepared by the NPC method (i.e. PCL/PLGA NPC) exhibit monophasic burst release profiles in which $\approx 80\%$ of the loaded drug is released within the first hour and the entire drug is released after 6 h. The *in vitro* release profiles of the PCL and PLGA NPC nanoparticles are found to be fairly similar with each other. Importantly, as the burst release period is significantly longer than the typical biofilm penetration time of the nanoparticles reported in the literature¹¹⁵, a majority of the antibiotic is to be released inside the biofilm.

Figure 4.2: *In vitro* antibiotic release profiles of the different nanoparticle formulations.

On the other hand, nanoparticles produced by the ESE method (i.e. PCL/PLGA ESE) exhibit biphasic extended release profiles. For the PLGA ESE nanoparticles, $\approx 80\%$ of the loaded drug is released after one day, which represents the initial fast release that is followed by a slower release resulting in the entire drug being released only after the 6th day. Attributed to the considerably lower drug loading, the PCL ESE nanoparticles exhibit a slower drug release profile than the PLGA ESE nanoparticles, where only $\approx 40\%$ of the loaded drug is released after one day and only $\approx 75\%$ of the drug is released after six days.

4.3.3 Blank nanoparticle susceptibility

After 6, 12, and 24-h exposures to the blank nanoparticles, the viable CFU of the exposed biofilm cells are found to remain closely similar to the viable CFU of the experimental control as shown in Fig. 4.3A, which indicates that the four blank

nanoparticles (i.e. PCL/PLGA NPC and PCL/PLGA ESE) do not possess any antibacterial activities towards the biofilm cells. The experimental uncertainties in the viable CFU based on the two replicates are approximately equal to ± 0.5 CFU. In addition, the presence of the blank nanoparticles does not alter the biofilm growth either as the viable CFU of the biofilm-derived planktonic cells presented in Fig. 4.3B are also equal to the viable CFU of the experimental control.

Figure 4.3: Viable bacterial cell counts from the blank nanoparticle susceptibility testing (A) biofilm cells, (B) biofilm and planktonic cells.

The antibacterial efficacy of the LEV-loaded nanoparticles can therefore be attributed exclusively to the antibiotic exposure and not caused by the nanoparticle inclusion or by the trace of chemicals used in the nanoparticle preparation. As the presence of the nanoparticles does not have any effects on the biofilm cells, the biofilm susceptibility testing is conducted by using only the LEV solution obtained from the *in vitro* release study without physically including the nanoparticles in the testing.

4.3.4 Effect of LEV encapsulation on its antibacterial activity

Figure 4.4: Effect of encapsulation on the LEV antibacterial activity.

The MIC and MBIC values of LEV against *E. coli* planktonic and biofilm cells are approximately equal to 0.03 and 0.15 $\mu\text{g/mL}$, respectively, which indicate that the biofilm mode of growth of *E. coli* bacterial cells results in a five times higher tolerance towards LEV than the planktonic mode.

To investigate whether the antibacterial activity of LEV is altered by its encapsulation into the nanoparticles, the antibacterial efficacies of the native LEV (i.e. free drug) and the LEV released from the PCL and PLGA NPC nanoparticles are examined against 1 day-old biofilm cells at an equal antibiotic concentration (i.e. 0.45 $\mu\text{g/mL}$). After taking into account the experimental uncertainties in the viable CFU of the two replicates, the results in Fig. 4.4 indicate that the eradication rates of the encapsulated LEV after 6, 12, and 24 h of antibiotic exposures are relatively similar to those of the native LEV, where up to 3 log (CFU/peg) (i.e. 99.9%) of the biofilm cells are eradicated. A similar result is found for the LEV released from the PCL and PLGA ESE nanoparticles but the data are not shown here for brevity. Hence, LEV can be safely encapsulated into the polymeric nanoparticles without affecting its antibacterial activity.

4.3.5 *In vitro* dilution rate to simulate the *in vivo* drug removal

In the biofilm susceptibility testing, the *in vivo* drug removal by various clearance mechanisms in the lung (e.g. absorption, phagocytosis, enzymatic degradation) is

simulated by a two-fold dilution of the LEV solution at a fixed time interval. This static dilution approach represents a simpler alternative to simulate the *in vivo* drug removal than the more conventional approach using a chemostat. This approach is nevertheless limited to biofilm studies in which the impact of the interaction between the drug-bearing particles and the biofilm cells on the biofilm mode of growth is minimal as is the case with the present study.

In this regard, the typical half-life of intravenously administered LEV in the lung interstitial fluid is approximately equal to 8 h¹¹⁶. In the present work, a longer half-life is simulated as LEV has been incorporated into nanoparticulate carriers that prolong the residence time of LEV in the biofilm infection sites. To determine the appropriate dilution rate, the effect of the two-fold dilution at two different time intervals (i.e. 12 and 24 h) on the LEV concentration variations during the 6 days time-kill period is examined in Fig. 4.5 by using the LEV solution released from the PLGA NPC and PLGA ESE nanoparticles.

The total amount of LEV used in the *in vitro* release study is set at 0.16 mg for both nanoparticles yielding a maximum LEV concentration of $\approx 2.3 \mu\text{g/mL}$. This concentration range is in the same order of magnitude as the maximum serum concentration ($\approx 5.5 \mu\text{g/mL}$) of 500 mg orally-administered LEV that is typically used in antibiotic therapies against common respiratory pathogens¹¹⁷. Moreover, the maximum LEV concentration equals ≈ 80 -fold of the MIC value and ≈ 15 -fold of the MBIC value of the *E. coli* bacterial cells.

For the PLGA NPC nanoparticles having a burst release profile, a two-fold dilution every 12 h in Fig. 4.5A causes the LEV concentrations to drop below the MBIC value after 2 days before reaching a practically zero value after 3.5 days. As a result, the biofilm susceptibility testing from Day 2 onwards will not likely reveal any useful information other than an uninhibited biofilm growth. In contrast, a two-fold dilution every 24 h keeps the LEV concentrations above the MBIC value for 5 days with a half-life of 24 h.

For the PLGA ESE nanoparticles having a slower release profile, a two-fold dilution every 12 h in Fig. 4.5B maintains the LEV concentrations above the MBIC value up to 4 days. Nonetheless, the LEV concentrations from Day 3 onwards are not much higher than the MBIC value hence they are unlikely to be effective. On the

contrary, a two-fold dilution every 24 h results in LEV concentrations that are at least 1.5 times above the MBIC value throughout the six days with a half-life of 24 h. For this reason, a twice dilution at 24 h intervals is selected as the *in vitro* dilution rate to simulate the *in vivo* drug removal. Nevertheless, dilution rates at shorter time-intervals can be selected at higher dose amounts.

Figure 4.5: Effect of the two-fold dilution rate on the day-to-day variations of the LEV concentrations for (A) PLGA NPC and (B) PLGA ESE nanoparticles.

4.3.6 Effects of antibiotic release profile on the biofilm susceptibility

Three distinct antibiotic release profiles (i.e. burst release by PCL/PLGA NPC, intermediate release by PLGA ESE, and slow release by PCL ESE) are employed to examine their effects on the antibacterial efficacy of the LEV-loaded nanoparticles. The antibacterial efficacies produced by the burst and the slow release profiles are

examined first. The total amount of LEV used is set at 0.16 mg for both nanoparticles. The day-to-day variations in the LEV concentrations of the two release profiles after a two-fold dilution every 24 h are presented in Fig. 4.6A, whereas the time-kill results are presented in Fig. 4.6B.

Figure 4.6: (A) LEV concentration variations and (B) time-kill results of the different antibiotic release profiles.

The LEV concentrations from the burst release profile decrease with time following a first-order kinetics and fall below the MBIC value on the 6th day. In contrast, the LEV concentrations from the slow release profile remain at least 3 times above the MBIC value throughout the 6 days. The main difference between the two release profiles lies in their LEV concentrations on Day 1, where the PCL/PLGA NPC nanoparticles release 2.25 $\mu\text{g/mL}$ of LEV (i.e. $15 \times \text{MBIC}$), whereas the PCL ESE nanoparticles release only 1.05 $\mu\text{g/mL}$ of LEV (i.e. $7 \times \text{MBIC}$). Nevertheless, both

concentrations likely can eradicate a majority of the young biofilm cells on Day 1 without difficulty as they are both high above the MBIC value. The eradication rates on the subsequent days after the biofilm cell defensive response, however, remain to be investigated.

The viable CFU of the positive control in Fig. 4.6B remain relatively constant between 6–7 log (CFU/peg) throughout the six days, which denote a sustained existence of the biofilm cells at a steady state, where the rate of the planktonic cells shed from the biofilm is equal to the adhering rate of new bacterial cells onto the biofilm. Importantly, the sustained existence of the positive control throughout the 6 days indicates that the technique employed in the present work to uphold the biofilm existence over a prolonged period is equally appropriate as the one conducted in a chemostat.

For both the burst and the slow release profiles, the LEV concentrations released on Day 1 as expected eradicate about four log(CFU/peg) (i.e. 99.99%) of the biofilm cells as shown in Fig. 4.6B. Importantly, even though the antibacterial efficacy of fluoroquinolone antibiotics is known to be concentration dependent, the higher initial LEV concentration in the burst release profile (i.e. 2.25 µg/mL) does not result in a higher eradication rate on Day 1 than that of the slow release profile (i.e. 1.05 µg/mL).

The presence of a plateau in the eradication rate as the antibiotic concentration is increased typically signifies the existence of surviving persister cells¹¹⁸. For this reason, the antibiotic susceptibility of 1 day-old biofilm cells is examined in a dose-kill study at LEV concentrations ranging from 0.15 to 3.10 µg/mL. The results in Fig. 4.7 indicate that the variations in the viable CFU after exposures to a wide range of LEV concentrations are statistically insignificant as they fall within the experimental uncertainties. The viable CFU is found on average to be approximately equal to 2 log (CFU/peg) indicating the survival of 0.01% subpopulation persister cells.

Even though the biofilm eradication rates on Day 1 are equal between the burst and the slow release profiles, their eradication rates on Day 2 are vastly different. For the slow release profile, the surviving persister cells can freely multiply producing new biofilm cells on Day 2 despite their exposure to 0.80 µg/mL of LEV, which is more than 5 times above the MBIC value. As a result, a similar number of biofilm colonies as the ones exist prior to the antibiotic exposure are formed on Day 2. For the burst

release profile, the biofilm growth on Day 2 is inhibited to a significantly larger extent due to the higher LEV concentration present at 1.10 $\mu\text{g/mL}$.

Figure 4.7: Viable bacterial cell counts of 1 day-old biofilm cells exposed to a wide range of LEV concentrations.

From Day 3 onwards, the biofilm growth continues at a relatively constant pace as the LEV concentration decreases exponentially with time in the burst release profile. The LEV concentration eventually drops below the MBIC value on Day 6 resulting in a sharp increase in the biofilm growth. The time needed for the biofilm to recover to its initial state is therefore around 5 days. On the other hand, the LEV concentrations are maintained at $\approx 0.50 \mu\text{g/mL}$ from Day 3 onwards in the slow release profile. As a result, the biofilm growth is slightly subdued on the subsequent days. Nevertheless, it is not significant enough to offset the rapid biofilm growth on Day 2. Hence, in terms of the speed of the biofilm recurrence, the burst release profile is unexpectedly found to be superior to the slow release profile though the LEV concentrations in the latter are already maintained above the MBIC value throughout the six days.

A similar trend in the biofilm growth is observed for the biofilm cells exposed to the intermediate release profile from the PLGA ESE nanoparticles. The result is not unexpected as the LEV concentrations from the intermediate release profile after the two-fold dilution, which is shown earlier in Fig. 4.5B, fall within the same range as that of the burst release profile throughout the six days. Fittingly, similar biofilm eradication rates are observed in Fig. 4.6B between the burst and the intermediate release profiles, where the variations in the viable CFU between the two release profiles still fall within the experimental uncertainties.

4.3.7 Antibiotic susceptibility of biofilm-derived planktonic cells

Two separate time-kill studies (i.e. real and pseudo time-kills) are conducted in which the LEV concentration is varied between 0.14 and 1.80 $\mu\text{g/mL}$. In the pseudo time-kill, the susceptibility testing is conducted against planktonic cells shed from 1 day-old biofilm cells that have not been exposed to antibiotics. In the real time-kill, the testing is conducted against planktonic cells shed from biofilm cells that have been exposed to antibiotics for a period of 1–6 days. For brevity, the biofilm-derived planktonic cells used in the pseudo and real time-kill studies are referred from this point onwards as fresh and exposed biofilm-derived planktonic cells, respectively.

Figure 4.8: Antibiotic susceptibility testing of biofilm-derived planktonic cells.

The results of the antibiotic susceptibility testing in Fig. 4.8 indicate that the growth of the fresh biofilm-derived planktonic cells is successfully inhibited in the range of the LEV concentrations investigated as reflected in $\text{OD}_{600} \leq 0.1$ that is observed for the most part. The results are well expected as the lowest LEV concentration investigated is close to 5 times of the MIC value. On the contrary, the exposed biofilm-derived planktonic cells exhibit a significantly higher antibiotic tolerance than the fresh ones, where a minimum of 0.60 $\mu\text{g/mL}$ of LEV ($\approx 20 \times \text{MIC}$) is needed to inhibit the planktonic cell growth.

4.4 Discussion

The time-kill results from the three antibiotic release profiles indicate that LEV concentration that is significantly higher than the MBIC value (i.e. $> 1.10 \mu\text{g/mL}$) is

needed to inhibit the biofilm growth caused by the multiplications of the surviving persister cells. The results hence signify the importance of the antibiotic exposure history as the surviving persister cells, as part of their defensive response, acquire a higher antibiotic tolerance after the initial antibiotic exposure. Nevertheless, the higher antibiotic tolerance acquired by the surviving persister cells after the initial dosing is often overlooked in many biofilm susceptibility studies because of the daily, or even more frequent, administrations of the new dose.

Simply maintaining the LEV concentrations slightly above the MBIC value is therefore inadequate to inhibit the biofilm growth as the MBIC value is determined from biofilm cells that have no previous antibiotic exposure. At the current LEV dose, the burst and the intermediate release profiles are more effective than the slow release profile in terms of slowing down the biofilm recurrence because of their higher initial antibiotic concentrations. Nevertheless, neither of them is effective in inhibiting the growth of the surviving persister cells over the 6 days unless new dosing are administered daily.

The question that remains is whether the higher antibiotic tolerance acquired by the surviving persister cells is dependent on their antibiotic exposure on Day 1. For this reason, the effect of increasing the LEV dose on the biofilm eradication rate is investigated, where the total amount of LEV used is increased from 0.16 mg to 0.30 mg. The PLGA ESE nanoparticles are employed as their intermediate release profile allows the LEV concentration to remain above 1.10 $\mu\text{g/mL}$ for a prolonged period while at the same time providing a high initial antibiotic concentration needed for the purpose of this study.

The day-to-day variations of the LEV concentrations and the corresponding time-kill results are presented in Fig. 4.9. Owing to the higher dose, the LEV concentration on Day 1 is equal to 3.20 $\mu\text{g/mL}$ and the LEV concentrations do not fall below 1.10 $\mu\text{g/mL}$ for three days. On Day 1, $\approx 99.99\%$ of the biofilm cells are eradicated indicating the survival of the 0.01% subpopulation persister cells. On Days 2 and 3, the LEV concentrations ($\geq 1.10 \mu\text{g/mL}$) can sufficiently inhibit the growth of the surviving persister cells. As the LEV concentrations fall below 1.00 $\mu\text{g/mL}$ from Day 4 onwards, the biofilm growth picks up its pace resulting in a complete recovery of the biofilm cells on Day 5.

Figure 4.9: LEV concentration variations and time-kill results at a higher LEV dose.

The results from Days 2 and 3 indicate that the new MBIC value of the surviving persister cells is approximately equal to 1.10 $\mu\text{g/mL}$, which is more than seven times above the MBIC value prior to the antibiotic exposure. Interestingly, even though the LEV concentration drops to 0.60 $\mu\text{g/mL}$ on Day 4, the biofilm growth is relatively benign as only approximately two log (CFU/peg) new biofilm colonies are formed. The intermediate release of the higher LEV dose hence eliminates the need for daily dosing as the biofilm growth is suppressed for up to four days.

The time-kill results at a lower LEV dose presented earlier in Fig. 4.6B, however, suggest that 1.10 $\mu\text{g/mL}$ of LEV on Day 2 is insufficient to inhibit the biofilm growth of the surviving persister cells, despite the similar number of viable biofilm colonies initially present. The main difference between the two studies lies in their LEV concentration on Day 1, where the one conducted at the lower dose is at 2.25 $\mu\text{g/mL}$, whereas the one conducted at the higher dose is at 3.20 $\mu\text{g/mL}$. The results therefore suggest that the higher antibiotic tolerance of the surviving persister cells is dependent on their initial antibiotic exposure, where the higher the LEV concentration on Day 1, the lower the increase in the antibiotic tolerance of the surviving persister cells.

The importance of a high initial antibiotic exposure on the subsequent biofilm eradication rates denotes the preference of having a biphasic intermediate release profile, where the fast release in the beginning ensures the high initial antibiotic concentration, which is followed by the slower release that maintains the antibiotic

concentration above the new MBIC value of the surviving persister cells for a prolonged period. In this regard, a similar impact can be achieved using the burst release profile provided that the initial dose is sufficiently high, while still below the toxicity limit, to sustain the antibiotic concentrations above the new MBIC value between dosing. To obtain a high initial antibiotic exposure in the slow release profile, however, is not practical as it requires a significantly larger amount of nanoparticles due to the lower drug loading, hence limiting their feasibility to be delivered by inhalation.

The antibiotic susceptibility of the biofilm-derived planktonic cells is investigated to examine whether they respond in the same manner as the surviving biofilm persister cells in terms of acquiring a higher antibiotic tolerance upon their exposure to antibiotics. The results indicate that the surviving persister biofilm cells transfer their increased antibiotic tolerance to their progeny. The exposed biofilm-derived planktonic cells, however, do not necessarily become invulnerable towards antibiotics like the persister cell as the persister cells have been found to not transfer their invulnerability towards antibiotics to their progeny¹¹⁹.

The lower antibiotic susceptibility of the exposed biofilm-derived planktonic cells, however, can also be possibly caused by the presence of floating planktonic cells that originate from the non-eradicated mature biofilm cells, which are not present in the fresh biofilm-derived planktonic cell population. In this regard, mature biofilm cells are known to exhibit a higher antibiotic tolerance than the young biofilm cells because their cells are less permeable and less metabolically active¹²⁰. Nevertheless, mature biofilm cells are typically embedded deeply in the biofilm matrix, such that the ones shed into the planktonic state predominantly consist of young biofilm cells that occupy the periphery¹²⁰. Therefore, the role of the mature biofilm cells in the increased antibiotic tolerance of the exposed biofilm-derived planktonic cells is likely to be minimal though it cannot be ruled out completely.

4.5 Chapter Summary

The results and discussions presented in this chapter are based on:

W.S. Cheow, M.W. Chang, and K. Hadinoto. Antibacterial efficacy of inhalable levofloxacin loaded polymeric nanoparticles against *E. coli* biofilm cells: The effect of antibiotic release profile. *Pharm Res* 27:1597–1609 (2010).

In addition, similar findings using another antibiotic, ciprofloxacin, have been presented in:

W.S. Cheow, M.W. Chang, and K. Hadinoto. Antibiotic loaded polymeric nanoparticle formulation for inhaled antibiotic therapy against bacterial biofilm. *J of Biomed Nanotechnol* 6:391–403 (2010).

The biofilm susceptibility testing of the antibiotic-loaded nanoparticles signify the importance of the initial antibiotic exposure on the biofilm eradication rates, where the surviving biofilm cells exhibit a higher antibiotic tolerance than the fresh biofilm cells depending on the initial antibiotic concentration. In this regard, the lower the initial antibiotic exposure, the higher the increase in the antibiotic tolerance of the surviving biofilm cells. Furthermore, the surviving biofilm cells transfer their increased antibiotic tolerance to their biofilm-derived planktonic cell progeny hence promoting the exacerbations. A successful inhaled antibiotic therapy against biofilm infections requires a biphasic extended release profile, where the fast antibiotic release in the beginning ensures a high initial antibiotic concentration and the slower extended release sustains a sufficiently high antibiotic concentration to inhibit the biofilm growth and to minimize the exacerbation.

The requirement for a high initial antibiotic concentration necessitates a high loading of the antibiotic in the nanoparticles. While not the focus of the present chapter, the low LEV loading in the nanoparticles (0.3–1.1%) represent a shortcoming in the present antibiotic delivery system, where a high amount of nanoparticles need to be delivered to meet the therapeutic level. Therefore, the crucial development of LEV-loaded nanoparticles with a higher drug loading is addressed in Chapter 5.

Chapter 5

Enhancing the Encapsulation Efficiency of Antibiotic-Loaded Nanoparticles

5.1 Introduction

In this chapter, the encapsulation efficiency of the LEV-loaded nanoparticles first introduced in Chapter 4 is improved upon by applying some modifications to the antibiotic-preparation methods. LEV is highly efficacious against pulmonary biofilm cells as it does not have the propensity for sputum binding, unlike aminoglycoside antibiotics¹²¹⁻¹²². However, the high solubilities of LEV in both organic solvents (e.g. DCM, ethanol, acetone) and water ($\approx 200\text{--}300$ mg/mL at pH $\approx 2\text{--}8$) render its encapsulation in polymeric nanoparticles highly inefficient¹²³. The low encapsulation efficiency of LEV is particularly true when the nanoparticles are prepared by standard methods, such as NPC and ESE, as previously presented in Table 4.1. For these methods, high drug solubility in either the organic phase or the aqueous phase, but not both, is required to achieve high drug encapsulation efficiency, as the drug must not leak out from its original phase upon precipitations of the polymer chains to form the nanoparticles.

In this regard, several investigators have employed standard nanoparticle preparation methods to encapsulate drugs that are highly soluble in both water and organic solvents ($\propto 10^2$ mg/mL), such as procaine hydrochloride, sodium cromoglycate, and phenobarbital, into polymeric nanoparticles^{106, 124-125}. Not unexpectedly, low drug encapsulation efficiency ($< 20\%$ w/w) and low drug loadings ($\approx 1\%$ w/w) were reported. For this reason, modifications to the standard method were subsequently performed, including (i) incorporation of salt additives, (ii) pH variation, and (iii)

using alternative solvents, which led to significant improvements in the encapsulation efficiency ($\approx 40\text{--}50\%$ w/w).

The objective of the work in the present chapter is to investigate the effects of modifications of the standard nanoparticle preparation methods on the encapsulation efficiency and drug loading of levofloxacin in PLGA nanoparticles. Three different nanoparticle preparation methods (i.e. NPC, ESE, and double-ESE (DESE)) are examined. PLGA is selected as the polymeric carrier because of its higher encapsulation efficiency as found in Chapter 4.

In addition to the encapsulation efficiency and drug loading, the nanoparticles are evaluated in terms of their size, production yield, and *in vitro* drug release profile. In this regard, small nanoparticle sizes (< 300 nm) are desirable to facilitate an effective sputum penetration, whereas sustained antibiotic release profiles can potentially reduce the dosing frequency and lower the risk of systemic toxicity. Moreover, the feasibility of transforming the LEV-loaded PLGA nanoparticle suspension into inhalable dry powder nanoparticle aggregates by spray drying is investigated.

5.2 Methodology

LEV-loaded nanoparticles were prepared by using the standard NPC, ESE, and DESE methods, and were characterized for their size, encapsulation efficiency, drug loading, morphology and *in vitro* release using methods detailed in Sections 3.2.1–3.2.3. To increase the encapsulation efficiency of LEV in the PLGA nanoparticles, the standard nanoparticles preparation methods were modified systematically.

5.2.1 Modified nanoparticles preparation methods

In the modified ESE methods, single oil or aqueous phase modifications were performed. On the other hand, DESE can be modified in two ways—single phase modification or multiple-phase modifications. In the single phase modification, only the inner aqueous phase or only the oil phase was modified, while in the multiple phase modifications, both the aqueous and oil phases were modified simultaneously. Aqueous and oil phase modifications were performed by using 50% (w/w) surfactant replacement (PEG, chitosan, Tyloxapol, or phosphatidylcholine (PC)), or 50% (v/v) solvent replacement (acetone or ethyl acetate), respectively.

5.2.2 Spray-drying experiment

The LEV-loaded PLGA nanoparticle suspension was transformed into dry powder nanoparticle aggregates using a Büchi B-290 mini spray dryer (Büchi, Switzerland). Drying adjuvants were used (i) to protect the structural integrity of the nanoparticles upon drying and (ii) to ensure the nanoparticle aggregates can be reconstituted into primary nanoparticles upon their exposure to an aqueous environment. The particle geometric size (d_G) and shape were characterized using SEM, whereas the particle effective density (ρ_e) was determined using a tap densitometer (Quantachrome, USA)¹²⁶.

5.3 Results and Discussion

5.3.1 Nanoparticles prepared by the standard methods

5.3.1.1 Physical characteristics

A summary of the LEV-loaded PLGA nanoparticles produced by using the three standard methods (i.e. NPC, ESE, and DESE in Runs A–C, respectively) is presented in Table 5.1. Despite the similar initial amount of PLGA used (80–100 mg), nanoparticles prepared by the NPC method exhibit a considerably smaller size (≈ 100 nm) compared to that of nanoparticles prepared by the ESE and DESE methods (≈ 200 nm). The sizes of the three nanoparticles produced nevertheless remain within the range ideal for sputum penetrations¹¹⁴.

Table 5.1: Physical characteristics of nanoparticles prepared by the standard methods

Run	Preparation method	Size (nm)	% Encapsulation	Drug loading (%)
A	NPC	80 ± 30	15	0.7
B	ESE	190 ± 50	16	1.1
C	DESE	190 ± 80	10	1.0

The particle size distribution of the nanoparticles produced by the ESE method is presented in Fig. 5.1A, where a highly uniform distribution is observed. Similarly, uniform size distributions are also observed for the nanoparticles produced by the NPC and DESE methods. A SEM image of the nanoparticles in Fig. 5.1B indicates spherical nanoparticles are produced and verifies their highly uniform size distribution. The zeta

potentials of the three nanoparticles are in the range of (–) 10–30 mV indicating colloiddally-stable nanoparticles.

Figure 5.1: (A) Particle size distribution of LEV-loaded PLGA nanoparticles indicating a highly monodisperse distribution, and (B) their SEM image for verification.

In addition to the smaller size, the NPC method results in a lower production yield ($\approx 65\%$) than the ESE and DESE methods ($\approx 90\%$), which can be attributed to greater particle losses during the centrifugation and washing steps as a result of the smaller particle size. Due to the high aqueous solubility of LEV, the % encapsulations of LEV in nanoparticles prepared by the standard methods are expectably low ($\leq 16\%$ w/w) as LEV diffuses out into the aqueous phase during the nanoparticle preparation steps.

For the DESE method, the % encapsulation is also low due to the high LEV solubility in the oil phase, which leads to the escape of LEV from the inner to the outer aqueous phase. Consequently, drug loadings of the nanoparticles are also low ($\leq 1.1\%$ w/w), where the NPC method results in the lowest drug loading at 0.70% (w/w). Experimental uncertainties in the % encapsulation and drug loading measurements are between 4–8% and 5%, respectively, based on three replicates.

The extremely low drug loading of the nanoparticles produced by the NPC method is caused by LEV diffusion out into the aqueous phase, together with the water-miscible acetone upon PLGA precipitations, which results in LEV being predominantly adsorbed onto the nanoparticle surface. Consequently, LEV is washed off more easily during the centrifugation step, compared to when LEV is encapsulated within the polymer matrix, resulting in a low drug loading. The dominant presence of

LEV on the surface of the nanoparticles produced by the NPC method is manifested in its fast release profile as presented in the next section. The use of water-immiscible DCM in the ESE and DESE methods prevents LEV from being predominantly adsorbed onto the nanoparticle surface, which is reflected in the higher drug loading.

In this regard, the NPC and ESE methods are commonly used to encapsulate hydrophobic drugs into nanoparticles, where the lack of drug solubility in the aqueous phase ensures high encapsulation efficiency. Thus, when highly water-soluble drugs, such as LEV, are encapsulated using the NPC and ESE methods, % encapsulations achieved are expectably low. The DESE method, on the other hand, is typically employed to encapsulate water-soluble, but not solvent-soluble, drugs, as the inner aqueous phase acts as a reservoir in which the drug is dissolved, whereas the oil phase serves as a dissolution barrier preventing the drug leakage from the inner to the outer aqueous phase. For LEV, which is highly soluble in both the aqueous and oil/organic phases, standard NPC, ESE, and DESE methods have been shown to be incapable of producing high % encapsulations. Hence, modifications to the standard methods are needed to enhance the % encapsulation of LEV.

5.3.1.2 *In vitro* drug release

In vitro release profiles of LEV from the nanoparticles produced in Runs A–C are shown in Fig. 5.2. Experimental uncertainties in the % drug released, based on three replicates, are approximately 5%. As previously mentioned, nanoparticles prepared by the NPC method exhibit a monophasic burst release profile as a result of LEV being predominantly present on the nanoparticle surface. Specifically, $\approx 80\%$ of the loaded LEV is released within the first hour and the entire loaded drug is completely released within 3 h.

In contrast, nanoparticles prepared by the ESE and DESE methods display biphasic release profiles, where a high burst release in the first hour ($\approx 40\text{--}50\%$) is observed due to the presence of LEV on the nanoparticle surface. The burst release is followed by a slower release of the remaining LEV that is contributed by LEV that is encapsulated within the PLGA matrix. Not all of the loaded LEV, only 80–90%, however, is released after 24 h. Nevertheless, the extended biphasic LEV release profiles produced by the ESE and DESE methods are more desirable than the burst release profile as they can ensure that LEV concentrations are maintained above the

level needed to inhibit the biofilm growth in-between dosing¹²⁷, as well as reducing the risk of systemic toxicity.

Figure 5.2: *In vitro* drug release profiles from nanoparticles prepared by the standard methods.

Thus, nanoparticles prepared by the ESE and DESE methods are deemed superior compared to those prepared by the NPC method, because of (i) the higher production yield and drug loading, and (ii) the sustained release profiles. For this reason, subsequent modification attempts to improve the % encapsulation and drug loading are focused on the ESE and DESE methods. A summary of modifications that are attempted and their corresponding results (i.e. size, % encapsulation, and drug loading) is presented in Tables 5.2–5.4. The modifications reported in the present work are not aimed towards the more usual parameters, such as stirring speed, pH change, starting polymer and drug concentrations, or the volume ratio of the aqueous to organic phase, because these parameters have been found in our preliminary study to be non-influential towards the LEV encapsulation process.

Table 5.2: Physical characteristics of nanoparticles prepared by the modified ESE methods.

Run	Modified Phase	ESE Modification	Size (nm)	% encapsulation	Drug loading (%)
B1	Organic	50% (v/v) DCM : ethyl acetate	≈ 200	≈ 15	≈ 1.0
B2		50% (v/v) DCM : acetone			
B3	Aqueous	50% (w/w) PVA : PC	240 ± 50	23	2.3

5.3.2 Nanoparticles prepared by the modified ESE methods

Three modifications to the standard ESE method are performed, which are (1-2) using alternative solvents composed of 50% (v/v) mixture of DCM and either acetone or ethyl acetate in Runs B1 and B2, and (3) addition of 50% (w/w) PC into the aqueous PVA solution in Run B3. For the effect of using alternative solvents, precipitations of PLGA chains to form solid nanoparticles are slower when completely water-immiscible solvents, such as DCM, are used as it takes longer for the polymer molecules to be exposed to the aqueous phase upon emulsifications. The slower PLGA precipitation rate provides an opening for the highly water-soluble LEV to leak out of the organic phase into the aqueous phase resulting in the low % encapsulation.

For this reason, varying the water-miscibility level of the solvent is thought to potentially have an impact on the % encapsulation. The solvent composition is therefore varied from 100% (v/v) DCM in the standard ESE method to 50% (v/v) of DCM and ethyl acetate in Run B1, and to 50% (v/v) of DCM and acetone in Run B2. The water-miscibility levels of these mixtures increase in the following order: 100% (v/v) DCM < 50% (v/v) DCM/ethyl acetate < 50% (v/v) DCM/acetone, which is attributed to the partial-miscibility of ethyl acetate and full-miscibility of acetone in water.

Completely replacing DCM with a highly water-miscible solvent, such as acetone, however, is not pursued as it denotes a shift from the ESE method to the NPC method, which has been found to produce less-than-ideal nanoparticles. The results of Runs B1 and B2 in Table 5.2 nonetheless suggest that the effects of the water-miscibility level of the solvent on the % encapsulation and drug loading are minimal, where their respective values remain in the 15% (w/w) and 1.0% (w/w) ranges.

Hence, instead of attempting to narrow down the time-scale of the drug leak-out, the next modification attempt aims to incorporate physicochemical barriers into the nanoparticles, so that the drug leak-out can be minimized. In this regard, adding phosphatidylcholine (PC) into the aqueous phase during PLGA nanoparticle preparation has been shown to result in adsorptions of the amphiphilic PC onto the nanoparticle surface through hydrophobic interactions³⁴.

As LEV is lipophilic¹²⁸, such that it also interacts with the hydrophobic tails of the PC similar to PLGA, incorporating PC onto the nanoparticle surface can potentially enhance the % encapsulation by entrapping LEV in the phospholipid layer. Indeed, two-fold increases in the % encapsulation and drug loading to 23% and 2.3% (w/w), respectively, are observed in Run B3, where PC is added to the 1.0% (w/v) aqueous PVA solution at 50% (w/w) PVA to PC ratio. Moreover, in spite of the higher drug loading, the PC inclusion slows down the LEV release rate due to the hydrophobic interactions as shown in Fig. 5.3. As a result, the nanoparticles exhibit the ideal extended biphasic release profile.

Figure 5.3: Effect of PC inclusion on the *in vitro* drug release profile.

5.3.3 Nanoparticles prepared by the modified DESE methods

For the DESE method, the modifications are first performed one-at-a-time on either the inner aqueous phase or the oil phase of the w/o/w nano-emulsion to identify formulation parameters (e.g. solvent, surfactant) that have the most significant impacts on the % encapsulation. Subsequently, simultaneous modifications involving the significant parameters are carried out in both the inner/outer aqueous and oil phases.

5.3.3.1 Single-phase modifications

The effects of modifying the inner aqueous phase on the % encapsulation and drug loading are investigated by substituting DI water used in the standard DESE

method with 100% (v/v) PBS in Run C1, and 0.1% (w/v) aqueous PEG and PVA solutions in Runs C2 and C3, respectively. The oil and outer aqueous phases remain unchanged from the standard DESE method.

First, owed to the higher observed solubility of LEV in PBS than that in DI water, the use of PBS as the inner aqueous phase is thought to be able to increase the % encapsulation, as a result of LEV that remains longer in the aqueous core upon emulsifications due to the lower degree of supersaturation. However, the results of Run C1 presented in Table 5.3 suggest otherwise, where the % encapsulation and drug loading are lowered to 6% and 0.6% (w/w), respectively, from 10% and 1.0% (w/w) in the standard DESE method. The nanoparticle size is nevertheless unaffected and remains in the ≈ 200 –300 nm range.

Table 5.3: Physical characteristics of nanoparticles prepared by the modified single-phase DESE methods.

Run	Modified Phase	DESE Modification	Size (nm)	% encapsulation	Drug loading (%)
C1	Inner aqueous	PBS core	190 ± 70	6	0.6
C2		0.1% PEG (w/v)	200 ± 70	8	0.8
C3		0.1% PVA (w/v)	360 ± 120	22	2.1
C4	Oil	Acetone	110 ± 40	22	2.4
C5		Ethyl acetate	140 ± 50	20	2.1

Second, the inclusion of a surfactant in the inner aqueous phase of the DESE method has been shown at the micro-scale to enhance the primary w/o emulsion stability, which increases the aqueous pore volume of the carrier particles, resulting in higher % encapsulation and drug loading¹²⁹. For this reason, the inclusion of surfactants in the aqueous core is now attempted at the nano-scale to examine whether a similar finding is observed.

Using PEG as the surfactant, the results of Run C2 indicate that % encapsulation and drug loading are lowered to 8% and 0.8% (w/w), respectively, without any significant impacts on the nanoparticle size. In contrast, using PVA as the surfactant in Run C3 yields nanoparticles having higher % encapsulation and drug loading (i.e. 22% and 2.1% w/w, respectively). However, the nanoparticle size is increased to ≈ 400 –500 nm range, which makes them less suitable for sputum penetration.

Third, the modifications are performed in the oil phase by replacing the 100% (v/v) DCM in the standard DESE method with 50% (v/v) mixture of DCM/acetone in Run C4 and DCM/ethyl acetate in Run C5. DCM cannot be completely replaced as the DESE method requires the presence of a completely water-immiscible solvent. In this regard, the type of solvent used as the oil phase has been reported to impact the % drug encapsulation in nanoparticles due to the different nanoparticle precipitation rates depending on the water-miscibility level of the solvent used¹³⁰.

Specifically, in the second emulsification step, the polymer precipitates at a rate proportional to the water-miscibility level of the solvent, where the more water-miscible the solvent is, the faster the polymer precipitates to form w/o/w nano-emulsion as a result of the faster exposure to the aqueous phase. The faster the polymer precipitates and encapsulates the drug in the process, less drug leaks out from the inner to the outer aqueous phase, which in theory should improve the % encapsulation.

For this reason, the effect of varying the water-miscibility level of the oil phase is investigated in Runs C4 and C5 by adding miscible acetone and partially-miscible ethyl acetate into the DCM. In contrast to the results of a similar study in the ESE method (i.e. Runs B1–B2), the results in Table 5.3 indicate that increasing the water-miscibility level of the oil phase does lead to improvements in the % encapsulation and drug loading to 20–22% and 2.1–2.4% (w/w), respectively, which is more than two-fold higher than the values obtained from the standard DESE method. The slightly higher drug loading achieved in Run C4 is attributed to the higher water-miscibility of the DCM/acetone mixture compared to the DCM/ethyl acetate mixture.

Importantly, the higher drug loading does not jeopardize the nanoparticle sizes, which in fact are slightly reduced to be below 200 nm. Despite the increased drug loading, the *in vitro* LEV release profiles of Runs C4 and C5 presented in Fig. 5.4 are similar to that of the standard DESE method in Run C, where 90% of the drug is released within 6 h.

Figure 5.4: Effect of varying the water-miscibility level of the oil phase on the *in vitro* drug release profile.

5.3.3.2 Multiple-phase modifications

To examine whether further improvements in the % encapsulation and drug loading can be achieved, simultaneous modifications involving the inner and outer aqueous phases, as well as the oil phase, are investigated in Runs C6–C10 presented in Table 5.4. 0.1% (w/v) aqueous PVA solution is used as the inner aqueous phase in Runs C6–C10 as it has been found in Run C3 to significantly improve the % encapsulation and drug loading, though it also results in a larger nanoparticle size.

Table 5.4: Physical characteristics of nanoparticles prepared by the modified multiple-phase DESE methods.

Run	Inner aqueous phase	Oil phase	Outer aqueous phase	Size (nm)	% encapsulation	Drug loading (%)
C6	0.1% (w/v) PVA	DCM : Acetone	Standard	170 ± 80	6	0.5
C7		DCM : Ethyl acetate		180 ± 80	10	1.0
C8		Standard	50% (w/w) PEG	290 ± 110	11	1.0
C9			50% (w/w) Chitosan	720 ± 290	8	0.8
C10			50% (w/w) Tyloxapol	720 ± 340	4	0.4

The effects of simultaneously modifying the inner aqueous phase and the oil phase, while keeping the outer aqueous phase unchanged (i.e. 2.0% (w/v) aqueous PVA solution), are first examined in Runs C6 and C7. DCM/acetone and DCM/ethyl acetate mixtures are used as the oil phase in Runs C6 and C7, respectively, as they have been found to increase the % encapsulation in Runs C4–C5. The results, however,

indicate that a synergistic effect is not observed when the inner aqueous phase and the oil phase are modified simultaneously.

In fact, less favorable results in terms of the % encapsulation and drug loading are observed in Runs C6–C7 compared to the single-phase modification results. Specifically, the % encapsulation is back to be $\leq 10\%$ (w/w) similar to that obtained using the standard DESE method. Similarly, the drug loading falls below 1.0% (w/w) and the nanoparticle size is back in the $\approx 200\text{--}300$ nm range. The insolubility of PVA in the replacement solvents (acetone and ethyl acetate) may have negated the positive effect of PVA on the % encapsulation.

Subsequently, the effects of simultaneously modifying the inner and outer aqueous phases, while keeping the oil phase remains unchanged (i.e. 100% (v/v) DCM), are investigated in Runs C8–C10. The outer aqueous phase is modified by adding new surfactants or polymers widely used in inhaled drug delivery formulation, such as PEG, chitosan, and tyloxapol, into the 2.0% (w/v) aqueous PVA solution. The presence of these materials at 50% (w/w) ratio in the outer aqueous phase is thought to have the potential to influence the drug leak-out rate.

The results nonetheless indicate that the % encapsulation, drug loading, and nanoparticle size are significantly worse off compared to the single-phase modifications in Runs C3–C5. Specifically, the % encapsulations are low between 4–11% (w/w) resulting in low drug loadings between 0.1–1.0% (w/w). Furthermore, the nanoparticle sizes in Runs C9 and C10 are excessively large at ≈ 700 nm, hence signifying the importance of either PVA or PEG in maintaining the colloidal stability of the w/o/w nano-emulsion formed.

To summarize, for the DESE method, simultaneous modifications involving multiple phases negatively affect the % encapsulation and drug loading. In contrast, single-phase modifications in Runs C3–C5 result in two-fold improvements in the % encapsulation and drug loading. However, the large nanoparticle size produced in Run C3 (i.e. ≥ 400 nm) is undesirable due to the lack of sputum penetration ability. For the ESE method, the PC inclusion in Run B3 similarly results in a two-fold improvement in the % encapsulation and drug loading, while maintaining the nanoparticle size in the 200–300 nm range.

Importantly, the nanoparticles produced in Run B3 exhibit a slower sustained LEV release profile, which make them more attractive than those produced in Runs C4 and C5, despite the similarities in terms of their size, % encapsulation, drug loading, and production yield. In addition, the ESE method is simpler than the DESE method owed to the single-emulsification step. For this reason, the PC-modified nanoparticles produced in Run B3 are selected as the optimal formulation for further spray-drying and antibacterial activity testing experiments.

5.3.4 Spray-drying of LEV-loaded PLGA nanoparticles

The following spray-drying conditions are employed, i.e. inlet temperature = 100°C, feed rate = 2.8 mL/min, atomizing flow rate = 350 L/hour, and feed concentration = 1.0% (w/w). Due to the high drying temperature, which is above the glass transition temperature of PLGA (i.e. $\approx 35^\circ\text{C}$), the inclusion of leucine and PVA as drying adjuvants is necessary to protect the structural integrity of the PLGA nanoparticles. The ratio of the nanoparticles to the drying adjuvants is maintained at 1:1. Dry powder nanoparticle aggregates produced at this spray drying condition possess d_G between 6 and 9 μm and low ρ_{eff} ($\approx 0.3 \text{ g/cm}^3$) owed to the hollow morphology as shown in Fig. 5.5A. Due to the hollow morphology, d_A of these particles is between 3 and 5 μm rendering them suitable for inhaled delivery.

Importantly, the drug loading of the dry powder nanoparticles is similar in magnitude to that of the suspension form indicating a minimal drug loss in the drying process. Furthermore, these nanoparticle aggregates readily reconstitute into individual nanoparticles upon their exposure to an aqueous environment. The nanoparticle size after the re-constitution in Fig. 5.5B, however, is about twice larger than their size prior to the dry powder transformation, which is due to the coalescence of the PLGA nanoparticles upon exposure to a high temperature above their glass transition temperature as can be seen in Fig. 5.5C.

Figure 5.5: (A) SEM image of dry powder nanoparticle aggregates prepared by spray-drying, (B) particle size distribution of the nanoparticles before the dry powder transformation and after re-constitution, and (C) SEM image of the re-dispersed nanoparticles.

5.4 Chapter Summary

The findings presented in this chapter have been published as:

W.S. Cheow and K. Hadinoto. Enhancing encapsulation efficiency of highly water-soluble antibiotic in poly(lactic-co-glycolic acid) nanoparticles: modifications of standard nanoparticle preparation methods. *Colloids Surfaces A* 370:79–86 (2010).

In the present chapter, LEV-loaded PLGA nanoparticles are prepared by employing the standard and modified NPC, ESE and DESE methods. In comparison to the standard methods, single-phase modifications involving (i) the addition of PC into the aqueous phase (ESE) and (ii) the use of alternative solvents (DESE) result in two-fold improvements in the % encapsulation and drug loading (i.e. $\approx$ 22–24% and 2.1–2.4% w/w, respectively). Significantly, the experimental results indicate that simultaneous modifications involving multiple phases do not lead to additive effects of the single-phase modifications. In other words, doubling of % encapsulation when two single-phase modifications are performed separately does not translate to quadrupled % encapsulation when the two modifications are performed simultaneously.

The optimal formulation, which is evaluated in terms of the nanoparticle size, % encapsulation, drug loading, *in vitro* drug release profile, and production yield, has been identified in the form of PLGA–PVA/PC nanoparticles prepared by the ESE method. Significantly, the nanoparticles can be straightforwardly transformed into the inhalable solid dosage form by spray-drying with the inclusion of drying adjuvants. The antimicrobial efficacy of the nanoparticles in both suspension and dry powder form are investigated against *P. aeruginosa* biofilm cells in the chapter that follows.

Chapter 6

Anti-Biofilm Efficacy of Lipid–Polymer Hybrid Nanoparticles Encapsulating Antibiotics

6.1 Introduction

In the present chapter, the *in vitro* anti-biofilm efficacy of lipid–polymer hybrid nanoparticles encapsulating antibiotics against *P. aeruginosa* biofilm cells is examined. Two types of pharmaceutical dosage forms of the nanoparticles – aqueous suspension and dry powder – are employed. The anti-biofilm efficacy of the hybrid nanoparticles is assessed with respect to that of (i) the non-encapsulated antibiotic solution (i.e. native) and (ii) non-hybrid polymeric nanoparticles (i.e. no lipid coat, Fig. 6.1) to determine the roles of lipid, if any, on biofilm eradication. The antibacterial efficacy is also examined against planktonic cells that are steadily shed from the biofilm colonies as part of the biofilm mode of growth. These biofilm-derived planktonic cells are responsible for disease exacerbations by forming new biofilm colonies upon their release.

Figure 6.1: Antibiotic-loaded hybrid lipid–polymer nanoparticles (i.e. PLGA–PVA/PC) and their non-hybrid polymer nanoparticle counterparts (i.e. PLGA–PVA).

6.2 Methodology

The hybrid and non-hybrid nanoparticles prepared by the ESE method, as presented in the previous chapter (Chapter 5) are investigated for their anti-biofilm efficacy against *P. aeruginosa* in both suspension and dry powder form.

6.2.1 Biofilm growth protocols and biofilm susceptibility testing

Figure 6.2: Antibiotic susceptibility testing methodology against biofilm cells and biofilm-derived planktonic cells.

P. aeruginosa biofilm cells were grown according to the same procedures used to determine MBIC (Section 3.2.4.2). In the biofilm susceptibility testing (illustrated in Fig. 6.2), the biofilm cells were exposed to 20 μ L of nanoparticle suspension, or 20 μ L of reconstituted nanoparticles, or 20 μ L of native solution at known LEV concentrations in a new microplate (i.e. challenge plate), which contains 180 μ L of MHB as the growth medium. In the control experiment, the biofilm cells were exposed to 200 μ L of MHB. After 6, 12, and 24 h of static incubation at 37°C, the pegs were washed to remove loosely adhering bacterial cells and adsorbed nanoparticles (if any) by immersing the pegs in a new microplate containing 200 μ L of PBS (i.e. wash plate). Next, the biofilm cells were recovered from the pegs by sonication for 5 min into a

new microplate of 200 μ L PBS (i.e. recovery plate). The recovered biofilm cells were enumerated by serial 10-fold dilutions and LB agar plating to determine the viable CFU/peg.

For the biofilm-derived planktonic cells, optical density at 600 nm (i.e. OD₆₀₀) obtained using a microplate reader (Synergy HT, Biotek, USA) were used to assess the cell growth inhibition, where OD₆₀₀ $\leq$ 0.1 denotes zero bacterial growth¹³¹. The MIC was determined following the procedures in Section 3.2.4.1. The biofilm susceptibility testing was carried out using two replicates performed on two separate days.

6.3 Results and Discussion

6.3.1 Blank nanoparticle susceptibility testing

The susceptibility testing results of blank nanoparticles are presented in Fig. 6.3. The blank nanoparticles exhibit no antibacterial activities against both biofilm cells and biofilm-derived planktonic cells. The zero antibacterial activities are observed for both the hybrid and non-hybrid nanoparticles in both their wet and dry forms. Hence, bacterial cell eradications reported in the next sections are attributed exclusively to the antibiotic exposures, and not contributed by other formulation ingredients (e.g. PVA, leucine, PC, solvent trace). It must be noted here that the 1 day-old biofilm model used here does not exactly mimic the biofilm colonies found in the infected lung sites, which consists mainly of mature biofilm cells that have experienced previous antibiotic exposures, hence may possess different antibiotic tolerance levels. The present results therefore serve primarily to provide preliminary insights into the role of lipid in biofilm eradications by hybrid nanoparticles.

Figure 6.3: Non-existent antibacterial activities of blank nanoparticles of both PLGA-PVA and PLGA-PVA/PC against (A) biofilm-derived planktonic cells and (B) biofilm cells of *P. aeruginosa* at two different nanoparticle suspension concentrations – H = 0.63 mg/mL and L = 0.13 mg/mL.

6.3.2 Anti-biofilm efficacy of hybrid versus non-hybrid nanoparticles

Anti-biofilm efficacy of the wet PLGA-PVA/PC and PLGA-PVA nanoparticles against 1 day-old *P. aeruginosa* biofilm cells are presented in Fig. 6.4A. The biofilm susceptibility testing is performed at LEV concentration $\approx 3.5 \mu\text{g/mL}$ over a 24 h period. The total amount of LEV released *in vitro* in 24 h and the drug loading value are used to calculate the corresponding amount of nanoparticle suspension or dry powder to be used in the biofilm susceptibility testing. The LEV concentration is selected to be above the MIC ($\approx 2 \mu\text{g/mL}$) as the biofilm mode of growth typically

leads to higher antibiotic tolerance. In the control experiment, the viable cell counts remain in the range of ≈ 7.5 – 8.0 CFU/peg over the 24 h period signifying constant biofilm cell populations.

Figure 6.4: Anti-biofilm efficacy of PLGA-PVA and PLGA-PVA/PC nanoparticles against *P. aeruginosa* biofilm cells at 3.5 µg/mL LEV concentration – (A) aqueous suspension; (B) dry powders.

After 6 and 12 h of LEV exposures, the viable cell counts, after taking into account the experimental uncertainties (± 0.4 CFU/peg), are comparable at ≈ 6.0 CFU/peg between cells that have been exposed to the native LEV solution and those that have been exposed to the nanoparticles. The similarity in the viable cell counts of the three runs, which indicate ≈ 90 – 95% biofilm eradication rates, is observed despite the cells being exposed to different LEV concentrations that are attributed to the different LEV release rates, albeit the same total LEV concentration (i.e. 3.5 µg/mL) is used over 24 h. For example, using the native solution, biofilm cells are exposed to 3.5 µg/mL of LEV from the start of the susceptibility testing, whereas using the nanoparticles, biofilm cells are exposed to 2.1 and 2.8 µg/mL of LEV only at the 6th h for the PLGA-PVA/PC and PLGA-PVA nanoparticles, respectively. The results indicate that biofilm eradication rates in the first 12 h are independent of the nanoparticle formulation, as well as the LEV concentration, provided that the concentration is above the MIC.

After 24 h of LEV exposures, however, the viable fraction of the biofilm cells exposed to the PLGA-PVA/PC nanoparticles are lower at ≈ 4.5 CFU/peg denoting 99.9% eradication rate. On the other hand, biofilm cells that are exposed to the native

solution and PLGA–PVA nanoparticles remain at ≈ 6.0 CFU/peg. As the amounts of LEV released after 24 h are identical, the variations in the viable cell counts at the 24th hour are likely caused by the difference in the nanoparticle formulations. Specifically, the presence of PC in the hybrid nanoparticle formulation, which affects nanoparticle size and colloidal stability, seems to impact the biofilm eradication rate.

Next, anti-biofilm efficacy of the dry nanoparticles is examined in Fig. 6.4B to see whether a similar trend is observed. The results indicate that the viable cell counts in Fig. 6.4B are highly comparable in magnitude to those presented in Fig. 6.4A. The dry nanoparticles therefore possess very similar anti-biofilm efficacy as the wet nanoparticles, which is not unexpected as the nanoparticle aggregate powders can fully reconstitute into nano-scale particles. Importantly, these results reaffirm the earlier finding that the anti-biofilm efficacy of the hybrid nanoparticles is higher than the polymeric nanoparticles. Interestingly, the higher anti-biofilm efficacy is only observed at the 24th hour in both studies, but not at earlier incubation times.

Figure 6.5: Dose-kill results of PLGA–PVA and PLGA–PVA/PC nanoparticles at time = 24 h.

In further investigations, the biofilm susceptibility testing is performed at a wide range of LEV concentrations above the MIC (i.e. 2.6–12.6 μg/mL). Significantly, the dose-kill curve after 24 h of LEV exposures (Fig. 6.5), which include the results of both the wet and dry nanoparticles, indicates that the PLGA–PVA/PC nanoparticles consistently produce higher anti-biofilm efficacy than the PLGA–PVA nanoparticles across the LEV concentration range investigated. The viable cell counts in the

presence of the PLGA–PVA/PC nanoparticles remain constant at ≈ 4 CFU/peg even at the highest LEV concentration investigated. A similar trend is observed for the PLGA–PVA nanoparticles, though at higher viable cell counts of ≈ 6 CFU/peg.

The dose-kill results hence indicate that increasing the LEV concentration above the MIC has minimal impacts on the eradication rates of 1 day-old biofilm cells. Importantly, the dose-kill results reaffirm our earlier finding that hybrid nanoparticles produce higher anti-biofilm efficacy than their non-hybrid counterparts. In this regard, the variation in the LEV release rates between the PLGA–PVA and PLGA–PVA/PVC nanoparticles is initially thought to be responsible for the difference in their anti-biofilm efficacy, as biofilm cells are known to possess adaptive defence mechanisms depending on their antibiotic exposure history, which may explain why the higher anti-biofilm efficacy is observed only at 24 h¹³². The dose-kill results, however, indicate that different levels of antibiotic exposures have minimal impacts on biofilm eradication, therefore ruling out the difference in the LEV release rates as the cause for the higher anti-biofilm efficacy of the hybrid nanoparticles.

6.3.3 The higher anti-biofilm efficacy of hybrid nanoparticles

As liposomes have been found to possess affinity towards biofilm cells, the presence of PC in the hybrid nanoparticles is thought to confer them similar biofilm affinity, where they are readily adsorbed onto the biofilm matrix and release the antibiotic in the vicinity of the biofilm cells resulting in higher biofilm eradication rates. Another possibility is that the biofilm-adsorbed nanoparticles may promote biofilm cell detachment into the planktonic state. To evaluate this hypothesis, the extent of nanoparticle adsorption onto the biofilm matrix is investigated using blank nanoparticles at the same suspension concentration as that employed in the biofilm susceptibility testing. Blank nanoparticles are employed because their presence has been found to have negligible impacts on the viable cell counts (Fig. 6.3). In other words, blank nanoparticles do not impact biofilm cell detachment, where the amount of shed biofilm cells in the presence of blank nanoparticles remains comparable to that of the control experiment.

As a result, nanoparticles adsorption onto biofilm can be qualitatively examined by comparing OD₆₀₀ of the wash and recovery plates in the presence of blank nanoparticles with that of the control experiment. By first assuming that nanoparticles

are readily adsorbed onto the biofilm matrix, when biofilm cells are exposed to blank nanoparticles, OD₆₀₀ of the wash plate consists of contributions from the loosely-attached biofilm cells and the nanoparticles, which are desorbed upon washing. On the other hand, OD₆₀₀ of the wash plate in the control experiment is contributed only by the biofilm cells, such that its value is anticipated to be lower, owed to less turbidity, than that obtained in the presence of blank nanoparticles.

Figure 6.6: OD₆₀₀ measurements of the suspensions recovered in the microplate wells upon exposure to blank nanoparticles – (A) wash plate; (B) recovery plate.

The results in Fig. 6.6A indicate that for runs in which the biofilm cells are exposed to either wet or dry blank PLGA-PVA/PC nanoparticles, OD₆₀₀ of the wash plate are comparable in magnitude to that of the control experiment, after taking into account the experimental uncertainties. These results can either indicate that (i) PLGA-PVA/PC nanoparticles are not desorbed upon washing owed to their strong interaction with the biofilm matrix, or (ii) they are not adsorbed onto the biofilm matrix in the first place and remain in the challenge plate. To find out, OD₆₀₀ of the recovery plate is examined in Fig. 6.6B as the sonication in the recovery step ensures detachment of all substances occupying the pegs. If the PLGA-PVA/PC nanoparticles are indeed adsorbed onto the biofilm matrix, OD₆₀₀ of the recovery plate will be higher than that of the control experiment.

The results, however, indicate that OD₆₀₀ of the recovery plate, in the presence of either wet or dry PLGA-PVA/PC nanoparticles, are comparable to that of the control experiment, which suggest that the PLGA-PVA/PC nanoparticles are not adsorbed onto the biofilm matrix in the first place. For comparison, a similar study is carried out

for the blank PLGA–PVA nanoparticles. In contrast, the results in Fig. 6.6A indicate that OD₆₀₀ of the wash plate in the presence of the wet PLGA–PVA nanoparticles is considerably higher than that of the control experiment, which seems to suggest that PLGA–PVA nanoparticles are adsorbed onto the biofilm matrix and subsequently desorbed upon washing. However, the higher OD₆₀₀ of the wash plate is only observed for the wet nanoparticles, but not for the dry nanoparticles.

It has to be pointed out that the drug loading of the PLGA–PVA nanoparticles is approximately half that of the PLGA–PVA/PC nanoparticles, such that a larger number of PLGA–PVA nanoparticles (i.e. doubled) are required to achieve the same LEV concentration. As the present OD₆₀₀ study is performed at the same nanoparticle suspension concentration as the susceptibility testing, it is plausible that the higher OD₆₀₀ of the wash plate in Fig. 6.6A in the presence of the wet PLGA–PVA nanoparticles is merely caused by a large number of nanoparticles present in the vicinity of the pegs, which are transferred to the pegs by capillary forces. If there was such a vast difference in the biofilm adsorption between the wet and dry PLGA–PVA nanoparticles, their anti-biofilm efficacy in Fig. 6.4 would not likely be comparable to each other. Moreover, OD₆₀₀ of the recovery plate, in the presence of either the wet or dry PLGA–PVA nanoparticles, are found to be comparable to that of the control experiment (Fig. 6.6B), which suggest minimal biofilm adsorption of the PLGA–PVA nanoparticles similar to the PLGA–PVA/PC nanoparticles.

To summarize, OD₆₀₀ results of the wash and recovery plates suggest that both the PLGA–PVA/PC and PLGA–PVA nanoparticles are not adsorbed, or weakly adsorbed at best, onto the biofilm matrix. Contrary to our initial supposition, the lipid inclusion into the polymeric nanoparticle does not lead to an improvement in the affinity of the nanoparticles towards biofilm cells. Nonetheless, the lipid inclusion does lead to higher anti-biofilm efficacy, though the reason for that is yet to be determined. In this regard, the roles of the LEV release profile and biofilm adsorption in enhancing the anti-biofilm efficacy have been found to be minimal.

Significantly, the higher anti-biofilm efficacy of the hybrid nanoparticles does not translate to their higher efficacy against biofilm-derived planktonic cells. OD₆₀₀ of the biofilm-derived planktonic cells in the challenge plate (Fig. 6.7), which are exposed to the PLGA–PVA and PLGA–PVA/PC nanoparticles for 24 h, are found to be

comparable to each other. For the two LEV concentrations investigated (i.e. 3.5 and 12.6 $\mu\text{g/mL}$), OD_{600} varies within a narrow range of 0.05–0.15 denoting minimal planktonic cell growth. Identical OD_{600} results are found for 6 and 12 h incubations (Fig. 6.8). These results suggest that the higher anti-biofilm efficacy of the PLGA–PVA/PC nanoparticles is not caused by either (i) enhancement of LEV antibacterial activity in the presence of lipid, or (ii) increased biofilm cell detachment into the planktonic state due to the presence of the nanoparticles, where the biofilm cells are not necessarily eradicated.

Figure 6.7: Antibacterial efficacy against biofilm-derived planktonic cells in the challenge plate at two LEV concentrations after 24 h incubation – High (H) = 12.6 $\mu\text{g/mL}$; Low (L) = 3.5 $\mu\text{g/mL}$.

Instead, the higher anti-biofilm efficacy is likely attributed to a larger number of biofilm cells in the matrix are being eradicated. To identify the exact cause of the higher biofilm eradication rate, however, a detailed study at the length scale of the biofilm matrix and at the time scale of the antibiotic molecular diffusion needs to be conducted. One possible scenario, which remains to be validated experimentally, is that interactions between the lipid coat of the hybrid nanoparticles and EPS produced by the biofilm cells may lead to lipid layer disruptions and eventually its disengagements. In this regard, lipid–EPS interactions in liposomes have been found to disrupt the phospholipid bilayer¹³³⁻¹³⁴. The presence of freed lipid molecules due to

lipid–EPS interactions may enhance LEV diffusion into the biofilm matrix as the lipid interacts with various components of the EPS matrix, which otherwise would bind the antibiotics, hence freeing the antibiotic to reach the bacterial cells resulting in the higher biofilm eradication rate. Further studies employing (i) different types of lipids and antibiotics, (ii) EPS-defective and EPS-overproducing *P. aeruginosa* strains, and (iii) mature biofilm colonies are needed to elucidate the exact roles of lipid in enhancing the *in vitro* anti-biofilm efficacy of lipid–polymer hybrid nanoparticles.

Figure 6.8: OD₆₀₀ measurements of the biofilm-derived planktonic cells in the challenge plate exposed to the PLGA–PVA and PLGA–PVA/PC nanoparticles at two different LEV concentration – High (H) = 12.6 µg/mL; Low (L) = 3.5 µg/mL.

6.4 Chapter Summary

The results discussed in this chapter have been published as:

W.S. Cheow, M.W. Chang, and K. Hadinoto. The roles of lipid in anti-biofilm efficacy of lipid–polymer hybrid nanoparticles encapsulating antibiotics. *Colloids Surfaces A* 389:158–165 (2011).

Lipid–polymer hybrid nanoparticles, which are constituted of PLGA nanoparticle core and phosphatidylcholine lipid coat, have been found to exhibit higher antibacterial

efficacy against 1 day-old *P. aeruginosa* biofilm cells than their non-hybrid PLGA nanoparticle counterparts (i.e. 0.1% versus 5% survival rates). The difference in the anti-biofilm efficacy between the hybrid and non-hybrid nanoparticles nevertheless is evident only after 24 h of antibiotic exposures, and not earlier. The higher anti-biofilm efficacy is observed across a wide range of antibiotic concentrations and for nanoparticles in both their aqueous suspension and dry powder forms. The higher antibacterial efficacy, however, is not observed against planktonic cells that are steadily shed from the biofilm matrix, which consequently rules out lipid-induced enhancement of the antibiotic activity as the factor responsible for the high anti-biofilm efficacy of the hybrid nanoparticles.

Despite the presence of lipid, the hybrid nanoparticles, unlike liposomes, have been found to be not superior in terms of their affinity towards biofilm cells. Therefore, increased biofilm cell detachment into the planktonic state, which might be induced by biofilm-adsorbed nanoparticles, is not the cause for the higher biofilm eradication rate either. Furthermore, the impact of antibiotic exposure levels on the biofilm eradication rate has been found to be minimal, hence ruling out the more sustained antibiotic release rate in the hybrid nanoparticles as the factor contributing to the higher anti-biofilm efficacy. Hence, it is thought that the presence of lipid may have enhanced the antibiotic diffusion into the biofilm matrix resulting in more effective biofilm cell eradications. Follow-up studies that extend beyond biofilm susceptibility testing are needed to identify the mechanisms responsible for the higher anti-biofilm efficacy of the hybrid nanoparticles.

The identification of the PLGA–PC nanoparticles prepared by the ESE method in Chapter 5 as the optimal formulation, and the superior anti-biofilm activity of the hybrid nanoparticles presented in this chapter put forward the questions of whether (i) the use of PC as the sole stabilizer (instead of a combination of PC and PVA) will further increase the drug loading, and (ii) whether the encapsulation of other antibiotics using PLGA–PC will similarly result in improved drug loading. These questions are addressed in the next chapter.

Chapter 7

Factors Affecting Antibiotic Encapsulation and Stability of Lipid–Polymer Hybrid Nanoparticles

7.1 Introduction

Drawing from the results in Chapter 5–6 that shows that (i) modification with lipid doubles the encapsulation efficiency of LEV in PLGA nanoparticles, and (ii) lipid–polymer hybrid nanoparticles result in higher anti-biofilm efficacy, follow-up experiments shows that total replacement of PVA with PC is possible. In this chapter, the robustness of the emulsification-based method in encapsulating antibiotics with different physical properties (i.e. aqueous solubility, lipophilicity, and ionicity) into PC-only hybrid nanoparticles is investigated. The use of the emulsification-based method means that both water-insoluble and water-soluble drugs can likely be encapsulated into lipid–polymer hybrid nanoparticles as both drug types have been encapsulated into polymeric nanoparticles, in the absence of lipids, by employing single and double-emulsification methods, respectively. In this regard, the use of the DESE method to encapsulate a water-soluble drug, which is not soluble in the oil phase, into hybrid nanoparticles is attempted for the first time in the present chapter.

The polymer PLGA and PC, a major constituent of lung surfactants¹³⁵, are employed as the polymer and lipid components, respectively. Two other fluoroquinolones, the preferred class of antibiotics used for the treatment of pulmonary infections¹³⁶, i.e. CIP and OFX, in addition to LEV, are used as the model antibiotics. One run using aminoglycoside antibiotic, tobramycin (TOB), is also performed for validation purposes. The ionicities of these antibiotics vary, with LEV and OFX being zwitterionic, whereas CIP and TOB are cationic. In addition, their aqueous solubilities

also vary ranging from 3.2–500 mg/mL with OFX being the least soluble and TOB the most soluble. The hybrid nanoparticles are evaluated in terms of their size, drug encapsulation efficiency, drug loading, *in vitro* release profile, and their stabilities in both aqueous and salt solutions (i.e. phosphate buffer saline, PBS). The hybrid nanoparticle characteristics are evaluated with respect to those of PLGA nanoparticles prepared using a non-lipid surfactant (i.e. PVA). While these antibiotic-encapsulating hybrid nanoparticles are developed with anti-biofilm therapy in mind, with some tailoring of the size and drug loading, they are applicable to other drug delivery needs.

7.2 Methodology

7.2.1 Preparation and characterization of PVA-free antibiotic-loaded hybrid nanoparticles

In contrast to the hybrid nanoparticles in the previous two chapters, the antibiotic-loaded hybrid nanoparticles here were prepared without PVA as the surfactant. The DESE method (Section 3.2.1.3) was employed to prepare hybrid nanoparticles loaded with CIP and TOB, which are both water-soluble, but not soluble in the oil phase. The DESE method was also used to prepare nanoparticles loaded with LEV, which is soluble in both aqueous and oil phases. To encapsulate OFX, which is only soluble in the oil phase, the single emulsification method i.e. ESE (Section 3.2.1.2), was employed. Table 7.1 summarizes the antibiotic solubilities in the oil and aqueous phases, as well as the preparation method and phase in which the antibiotics were dissolved.

Table 7.1: Summary of the different nanoparticle preparation methods used for different antibiotics.

Antibiotic	Aqueous solubility	Solubility in oil phase	Preparation technique	Dissolution media
LEV	Soluble	Soluble	DESE	DI water
CIP	Insoluble	Insoluble	DESE	Acetic acid solution
TOB	Soluble	Insoluble	DESE	DI water
OFX	Sparingly soluble	Soluble	ESE	DCM

Specifically, when PC was used as the surfactant, it was dissolved in the oil phase. When SA and TPGS were included in the formulation for lipid charge reversal and stabilization, respectively, they were co-dissolved with PC in the oil phase. To prepare CIP-loaded hybrid nanoparticles, 9 mg of CIP was dissolved in an acetic acid solution

(pH $\approx$ 3) acting as the internal aqueous phase (w_1). The pH adjustment of the internal aqueous phase was necessary due to the low CIP solubility at pH $\approx$ 5–9¹³⁷. OFX was directly dissolved in the oil phase with all other parameters unchanged in order to have a consistent methodology. For non-hybrid nanoparticles, PVA was used as the surfactant and it was dissolved in the external aqueous phase (w_2) at 2% (w/v).

The physical characteristics of the hybrid nanoparticles were determined using standard characterization methods presented in Sections 3.2.2 and 3.2.3, while the nanoparticle production yield was determined from Eq. 7.1.

$$\text{Yield} = \frac{\text{Mass of nanoparticles recovered}}{\text{Mass of polymer added} + \text{mass of lipid added}} \quad (7.1)$$

7.3 Results and Discussion

7.3.1 Optimal lipid amount in the hybrid nanoparticle preparation

In addition to promoting biocompatibility and acting as physical barrier that minimizes drug leakage from the nanoparticles, the PC lipid layer also functions as a surfactant stabilizer of the o/w or $w_1/o/w_2$ emulsions, and subsequently functions as stabilizer of the nanoparticle suspension upon evaporation of the oil phase. Therefore, the optimal amount of PC required to prepare the hybrid nanoparticles must first be determined. Fig. 7.1 presents the size and production yield of drug-free nanoparticles produced by the ESE method when the mass of PC added into the formulation is varied as a percentage of the PLGA mass (i.e. W_{PC}/W_{PLGA}).

Figure 7.1: Size and production yield of hybrid nanoparticles prepared with 5–90% W_{PC}/W_{PLGA} .

At W_{PC}/W_{PLGA} values of 5–15%, the particles formed are large with sizes between 800–1000 nm. However, increasing W_{PC}/W_{PLGA} above 15% sharply decreases the nanoparticle size, though no further decrease in size is observed even when W_{PC}/W_{PLGA} is increased up to 90%. When the amount of PC added into the formulation is insufficient, the low emulsion stability causes the oil droplets to coalesce resulting in the formation of large particles. Thus, in terms of the nanoparticle size, 30% W_{PC}/W_{PLGA} is determined as the optimal PC amount.

Importantly, 30% W_{PC}/W_{PLGA} also represents the optimal amount of PC to contribute to a reasonable production yield of about 80%. Below 30% W_{PC}/W_{PLGA} , the coalescence of oil droplets contributes to polymer clumping that lowers the yield. On the other hand, not all the lipids are incorporated into the nanoparticles when $W_{PC}/W_{PLGA} = 90\%$ is employed therefore lowering the yield to about 45%. Thus, the optimal PC amount that yields reasonable nanoparticle size and production yield is 30% W_{PC}/W_{PLGA} , which translates to ≈ 30 mg of PC. This PC amount is used in the subsequent experiments for both the ESE and DESE methods.

Significantly, compared to the amount of PVA needed in the non-hybrid nanoparticle preparation, which is approximately 240 mg or 2% (w/v) in the aqueous phase, the hybrid nanoparticles evidently results in less material wastage, as not only a smaller amount of surfactant is used, but also the majority of lipid is incorporated into the hybrid nanoparticles, whereas only a minute amount of PVA ($\approx 2\%$ w/w nanoparticles) remains adsorbed on the non-hybrid nanoparticles⁹³.

7.3.2 Role of drug-lipid charge interaction

For the fluoroquinolones, the initial results reveal that only LEV and OFX are successfully encapsulated in the PLGA–PC nanoparticles, while attempts to encapsulate CIP fail to produce any nanoparticles, but instead they result in the formation of polymer and lipid aggregates. Since the CIP formulation involves a prior dissolution in acetic acid solution to form the internal aqueous phase, it is reasonable to assume that the presence of acetic acid may have affected the role of PC as a surfactant stabilizer.

Omitting CIP from the formulation (i.e. blank nanoparticles are prepared with acetic acid solution as the internal aqueous phase), however, successfully leads to production of nanoparticle suspension. Thus, the failure to form hybrid nanoparticles appears to be specific to the combination of CIP as drug and PC as surfactant, and is not caused by the acetic acid inclusion. In order to further elucidate the cause behind the failure in forming CIP-encapsulated hybrid nanoparticles, PC is replaced with PVA in the subsequent experiment. Using PVA, CIP-encapsulated nanoparticles are successfully produced, thus validating our earlier conjecture that simultaneous uses of PC and CIP leads to failed nanoparticle formation.

One major difference between CIP, which fails to be encapsulated, and the other successfully encapsulated antibiotics, is the ionicity of CIP. Table 7.2 shows the ionicities of the antibiotics and surfactants used in the nanoparticle formulation. While LEV and OFX, both of which can be encapsulated in the hybrid nanoparticles are zwitterionic, CIP is cationic. On the same note, ionicities of the surfactants also vary, where PVA is non-ionic and PC is anionic. Taking into account the failed nanoparticle formation when PC and CIP are used, whereas nanoparticles are successfully produced using PVA and CIP, the possibility of ionic interaction between the anionic PC and cationic CIP as the cause for the failed nanoparticle formation cannot be ignored.

Table 7.2: Summary of the ionicities of the antibiotics and surfactants used.

Category	Compound	Ionicity
Antibiotic	LEV	Zwitterionic
	CIP	Cationic
	OFX	Zwitterionic
Surfactant	PC	Anionic
	PVA	Non-ionic
	PC/SA	Cationic

For this reason, a trial experiment to encapsulate a different cationic antibiotic, TOB, is performed. Unsuccessful attempts to prepare TOB-encapsulated nanoparticles using PC as the surfactant corroborate the view that drug-lipid ionic interaction is the root cause for the failure in forming nanoparticles. Therefore, CIP-encapsulated nanoparticles theoretically should be able to be produced once the drug-lipid ionic interaction is neutralized by charge reversal of either the drug or lipid components.

In this regard, the anionic PC can be converted into cationic PC by replacing 10% (w/w) of the total PC amount (≈ 3 mg) with SA, which is a cationic lipid commonly used in a small amount to prepare cationic liposomes. Importantly, SA has been shown to have a negligible toxicity in *in vivo* studies¹³⁸⁻¹⁴⁰. As anticipated, the reversal of the lipid charge from anionic to cationic by the addition of 10% (w/w) SA results in a successful formation of CIP-encapsulated hybrid nanoparticles (i.e. PLGA–PC/SA). Hence, from the series of experiments performed, it can be concluded that ionic interactions between CIP and PC are indeed responsible for the failure of nanoparticle formation in the original formulation.

7.3.3 Evaluations of hybrid versus non-hybrid nanoparticles

Owing to the non-ionic nature of PVA, encapsulation of drugs of different ionicities into the non-hybrid nanoparticles is more straightforward than that into the hybrid nanoparticles, where drug-lipid ionic interaction must be taken into account. Nonetheless, % encapsulation and consequently the drug loading for the non-hybrid nanoparticles is anticipated to be lower than that for the hybrid nanoparticles due to the absence of the lipid barrier in the non-hybrid nanoparticles. Table 7.3 presents a summary of the zeta potential, size, and % encapsulation of the PLGA–PC nanoparticles encapsulating LEV and OFX, and the PLGA–PC/SA nanoparticles encapsulating CIP. The results of their non-hybrid nanoparticle counterparts (i.e. PLGA/PVA) are also presented in Table 7.3 for comparison.

Table 7.3: Characteristics of the antibiotic-encapsulated nanoparticles prepared using different surfactants.

Surfactant	Antibiotic	Zeta potential (mV)	Size (nm)	% encapsulation
PC or PC/SA	LEV	–26	420 $\pm$ 150	19
	CIP	+26	260 $\pm$ 90	4
	OFX	–22	360 $\pm$ 130	25
PVA	LEV	–12	300 $\pm$ 100	10
	CIP	–9	120 $\pm$ 40	4
	OFX	–12	270 $\pm$ 90	12

With the exception of the CIP-encapsulated PLGA–PC/SA nanoparticles, all of the nanoparticles produced possess negative zeta potential. In general, the zeta potential magnitude of the PLGA–PC nanoparticles at (–) 22–26 mV is higher than that of the PLGA–PVA nanoparticles at (–) 9–12 mV, which is attributed to the non-

ionic nature of PVA as opposed to the anionic nature of PC. The zeta potential of the CIP-encapsulated PLGA-PC/SA nanoparticles, on the other hand, is (+) 26 mV because of the cationic lipid coat.

In terms of the nanoparticle size, the PLGA-PC and PLGA-PC/SA nanoparticles are generally larger than the PLGA-PVA nanoparticles by ≈ 100 nm for the three antibiotics, which is likely due to the lipid coat. SEM image of the OFX-loaded PLGA-PC nanoparticles in Fig. 7.2A indicate that the nanoparticles are spherical with clearly defined boundaries despite the freeze drying step prior to imaging. The nanoparticle size observed in the SEM micrograph agrees well with that measured by PCS. As the other formulations result in similar morphologies, their SEM images are not presented here. Curiously, the nanoparticle size decreases from $\text{LEV} > \text{OFX} > \text{CIP}$ regardless of whether PC or PVA is used and whether the ESE or DESE method is employed.

Figure 7.2: (A) SEM image of the OFX-encapsulated PLGA-PC nanoparticles and (B) drug loading values of antibiotic-encapsulated PLGA-PC and PLGA-PVA nanoparticles plotted against antibiotic solubility.

In terms of % encapsulation, the results indicate that % encapsulation is in the order of $\text{OFX} > \text{LEV} > \text{CIP}$ for both the hybrid and non-hybrid nanoparticles with values ranging from 4–20% and 4–12%, respectively. The experimental uncertainty in % encapsulation is approximately $\pm 7\%$. These values of % encapsulation result in drug loading between 0.5% and 2.7% (w/w). When drug loadings of the three antibiotics are plotted as a function of their aqueous solubility in Fig. 7.2B, the drug loading is found to be inversely proportional, in an almost linear trend, to the solubility. The aqueous

solubility of CIP presented in Fig. 7.2B is taken to be its solubility in the internal aqueous phase (i.e. aqueous acetic acid solution).

This trend in the drug loading, which is observed in the PLGA-PC, PLGA-PC/SA, and PLGA-PVA nanoparticles, is not unexpected considering that % encapsulation, which dictates the drug loading, is governed by the propensity of the antibiotic to leak out of the nanoparticles during the preparation step, which depends on the aqueous solubility. Thus, OFX, with the lowest aqueous solubility (≈ 3.2 mg/mL), remains in the oil phase upon the emulsification step, such that a majority of OFX is encapsulated in the PLGA matrix resulting in the highest drug loading. In contrast, CIP with the highest solubility (≈ 120 mg/mL) rapidly leaks out of the nanoparticles upon the second emulsification step causing the lowest drug loading. Not unexpectedly, LEV with the intermediate solubility (≈ 30 mg/mL) results in the intermediate drug loading.

In addition, the drug loadings of the PLGA-PC nanoparticles (i.e. 2.0–2.7%) are consistently double that of the PLGA-PVA nanoparticles (i.e. 1.0–1.3%), for both OFX and LEV. The drug loading of the CIP-encapsulated nanoparticles, however, remains low at 0.4% regardless of whether PVA or PC/SA is used as the surfactant. The lack of improvement in the drug loading of the CIP-encapsulated PLGA-PC/SA nanoparticles is consistent with an earlier report that found LEV and OFX to be more lipophilic compared to CIP¹⁴¹. Thus, for lipophilic LEV and OFX, the lipid coat minimizes the loss of drugs during the preparation as the drugs interact with the lipid.

To summarize, the nanoparticle size is governed by the antibiotic type, and is less influenced by the preparation method or the surfactant used, as similar findings are observed for both hybrid and non-hybrid nanoparticles. The hybrid nanoparticles are larger and exhibit larger zeta potentials than their non-hybrid counterparts independent of the antibiotic type. The drug loading is governed by the drug solubility in the aqueous phase, which again is observed in both hybrid and non-hybrid nanoparticles. With the exception of the CIP-encapsulated nanoparticles, the hybrid nanoparticles possess higher % encapsulation, and consequently drug loading, compared to the non-hybrids, which is attributed to the presence of the lipid coat.

7.3.4 Colloidal stability and *in vitro* drug release

Despite their superior characteristics, hybrid nanoparticles prepared with only PC as the surfactant have been reported to be unstable in buffer solutions, unlike the non-hybrids that are stable in both aqueous and salt solutions³². Not unexpectedly, the PLGA–PC and PLGA–PC/SA nanoparticles prepared earlier are found to be unstable in PBS. To ensure that the hybrid nanoparticles remain relevant *in vivo*, TPGS, which is a biocompatible and biodegradable surfactant, is incorporated into the formulation following the approach of Fang *et al.*³² that used PEG-conjugated PC to obtain stable hybrid nanoparticles in PBS. TPGS, an amphiphile, can be added during the preparation step either (i) into the oil to be incorporated into the PLGA matrix, or (ii) into the external aqueous phase as an emulsifier on the nanoparticle surface. However, as PC is expected to dominate the nanoparticle surface, thus potentially limiting the adsorption of TPGS onto the same surface if TPGS is added into the aqueous phase, the present formulation opts to incorporate TPGS into the oil phase.

By including a small amount of TPGS, i.e. 10% (w/w PLGA) into the oil phase (≈ 9 mg), the initially unstable hybrid nanoparticles are conferred stability in PBS by virtue of the stabilizing property of TPGS. The TPGS inclusion is thought to result in hybrid nanoparticles, whose surface, in addition to PC, is also enriched with TPGS. The orientation of the TPGS on the hybrid nanoparticle surface is such that the polar PEG₁₀₀₀ component extends outwards into the aqueous phase, while the lipophilic tocopherol succinate component remains adsorbed onto the polymer matrix. The projection of the long and bulky PEG₁₀₀₀ chain of TPGS into the aqueous phase enhances the colloidal stability of the hybrid nanoparticles, as opposed to the relatively small choline head group of the PC lipid.

Table 7.4: Characteristics of antibiotic-encapsulated PLGA–PC or PLGA–PC/SA nanoparticles with TPGS.

Surfactant	Antibiotic	Zeta potential (mV)	Size (nm)	% Encapsulation	Drug loading (%)
PC/TPGS	LEV	–11	410 $\pm$ 190	16	1.8
PC/SA/TPGS	CIP	+10	300 $\pm$ 130	6	0.5
PC/TPGS	OFX	–13	300 $\pm$ 100	10	1.1

Table 7.4 presents a summary of the zeta potential, size, % encapsulation, and drug loading of the hybrid nanoparticles stabilized by TPGS. Due to the cationic nature

of TPGS, the magnitude of the negative zeta potential of the nanoparticles prepared with PC decreases from $\approx (-) 20$ mV to $(-) 10$ mV, hence they are more comparable to the PLGA–PVA nanoparticles in term of the zeta potential. Interestingly, the zeta potential of the CIP-encapsulated PLGA–PC/SA/TPGS nanoparticles decreases in magnitude as well from $+26$ to $+10$ mV, despite the cationic nature of both PC/SA and TPGS. In terms of the nanoparticle size, they do not differ significantly with and without TPGS and remain in the ≈ 300 – 400 nm range.

The drug loadings of LEV and CIP-encapsulated nanoparticles remain similar to those without TPGS at 1.8% and 0.5%, respectively. In contrast, the drug loading of the OFX-encapsulated nanoparticles decreases considerably from 2.7% to 1.1% upon TPGS inclusion. Appropriately, similar trends are observed in the % encapsulation that dictates the drug loading. Importantly, the present results strongly indicate that CIP is not suitable to be encapsulated into PLGA nanoparticles, regardless of the formulation, as manifested in the persistent low drug loading (≈ 0.4 – 0.5%) resulted from the poor % encapsulation (≈ 4 – 6%).

The drug loading is therefore no longer inversely proportional to the aqueous solubility of the antibiotics as the drug loading of the OFX-encapsulated nanoparticles is now lower than that of LEV. In this regard, while both CIP and LEV-encapsulated nanoparticles are prepared with the DESE method, where the antibiotics are dissolved in the internal aqueous phase, OFX is dissolved in the oil phase together with TPGS and PLGA in the ESE method. The competition for space between OFX and TPGS in the precipitated polymer matrix upon emulsification may have lowered the amount of OFX being encapsulated.

The *in vitro* drug release profiles of the OFX and LEV-encapsulated PLGA–PC/TPGS nanoparticles are presented in Figs. 7.3A and 7.3B, respectively. The release profiles of their non-hybrid counterparts are presented for comparison. The release profile of the CIP-encapsulated nanoparticles is not presented in Fig. 7.3 as the low drug loading results in an extremely slow release (i.e. $\approx 20\%$ released over a five-day-period). The results indicate that the lipid coat in the hybrid nanoparticles does not affect the OFX release resulting in a nearly identical release profile to that of the non-hybrid nanoparticles, where the encapsulated OFX are completely released in 15 h following a 5 h burst release of 90% of the encapsulated OFX.

Figure 7.3: *In vitro* drug release profiles of (A) OFX-encapsulated and (B) LEV-encapsulated PLGA-PC/TPGS nanoparticles.

In contrast, the LEV release from the hybrid nanoparticles is markedly different from that of the non-hybrid counterparts. While LEV is almost completely released from the PLGA-PVA nanoparticles in 5 h, only 70% of the encapsulated LEV is released from the PLGA-PC/TPGS nanoparticles after the same period. Afterwards, LEV is continuously released for the next 20 h to reach about 90% of LEV released. The LEV release is determined by the net effect of two factors (i) a lower release is attributed to the greater extent of interaction between lipid and LEV, as well as (ii) higher release due to higher lipid membrane permeability, both a direct consequence of the higher lipophilicity of LEV.

7.4 Chapter Summary

The results and discussions presented in this chapter have been published as:

W.S. Cheow and K. Hadinoto. Factors affecting drug encapsulation and stability of lipid-polymer hybrid nanoparticles. *Colloids Surfaces B* 85:214–220 (2011).

In summary, the experimental results demonstrated that stable lipid-polymer hybrid nanoparticles encapsulating LEV, CIP, and OFX antibiotics can be prepared by simple modifications of the emulsification-solvent-evaporation methods, where the optimal lipid PC amount to prepare hybrid nanoparticles has been determined. Specifically, it has been established that (i) ionic interactions between oppositely charged drug and lipid can prevent nanoparticle formation, which can be remedied by reversing the charge of the lipid through counterionic surfactant (e.g. SA) addition, (ii)

hybrid nanoparticles are larger than their non-hybrid counterparts and their size is governed by the type of drug being encapsulated, instead of being influenced by the preparation method and the surfactant used, (iii) drug loading of the hybrid nanoparticles is influenced by both the aqueous solubility and lipophilicity of the drug, where compared to the non-hybrid nanoparticles, the drug loading of the hybrid nanoparticles are twice as high, with the exception of CIP-encapsulated nanoparticles due to CIP being less lipophilic, (iv) the impact of the lipid coat on the *in vitro* drug release profile highly depends on the drug lipophilicity, and lastly (v) unlike their non-hybrid counterparts, hybrid nanoparticles prepared with PC as the sole surfactant are unstable in buffer solutions, but the stability can be conferred by the addition of TPGS. The TPGS inclusion, however, adversely impacts the drug loading of the OFX-encapsulated nanoparticles, while not affecting the CIP and LEV-encapsulated nanoparticles, due to their different preparation methods.

In perspective, the current drug loading, although an improvement from the LEV-loaded nanoparticles presented in Chapter 4, still needs to be significantly improved to render the hybrid nanoparticle formulation clinically useful. Towards this aim, further modifications to improve the drug loading by, for example, developing antibiotic nanoparticles, might prove effective. The antibiotic release profile can be tailored for specific purpose (in this case, for inhaled anti-biofilm therapy) by surface functionalization. The first step towards such nanoparticles with (i) high drug loading and (ii) controlled antibiotic release profile is the preparation of antibiotic nanoparticles, which is explored in the next chapter.

Chapter 8

Drug-Polyelectrolyte Complexation to Prepare High Drug Loading Antibiotic Nanoparticle

8.1 Introduction

The widespread use of lipid and polymer-based nanoparticles is attributed to their well-known preparation methods and the fact that their physicochemical characteristics can be readily modified for sputum penetration and controlled drug release purposes. Nevertheless, encapsulation of antibiotics into these nano-carriers is usually poor, particularly for water-soluble antibiotics, resulting in extremely low antibiotic loading ($< 20\%$) and high amount of drug wastage ($> 50\%$)¹⁴² as presented in the preceding chapters. The low antibiotic loading is an issue because in COPD therapy, high antibiotic dosage is required due to the high antibiotic tolerance of bacterial biofilm².

Therefore, in terms of the potential for widespread clinical implementations, lipid and polymer-based nano-carriers are less than ideal because of the anticipated high cost and high dosing frequency due to the inefficient antibiotic encapsulation. A better nano-carrier platform is actually represented by the antibiotic nanoparticles themselves. Instead of encapsulating the antibiotic into nano-carriers, a core-shell nanoparticle structure made up of (i) antibiotic nanoparticle as the core and (ii) lipid or polymer layer as the shell potentially represents a more clinically effective nanoscale antibiotic delivery system. Even though the use of a core-shell nanoparticle structure for drug delivery is not new¹⁴³, a core-shell structure, where the core is made up of the drug itself, instead of polymers or inorganic materials, has only been investigated recently¹⁴⁴⁻¹⁴⁵. The antibiotic nanoparticle core ensures high antibiotic loading, whereas the lipid or polymer shell can be functionalized for a wide range of

therapeutic functions. Specifically, for inhaled anti-biofilm therapy, the polymer shell can serve as a release modulator, hence providing tailored antibiotic release profiles.

In the present chapter, nanoplexes (see Section 2.3) of two fluoroquinolone antibiotics, i.e. OFX and LEV with antibiotic loading $\geq 60\%$ (w/w) are prepared by self-assembly complexation with dextran sulfate (DXT). DXT is the water-soluble sodium salt form of dextran, which is a natural polysaccharide produced by *Leuconostoc mesenteroides* bacterial species. Instead of using synthetic PE, low molecular weight DXT (5000 Da), which is nontoxic¹⁴⁶, widely available at a low cost, and regularly used in biomedical fields, is purposely used in order to make the preparation method as appealing as possible. This would hopefully promote the widespread applications of antibiotic nanoplex in future smart drug delivery systems.

Herein the effects of four complexation process variables, i.e. (i) drug to DXT charge ratio, (ii) pH, (iii) drug, and (iv) salt concentrations on the complexation efficiency, size, colloidal stability, yield, and antibiotic loading of the nanoplex are investigated. The effect of drug hydrophobicity is also investigated, where LEV represents the more hydrophobic amphiphile than OFX based on their partition coefficient values in water-octanol mixture¹⁴⁷. The optimal preparation conditions for each drug are identified. Antimicrobial activities of OFX and LEV nanoplexes are examined *in vitro* against *P. aeruginosa* planktonic cells to examine whether the complexation adversely affects the antibiotic activity. Lastly, the drug dissolution profiles from the nanoplex and the physical stability of the nanoplex powders after one-month storage are reported.

8.2 Methodology

The nanoplex were prepared according to the method in Section 3.2.5, and subsequently characterized in terms of complexation efficiency, yield, drug loading, and size (Section 3.2.6). The effect of the drug to DXT charge ratio ($R_{drug/DXT}$) was assessed at a fixed drug concentration. The DXT concentration was varied from 0.45 to 16 mg/mL resulting in $R_{drug/DXT}$ between 0.4 and 1.3. $R_{drug/DXT}$ was calculated from the number of charge groups per molecule of the drug and DXT. There are twenty-four $-\text{OSO}_3^-$ groups per DXT molecule and one $-\text{N}(\text{CH}_3)\text{H}^+$ group per fluoroquinolone molecule resulting in charge densities of 4.8×10^{-6} and 2.8×10^{-6} mol-charge/mg for the DXT and fluoroquinolones, respectively. Two concentrations of drug (i.e. 1 and 10

mg/mL) and salt (i.e. 0.1 and 0.3 M) were used. The pH effect was examined by varying the AA concentrations between 0.01, 0.1, and 0.2% (w/v) resulting in pH = 3.9, 3.4, and 3.2, respectively. Lastly, the nanoplex suspension was transformed into dry powders by freeze or spray drying to study its physical stability (Section 3.2.8).

8.3 Results and Discussion

8.3.1 Effects of complexation process variables

8.3.1.1 OFX nanoplex

The CE, nanoplex size, drug loading, and yield reported here are the average values of the replicates, where the error bars represent the experimental uncertainties between the different replicates. The effects of OFX, AA, and NaCl concentrations on CE at different $R_{OFX/DXT}$ are presented in Fig. 8.1A. In the reference runs, OFX, AA, and NaCl concentrations are fixed at 10 mg/mL, 0.1% (w/v) (i.e. pH = 3.4), and 0.1 M, respectively. At the reference conditions, CEs are relatively constant in the range of 65–70% across $R_{OFX/DXT}$. In an attempt to improve CE, the effect of pH is examined by increasing the AA concentration to 0.2% (w/v) (i.e. pH = 3.2), while keeping OFX and NaCl concentrations constant.

The results indicate that higher CEs ($\approx 95\%$) are obtained for $R_{OFX/DXT}$ up to ≈ 1.0 denoting almost complete complexation of OFX. The slightly lower pH at 0.2% (w/v) AA concentration leads to an increased number of charged OFX molecules¹⁴⁸, which leads to higher CEs compared to that obtained at 0.1% (w/v) AA concentration. As $R_{OFX/DXT}$ is varied at a constant OFX concentration in these runs, the constant CEs between $0.4 \leq R_{OFX/DXT} \leq 1.0$ indicate that the same amount of OFX participates in the complexation with DXT, regardless of the DXT amount in the feed. In other words, excess DXT is not needed to obtain high percentage complexation of OFX. Lower CE ($\approx 75\%$), however, is observed at $R_{OFX/DXT} = 1.3$, which is not unexpected as less DXT is available to form nanoplex with OFX at $R_{OFX/DXT} > 1.0$. At the lower pH, CEs are more sensitive to $R_{OFX/DXT}$ as DXT concentration becomes the rate limiting variable when OFX are sufficiently charged.

Figure 8.1: Effects of $R_{OFX/DXT}$, OFX, AA, and NaCl salt concentrations on (A) CE and (B) nanoplex size.

The effect of increasing AA concentration on CE, however, is much less at 1 mg/mL OFX concentration, which is reflected in the resultant CEs that remain below 70%. At a lower OFX concentration, there are fewer charged drug molecules in the vicinity of each other, such that the complex aggregate formation by hydrophobic interactions of the drug molecules is not as significant, resulting in the lower CEs. Nevertheless, the trend in CE as a function of $R_{OFX/DXT}$ is similar to that observed at 10 mg/mL OFX concentration, where lower CE ($\approx 55\%$) is observed at $R_{OFX/DXT} = 1.3$. The study of the pH effect is not performed at 0.01% (w/v) AA concentration because of the poor solubility of OFX at this condition.

Next, the effect of NaCl concentration on CE is examined while keeping OFX and AA concentrations at their reference values. Increasing NaCl concentration to 0.3 M produces considerably lower CEs ($\approx 45\text{--}55\%$) across $R_{\text{OFX/DXT}}$. At a higher salt concentration, the charge screening effect of the salt is intensified, such that, in addition to promoting the interactions of like-charged molecules, the presence of salt can also diminish the interactions between the oppositely-charged OFX and DXT molecules resulting in the lower CEs. When the study is repeated at 1 mg/mL OFX concentration, nanoplex formation does not take place at all across $R_{\text{OFX/DXT}}$, because the charged OFX molecules, which are at a lower amount, are completely screened out by the salt. The results therefore signify the importance of not only drug concentration, but also its ratio to the salt concentration.

The effects of OFX, AA, and NaCl concentrations on the nanoplex size measured by PCS are presented in Fig. 8.1B. As the nanoplex is intended for anti-biofilm therapy, therefore it must be able to penetrate the sputum, a demarcation line at 400 nm representing the upper threshold of the sputum mesh size is drawn in Fig. 8.1B. For the runs that yield the highest CEs (i.e. OFX = 10 mg/mL, AA = 0.2% (w/v), and NaCl = 0.1 M for $R_{\text{OFX/DXT}} \leq 1.0$), the nanoplexes produced are smaller than 400 nm. The nanoplex size is observed to decrease with decreasing $R_{\text{OFX/DXT}}$ (i.e. more DXT) from ≈ 80 nm at $R_{\text{OFX/DXT}} = 0.4$ to ≈ 330 nm at $R_{\text{OFX/DXT}} = 1.0$.

A small nanoplex size is critical at this stage as the intended application of the nanoplex requires it to undergo subsequent functionalization, which would certainly leads to a size increase. On this note, the individual nanoplex size at $R_{\text{OFX/DXT}} = 0.4$ has been verified by the FE-SEM image in Fig. 8.2A to be smaller than 100 nm. On the other hand, the PCS results of the nanoplex size at $R_{\text{OFX/DXT}} = 0.6$ and 1.0 are considerably larger at $\approx 270\text{--}330$ nm. However, a closer look at the FE-SEM image in Fig. 8.2B for $R_{\text{OFX/DXT}} = 1.0$ reveals that the individual nanoplex size is actually smaller than 100 nm, which is comparable to that at $R_{\text{OFX/DXT}} = 0.4$. The larger nanoplex size reported by PCS is because the size reflects the nanoplex agglomerates, instead of the individual nanoplex. Therefore, agglomeration of the nanoplex in its aqueous suspension form is more prevalent at $R_{\text{OFX/DXT}} = 0.6$ and 1.0.

Figure 8.2: FE-SEM images of OFX nanoplex at (A) $R_{OFX/DXT} = 0.4$ and (B) 1.0, respectively.

It has to be mentioned, however, that PCS is expected to produce a larger nanoplex size than the visual observation because of the swelling of DXT in an aqueous medium. The impact of DXT swelling alone, without agglomeration, however, is unlikely to cause the size reported by PCS to be more than three times larger than that of the FE-SEM image. The zeta potentials of the nanoplexes, which do not distinguish the individual nanoplex from the agglomerates, are negative at $\approx (-) 70 \pm 10$ mV owed to the surface presence of anionic DXT. Moreover, the zeta potentials are relatively independent of $R_{OFX/DXT}$.

On a side note, for the runs that yield lower CEs, nanoplexes with sizes below 400 nm are produced in a few runs, for example in the runs at the reference conditions. A majority of the nanoplexes produced (i.e. ten out of thirteen datapoints), however, are larger than or very close to 400 nm. Furthermore, the size of the larger nanoplexes does not exhibit a definite trend as a function of $R_{OFX/DXT}$. Therefore, the optimal preparation conditions (i.e. OFX, AA, NaCl concentrations, and $R_{OFX/DXT}$) in terms of the resultant CE and nanoplex size have been identified.

The drug loading and yield are now examined in Fig. 8.3 to identify the best overall conditions. The drug loading is shown to increase from $\approx 60\%$ at $R_{OFX/DXT} = 1.0$ to $\approx 75\text{--}80\%$ at $R_{OFX/DXT} = 0.4$ and 0.6. As the amount of OFX that forms complex with DXT is the same in these runs (i.e. similar magnitude of CEs), the variation in the drug loading ought to be contributed by the DXT amount, where the higher the OFX loading, the lower the DXT amount. The higher OFX loading at $R_{OFX/DXT} < 1.0$ suggest

that fewer DXT molecules actually form complex with OFX molecules when DXT is present in excess in the feed.

Figure 8.3: Effects of $R_{\text{OFX/DXT}}$ on drug loading and yield at OFX = 10 mg/mL, AA = 0.2% (w/v), and 0.1 M NaCl.

Consequently, the yield is shown to decrease with decreasing $R_{\text{OFX/DXT}}$ from $\approx 90\%$ at $R_{\text{OFX/DXT}} = 1.0$ to $\approx 40\%$ at $R_{\text{OFX/DXT}} = 0.4$ because of an increasing amount of non-complexed DXT at $R_{\text{OFX/DXT}} < 1.0$. In other words, once nearly 100% complexation of OFX is achieved, an abundant presence of DXT in the feed does not necessarily translate to its increased inclusion in the complexation with OFX. Interestingly, the nanoplexes that are constituted of fewer DXT (i.e. $R_{\text{OFX/DXT}} < 1.0$) exhibit less tendency to agglomerate than that produced at $R_{\text{OFX/DXT}} = 1.0$. The results therefore suggest that the nanoplex agglomeration is not dictated by the magnitude of electrostatic stabilization contributed by DXT. Instead, the agglomeration is possibly driven by other physical forces, such as hydrophobic and van der Waals interactions.

To summarize, all four process variables – $R_{\text{OFX/DXT}}$, pH, drug concentration, and its ratio to the salt concentration – have equally important roles in the nanoplex preparation. The run at OFX = 10 mg/mL, AA = 0.2% (w/v), NaCl = 0.1 M, and $R_{\text{OFX/DXT}} = 0.4$ represent the best overall run to produce OFX nanoplex attributed to its high CE ($\approx 96\%$), small size (≈ 80 nm), and high OFX loading ($\approx 80\%$). The low yield of this run (40%) is not a major concern because it is contributed by the excess of non-complexed DXT, and not because of the non-complexation of the more valuable OFX.

Furthermore, as done in our experiments, the excess DXT can be recovered to be reused in a new batch after readjusting its pH and salt concentration.

8.3.1.2 LEV nanoplex

The effects of drug, AA, and NaCl concentrations at different $R_{LEV/DXT}$ on the CE and size of the LEV nanoplex are presented in Fig. 8.4A. Overall, the trends in the CE of the LEV nanoplex as a function of the complexation variables (Fig. 8.4A) are highly similar to those observed with the OFX nanoplex (Fig. 8.1A), where CEs are higher at a higher drug concentration (LEV = 10 mg/mL) and lower pH (AA = 0.2% w/v). Also similar to the OFX nanoplex results, a higher NaCl concentration of 0.3 M leads to lower CEs ≈ 60 – 65% for LEV = 10 mg/mL, and nanoplex are not produced for LEV = 1 mg/mL, reiterating the importance of the drug to salt concentration ratio.

Compared to the OFX nanoplex, higher CEs for the LEV nanoplex are noticeable. For example, CEs obtained at the reference conditions (i.e. LEV = 10 mg/mL, AA = 0.1% (w/v), and NaCl = 0.1 M) are $\approx 75\%$, which are slightly higher than ≈ 65 – 70% for the OFX nanoplex. As LEV is more hydrophobic than OFX, LEV nanoplex forms more readily owed to the stronger hydrophobic interactions of the drug molecules resulting in the higher CEs. For the same preparation conditions, CEs of the LEV nanoplex are generally higher by ≈ 5 – 10% .

Not unexpectedly, the optimal CE for the LEV nanoplex are also obtained at LEV = 10 mg/mL, AA = 0.2% (w/v), and NaCl = 0.1 M, where CEs are $\approx 95\%$ in the range of $R_{LEV/DXT}$ investigated. The fact that CE remains high at $R_{LEV/DXT} = 1.3$, which is not observed in the OFX nanoplex, suggests that nearly complete complexation of LEV can be achieved with less DXT. The lower sensitivity of CEs to $R_{LEV/DXT}$ is also observed at LEV = 1 mg/mL, where CEs are constant in the range of ≈ 65 – 75% across $R_{LEV/DXT}$. On a separate note, with regard to the pH effect, LEV nanoplex are formed at AA = 0.01% (w/v), however, the yield is too low to be quantified due to too few charged LEV molecules at pH = 3.9. Only at AA = 0.1 and 0.2% (w/v) (i.e. pH = 3.2–3.4), LEV nanoplexes are produced with reasonable yield.

In terms of their size, a majority of the LEV nanoplexes (i.e. ten out of sixteen data points) are larger than 400 nm (Fig. 8.4B). For the runs that produce the optimal CEs, the two smallest nanoplex sizes are ≈ 180 and 340 nm obtained at $R_{LEV/DXT} = 1.3$

and 1.0, respectively. FE-SEM images of the LEV nanoplex, however, are similar to those of the OFX nanoplex in Fig. 8.2 (hence they are not shown here for brevity), where the size of the individual LEV nanoplex is shown to be smaller than 100 nm for all $R_{LEV/DXT}$. The zeta potential of the LEV nanoplex is also comparable to that of the OFX nanoplex. The consistently large nanoplex size obtained from PCS suggests that the LEV nanoplex is more prone to agglomeration due to the stronger hydrophobic interactions. As a result, unlike the OFX nanoplex, the LEV nanoplex size does not exhibit a definite trend as a function of $R_{LEV/DXT}$.

Figure 8.4: Effects of $R_{LEV/DXT}$, LEV, AA, and NaCl salt concentrations on (A) CE and (B) nanoplex size.

For the runs that produce the highest CEs, the trends in the drug loading and yield of the LEV nanoplex in Fig. 8.5 are also similar to those observed with the OFX nanoplex. The drug loading increases from $\approx 60\%$ at $R_{LEV/DXT} = 1.3$ to $\approx 90\%$ at $R_{LEV/DXT} = 0.4$, whereas the yield decreases from $\approx 90\%$ at $R_{LEV/DXT} = 1.3$ to $\approx 40\%$ at $R_{LEV/DXT} = 0.4$. As the amount of LEV is constant in these runs, the higher drug loading at lower $R_{LEV/DXT}$ indicates that less DXT is present in the nanoplex when DXT is abundantly present in the feed resulting in the lower yield. The physical explanation for the decrease in the complexed DXT amount at $R_{drug/DXT} < 1.0$ for both OFX and LEV is currently not known and is being investigated at different drug concentrations and for a wider range of $R_{drug/DXT}$.

Figure 8.5: Effects of $R_{LEV/DXT}$ on drug loading and yield at LEV = 10 mg/mL, AA = 0.2% (w/v), and 0.1 M NaCl.

To summarize, the optimal preparation conditions for the LEV nanoplex have been identified – LEV = 10 mg/mL, AA = 0.2% (w/v), NaCl = 0.1 M, and $R_{LEV/DXT} = 1.3$ – where nearly 100% CE, small agglomerate size (≈ 180 nm), reasonably high LEV loading ($\approx 60\%$), and yield of 90% are obtained. Compared to OFX, the higher hydrophobicity of LEV results in less DXT amount needed to achieve nearly 100% LEV complexation, however, at the same time, the higher hydrophobicity causes the LEV nanoplex to exhibit a stronger tendency to agglomerate.

8.3.2 Therapeutic characteristics of the OFX and LEV nanoplexes

8.3.2.1 Antibiotic dissolution profile and antimicrobial activity

The *in vitro* dissolution profiles of OFX and LEV from the nanoplex prepared at the optimal conditions are presented in Fig. 8.6. The results indicate that OFX and LEV are not released from the nanoplex in an aqueous medium, and only released in a salt solution. The salt-induced drug release phenomenon is depicted in the photographs in Fig. 8.6A. When water is used as the dissolution medium, the nanoplex suspension remains turbid after 24 h indicating that the nanoparticles still exist. In contrast, when PBS, which contains 0.14 M NaCl, is used as the dissolution medium, the nanoplex suspension is clear after 24 h indicating that the nanoplex has disintegrated and consequently the drugs are released. Importantly, the drug is also released in 0.14 M aqueous NaCl solution. When a lower NaCl concentration is present, however, the drug is not released.

Figure 8.6: (A) OFX and LEV are not released after 24 h from the nanoplex in water, only in PBS; (B) OFX and LEV release profiles from the nanoplex in PBS.

The presence of salt is therefore believed to neutralize the electrostatic interaction between the drug and DXT molecules in the nanoplex leading to de-complexation. One benefit of having the salt-induced release of the drug is that the nanoplex can be stored in its aqueous suspension form for a longer period between processing steps, without having concerns of drug leakage, which is prevalent in the lipid or polymer-based nano-carriers. The dissolution profiles of the OFX and LEV nanoplexes in PBS in Fig. 8.6B indicates that nearly 100% of the complexed antibiotics can be released within 3 h. The rapid antibiotic release renders the nanoplex appropriate for anti-biofilm therapy in which high antibiotic dosing is needed to suppress the emergence of

antibiotic-resistant strains. Lastly, MIC values of the OFX and LEV nanoplexes tested against *P. aeruginosa* planktonic cells are 2.0 and 0.5 µg/mL, respectively, which are of comparable magnitude to the MIC values of the native antibiotics. The complexation with DXT therefore does not adversely affect the antimicrobial activities of OFX and LEV.

8.3.2.2 Physical stability of nanoplex powders

Herein it is demonstrated that the nanoplex can be readily transformed into a dry powder form, which is preferred over the aqueous suspension form attributed to its less susceptibility to microbial contaminations and temperature or pH fluctuations, during processing or storage, resulting in more stable products. The OFX and LEV nanoplexes are readily transformed into dry powders by either freeze drying or spray drying denoting their physical robustness towards both low and high temperature drying. Nanoplex powders prepared by freeze drying are reported here.

PXRD patterns of the freshly freeze-dried LEV and OFX nanoplex powders are presented in Fig. 8.7A and 8.7B, respectively. Broad diffuse peaks at $2\theta \approx 26^\circ$ are visible suggesting that both nanoplex powders are amorphous, in contrast to the raw crystals of the antibiotics. The amorphous form is caused by a combination of rapid precipitation of the drug molecules, upon charge neutralization, and their electrostatic interactions with DXT, which prevent them from assembling into ordered crystalline structures. As the amorphous form is known to recrystallize over time, their PXRD patterns are re-examined after one month storage. The nanoplex powders are found to remain amorphous after one month denoting their physical stability (data not shown).

Next, thermal behaviours of the amorphous nanoplex powder are examined. For the raw OFX and LEV crystals, the endothermic peaks observed at 268°C and 226°C in the DSC thermograms (Fig. 8.7C) are attributed to the melting of the OFX and LEV crystals, respectively, as no mass change is observed in the TGA thermograms at these temperatures (Fig. 8.7D). The melting is immediately followed by large mass losses in the TGA thermograms at 278°C indicating OFX and LEV decompositions. Amorphous DXT does not display a melting endotherm and decomposes at 220°C as reflected by the exothermic peak and a large mass loss at the said temperature.

Figure 8.7: PXRD patterns of (A) OFX and (B) LEV amorphous nanoplex powders compared to their raw crystals; (C) DSC and (D) TGA thermograms of the nanoplex powders, raw drug crystals, and DXT.

In contrast to the raw crystals, the OFX and LEV nanoplex powders do not exhibit the endothermic melting peaks denoting their non-crystalline state. Instead, the nanoplex powders exhibit glass transition endotherms around 238–241 $^{\circ}\text{C}$ and decompose shortly after at 248 $^{\circ}\text{C}$. The high glass transition temperature contributes to the high stability of the amorphous nanoplex powders. Their stability is also reflected in the DSC thermograms by the absence of OFX and LEV recrystallization peaks preceding the glass transition points. Importantly, dissolution profiles of OFX and LEV are not affected at all by the dry powder transformation (data not shown). In addition, repeating the antimicrobial testing using the nanoplex powders results in similar MIC values.

8.4 Chapter Summary

The nanoplexation method presented in this chapter has been published as:

W.S. Cheow and K. Hadinoto, Green preparation of antibiotic nanoparticle complex as potential anti-biofilm therapeutics via self-assembly amphiphile-polyelectrolyte complexation with dextran sulfate. *Colloids Surfaces B* 92:55–63 (2012).

OFX and LEV nanoplexes with high drug loading in the range of 60–80% have been successfully prepared by self-assembly drug–PE complexation. The nanoplex preparation method involves only aqueous mixing of the drug and PE solutions at ambient conditions. The simplicity of the method represents a significant improvement over existing drug nanoparticle preparation methods, as it does not involve specialized equipment or intricate organic solvent removal process. The optimal preparation conditions (i.e. drug to PE charge ratio, pH, drug, and salt concentrations) have been identified for both OFX and LEV nanoplexes. In general, the effects of the complexation process variables on the nanoplex characteristics (i.e. size, stability, drug loading) are similar for OFX and LEV nanoplexes. Two key differences, which are both attributed to LEV's higher hydrophobicity, are in (i) the slightly higher complexation efficiency of the LEV nanoplex in general, and (ii) the optimal $R_{drug/DXT}$ – in terms of the resultant size, drug loading, and yield – of the LEV nanoplex that is higher than that of the OFX nanoplex.

At the optimal preparation conditions, nearly 100% of the drug molecules are transformed into the nanoplex hence minimizing drug wastage. In addition, the nanoplex size is < 400 nm and is hence ideal for anti-biofilm therapy. In terms of the therapeutic characteristics, OFX and LEV are only released from the nanoplex in aqueous salt solutions and their antimicrobial activities are preserved upon complexation. Moreover, the nanoplex is stable in both the aqueous suspension and amorphous dry powder forms. Looking ahead, both nanoplexes are highly charged owed to the DXT presence on the surface making them readily functionalized for a wide range of therapeutic functions. The high drug loading and amorphous state of the nanoplex prepared point towards a possibility of employing this method to prepare amorphous antibiotic nanoparticle for enhancing the dissolution rate of a sparingly soluble antibiotic. This is explored in the next chapter.

Chapter 9

Complexation to Prepare Amorphous Nanoparticle of Sparingly Soluble Antibiotic

9.1 Introduction

In this chapter, the drug-polyelectrolyte nanoplexation technique is applied to a sparingly soluble antibiotic to prepare an amorphous nanoparticle by virtue of the drug-drug hydrophobic interaction, and the drug-polyelectrolyte electrostatic interaction (see Fig. 9.1). CIP, a potent amphiphilic antibiotic, is employed as the model sparingly soluble drug. CIP has a limited aqueous solubility of 0.12 mg/mL at physiological temperature and neutral pH condition¹⁴⁹. At pH below the pK_{a1} of CIP (≈ 6), however, CIP is present in its cationic form and becomes freely soluble¹³⁷. DXT is employed as the oppositely charged polyelectrolyte for complexation with CIP to produce amorphous nanoscale CIP–DXT complex (i.e. CIP nanoplex). Upon the dry powder transformation to prepare solid dosage form of the drug, the high glass transition temperature of DXT is anticipated to kinetically stabilize the amorphous CIP nanoplex¹⁵⁰.

First, the effects of three CIP nanoplex preparation parameters (i.e. CIP concentration, CIP to DXT charge ratio ($R_{CIP/DXT}$), and salt concentration) on the complexation efficiency, production yield, CIP loading, and morphology (e.g. size, shape) of the nanoplex are investigated to identify the optimal CIP nanoplex formulation. Second, the dissolution rate under a sink condition, saturation solubility, supersaturated solution generation, and stability during storage of the optimal nanoplex formulation – in both suspension and dry powder forms – are examined with respect to those of raw CIP crystals. A dissolution rate comparison with the hydrochloride salt

form of CIP is also performed. Lastly, antimicrobial activity of CIP nanoplex is evaluated *in vitro* against *P. aeruginosa* bacteria to assess the impact of the complexation process, if any, on the antimicrobial activity of CIP.

Figure 9.1: Amorphous nanoplex formation via self-assembly drug-PE complexation.

9.2 Methodology

The CIP nanoplex were prepared according to the method in Section 3.2.5, at an acetic acid concentration of 0.3%. The effect of CIP concentration was investigated at two fixed CIP concentrations of 1 mg/mL and 10 mg/mL, whereas the DXT concentration was varied from 0.45 mg/mL to 16 mg/mL resulting in CIP to DXT charge ratios ($R_{CIP/DXT}$) between 0.4 and 1.3. The charge ratio was determined from

calculations involving the molecular weight (331 g/mol and 5000 g/mol for CIP and DXT, respectively) and the number of charge groups per molecule for both CIP and DXT. There are twenty-four $-\text{OSO}_3^-$ groups per DXT molecule and one $-\text{NH}_2^+$ group per CIP molecule, hence resulting in charge densities of 4.8×10^{-6} mol-charge/mg DXT and 3.0×10^{-6} mol-charge/mg CIP. Lastly, the effect of salt concentration was investigated using 0.1 M and 0.3 M NaCl.

Prepared CIP nanoplex were physically characterized according to the methods in Section 3.2.6, and the dissolution rate as well as saturation solubility were determined following the methods detailed in Section 3.2.7. Furthermore, the physical stability of the nanoplex following transformation into a dry powder form by freeze drying was determined by PXRD, DSC, and TGA (Section 3.2.8). Finally, the antimicrobial activity of the nanoplex was determined to investigate the effect of nanoplexation on CIP efficacy (Section 3.2.4.1).

9.3 Results

9.3.1 Optimal CIP nanoplex suspension formulation

The effects of CIP concentration, $R_{\text{CIP/DXT}}$, and NaCl concentration on CE are presented in Fig. 9.2. First, CE at CIP = 10 mg/mL is higher ($\approx 85\text{--}95\%$) than at CIP = 1 mg/mL ($\approx 40\text{--}80\%$) independent of $R_{\text{CIP/DXT}}$ and NaCl concentration. Second, at given CIP and NaCl concentrations, CE is relatively constant for $0.4 \leq R_{\text{CIP/DXT}} \leq 1.0$ and only starts to decrease slightly when $R_{\text{CIP/DXT}}$ is raised to 1.3, which is not unexpected as less DXT is available to complex with CIP at $R_{\text{CIP/DXT}} > 1.0$. The decrease in CE at $R_{\text{CIP/DXT}} = 1.3$ is more significant at CIP = 10 mg/mL than at CIP = 1 mg/mL for both NaCl concentrations. Third, the effect of NaCl concentration on CE is evident only at CIP = 1 mg/mL, where CE obtained at 0.1 M NaCl is higher ($\approx 70\text{--}80\%$) than that at 0.3 M NaCl ($\approx 40\text{--}50\%$) for all $R_{\text{CIP/DXT}}$. In contrast, CE is minimally affected by the NaCl concentration at CIP = 10 mg/mL. Importantly, in the absence of NaCl, no precipitates are formed when the CIP solution is added to the DXT solution.

Figure 9.2: Complexation efficiencies (CE) of CIP nanoplex prepared at different CIP (mg/mL) and NaCl (M) concentrations for $0.4 \leq R_{CIP/DXT} \leq 1.3$.

Next, the production yield of CIP nanoplex prepared at CIP = 10 mg/mL is examined in Fig. 9.3A as a function of $R_{CIP/DXT}$ and NaCl concentration. For both NaCl concentrations, the production yield gradually increases from ≈ 40 –60% at $R_{CIP/DXT} \leq 0.6$ to 80% at $R_{CIP/DXT} \geq 1.0$. As the complexation is performed at a fixed CIP concentration, the trend in the production yield as a function of $R_{CIP/DXT}$ is well anticipated because CE is relatively constant at $\approx 90\%$ for $0.4 \leq R_{CIP/DXT} \leq 1.0$, which denotes an increasing presence of non-complexed DXT at lower $R_{CIP/DXT}$, where DXT is supplied in excess. Similar to the trend in CE, the effect of NaCl concentration on the production yield is also insignificant.

Figure 9.3: (A) Production yield of CIP nanoplex as a function of $R_{CIP/DXT}$ and NaCl concentrations; (B) % CIP and size of CIP nanoplex prepared at 0.1 M NaCl as a function of $R_{CIP/DXT}$ (CIP = 10 mg/mL).

% CIP and size of the CIP nanoplex produced are presented in Fig. 9.3B as a function of $R_{CIP/DXT}$. Similar results are obtained using 0.1 and 0.3 M NaCl hence only the results at 0.1 M NaCl are presented for brevity. After taking into account the experimental uncertainties, % CIP is relatively constant as a function of $R_{CIP/DXT}$ at $\approx 70 \pm 10\%$ (w/w), which is consistent with the trends in the CE and production yield. The CIP nanoplex size measured by PCS at $R_{CIP/DXT} \leq 0.6$ is ≈ 500 – 600 nm, whereas at $R_{CIP/DXT} \geq 1.0$ the size is smaller at ≈ 400 nm. Thus, in terms of size, CIP nanoplex formed at $R_{CIP/DXT} \geq 1.0$ are more desirable as the smaller size theoretically leads to higher dissolution rate and saturation solubility. FE-SEM images of the freeze-dried CIP nanoplex at $R_{CIP/DXT} = 1.3$ and 1.0 are presented in Fig. 9.4. Nearly spherical CIP nanoplex having fairly uniform sizes between 200 and 300 nm are observed at $R_{CIP/DXT} = 1.3$. The CIP nanoplex size observed in the FE-SEM images is smaller than that measured by PCS due to the swelling of DXT in an aqueous environment. At $R_{CIP/DXT} = 1.0$, however, the CIP nanoplex produced are not as spherical and exhibit a higher tendency to coalesce. Therefore, higher $R_{CIP/DXT}$ (i.e. less DXT) results in a better quality of the CIP nanoplex produced.

Figure 9.4: FE-SEM images of CIP nanoplex prepared at CIP = 10 mg/mL, 0.1 M NaCl for $R_{CIP/DXT} = 1.3$ and 1.0.

CIP nanoplex exhibits highly negative zeta potentials at (–) 40–70 mV denoting their high colloidal stability in the aqueous suspension form. No clear trend is observed in the zeta potential at different $R_{CIP/DXT}$ (data not shown). However, after a one month period during which the CIP nanoplex suspension is stored in ambient conditions, the CIP nanoplex are observed visually, and verified by PCS measurement (data not shown), to have transformed into larger agglomerates suggesting the loss of their

colloidal stability. Hence, CIP nanoplex must be transformed into the dry powder form to ensure their long-term storage stability.

Based on the CE, production yield, % CIP, and morphology, CIP nanoplex produced at (i) CIP = 10 mg/mL (higher CE), (ii) 0.1 M NaCl (less salt as its effect is minimal at CIP = 10 mg/mL), and (iii) $R_{CIP/DXT} = 1.3$ (better morphology) are considered optimal, therefore, they are selected for the subsequent characterizations in the dry powder form. On this note, in determining the optimal nanoplex formulation, though it is not applicable to the present results, the production yield usually takes a back seat to CE as the drug typically possesses a significantly higher value than the polyelectrolyte.

9.3.2 Physical characteristics of CIP nanoplex powders

PXRD patterns of freeze-dried CIP nanoplex and raw CIP crystals are presented in Fig. 9.5. Whereas raw CIP displays sharp-peak characteristics of a crystal, CIP nanoplex powders display broad diffuse peaks at $2\theta \approx 26^\circ$ suggesting that the CIP nanoplex exist in the amorphous state. The CIP nanoplex powders remain in their amorphous state after storage of one month at 25°C and 55% relative humidity hence signifying their storage stability. FE–SEM image of the freeze-dried CIP nanoplex taken after one month also indicates that the nanoplex morphology is preserved and CIP recrystallization is not observed (image not shown).

Figure 9.5: PXRD patterns of freeze-dried CIP nanoplex and raw CIP crystals.

DSC and TGA thermograms of the CIP nanoplex powders and the two parent materials, i.e. DXT and crystalline CIP, are presented in Figs. 9.6A and 9.6B, respectively. For the crystalline CIP, the endothermic peak observed at 264°C in the DSC thermograms is attributed to the melting of CIP as no mass change is observed in the TGA thermograms, which is immediately followed by a large mass loss at 280°C indicating CIP decomposition. Amorphous DXT expectedly does not display a melting endotherm and decomposes at 219°C as reflected by the exothermic peak and a large mass loss at the said temperature.

Figure 9.6: (A) DSC and (B) TGA thermograms of freeze-dried CIP nanoplex, raw CIP crystals, and DXT.

In contrast, CIP nanoplex powders exhibit a glass transition endotherm at $\approx 230^\circ\text{C}$ and decompose shortly after the glass transition. CIP nanoplex powders do not exhibit an endothermic melting peak of crystalline CIP denoting their highly amorphous state owed to CIP complexation with DXT. The CIP–DXT complexation is evident in the decomposition temperature of CIP nanoplex that occurs at 235°C . Although CIP nanoplex contain approximately 20% (w/w) DXT, decomposition of the DXT fraction of the nanoplex is not observed at 219°C , which is the onset of decomposition of the native DXT. In addition to the PXRD results and the FE–SEM observation, the stability of the amorphous CIP nanoplex powders is also reflected in the DSC thermograms by the absence of CIP recrystallization peaks preceding the glass transition point.

9.3.3 CIP nanoplex dissolution rate, saturation solubility, and MIC

The dissolution testing indicates that CIP is not released from CIP nanoplex suspended in aqueous medium. CIP is only released upon exposure to salt medium. The effect of salt concentration on CIP nanoplex dissolution is therefore investigated and the results are presented in Fig. 9.7A. NaCl is chosen as the representative salt because PBS, the commonly used dissolution medium in drug release studies, is mostly composed of NaCl ($\approx 80\%$ w/w). When NaCl concentration is increased from 0 M to 0.15 M, the amount of CIP present in the supernatant gradually increases. However, beyond 0.15 M, increasing NaCl concentration no longer increases the amount of CIP released, most probably because the maximum amount of CIP release ($\approx 100\%$) has been achieved. Incidentally, 0.15 M NaCl is close to the concentration of NaCl in PBS (0.14 M).

Figure 9.7: (A) Effect of NaCl concentration on CIP nanoplex dissolution; (B) *in vitro* dissolution rates of CIP nanoplex suspension and powders.

Having ensured that CIP nanoplex dissolution in PBS will not be artificially retarded due to insufficient NaCl concentration, *in vitro* dissolutions of CIP nanoplex in both suspension and dry powder forms are presented in Fig. 9.7B, where they are compared with that of the raw CIP crystals. The dissolution rate of CIP nanoplex is rapid, achieving nearly 100% dissolution within 3 h. No significant difference is observed between the dissolution rates of the CIP nanoplex suspension and powders. For comparison, the time required for 50% dissolution for CIP nanoplex is < 1 h, which is roughly half of that of the raw CIP crystals (≈ 2 h). In addition, not all raw CIP crystals are dissolved, even after 4 h. The higher dissolution rate of CIP nanoplex

than the raw CIP crystals are attributed to the smaller size and amorphous state of the nanoplex. Importantly, the dissolution rate of CIP nanoplex is similar to that of CIP hydrochloride salt (results not shown), supporting the potential application of the present CIP nanoplex as a rapidly dissolving formulation.

Saturation solubilities of CIP nanoplex in their suspension and dry powder forms after 24 and 48 h incubation are presented in Fig. 9.8A and again compared with that of the raw CIP crystals. Attributed to their amorphous state and nanoscale size, saturation solubilities of CIP nanoplex (≈ 0.3 mg/mL) are approximately twice that of the raw CIP crystals (≈ 0.14 mg/mL) for both the suspension and dry powder forms. The saturation solubilities at 24 h and 48 h are identical in magnitude suggesting that equilibrium is achieved by 24 h.

Figure 9.8: (A) Saturation solubility and (B) dissolution behaviour under supersaturated conditions of CIP nanoplex.

The addition of CIP nanoplex at a concentration equal to 10 times of the CIP saturation solubility results in the generation of a highly supersaturated solution of CIP (≈ 1.2 mg/mL) that exists for about 5 min (Fig. 9.8B). Afterwards, the CIP concentration in the solution gradually decreases over a 20-min-period to 0.3 mg/mL, which is the saturation solubility of amorphous CIP nanoplex previously measured. The decrease in the CIP supersaturation level is due to CIP crystallization from the supersaturated solution. When CIP nanoplex are added at a concentration equal to 25 times of the saturation solubility, a lower supersaturation level in the solution (≈ 0.5 mg/mL) is generated, and is closely followed by a decrease in the CIP solution concentration to 0.3 mg/mL in less than 10 min. The lower and more rapid decrease of

the supersaturation level is due to faster CIP crystallization from the supersaturated solution. In contrast, the addition of raw CIP crystals under identical conditions does not result in the generation of a supersaturated solution.

9.3.4 Superior characteristics of amorphous CIP nanoplex

CIP nanoplex exhibit a higher dissolution rate and higher saturation solubility than raw CIP crystals. In addition, unlike raw CIP crystals, the amorphous CIP nanoplex, upon their dissolution at concentrations above the CIP saturation solubility, are able to generate a supersaturated drug solution. The higher dissolution rate of CIP nanoplex under sink condition is well expected and understood to be caused by the considerable size difference between the nanoplex (≈ 500 nm) and the raw CIP crystals (≈ 50 μ m) that leads to significant difference in their surface area to volume ratios ($\propto 10^2$). On the contrary, factors contributing to the higher saturation solubility of the nanoplex are not as clearly understood. In particular, contributions from the amorphous state and the nanoscale size of the nanoplex need to be examined.

For this purpose, saturation solubility of the nanoplex is calculated by the Ostwald-Freundlich equation (Eq. 2.1). A reasonable estimation for γ can be obtained by the following relationship¹⁵¹.

$$\gamma = -\frac{0.33k_B T}{(V_m / N_A)^{2/3}} \left[\ln\left(\frac{S_o}{55.6}\right) + 5 \right] \quad (9.1)$$

where k_B represents the Boltzmann constant (N.m/K), N_A the Avogadro's number (mol^{-1}), and S_o the aqueous molar solubility of the drug (mol/L). Substituting values of $T = 37^\circ\text{C}$ ($= 310$ K), $S_o = 0.14$ mg/mL ($= 4.2 \times 10^{-4}$ mol/L), and $V_m = 227 \times 10^{-6}$ m^3/mol ¹⁵² into Eq. (9.1) results in $\gamma = 18.4 \times 10^{-3}$ N/m. The γ values of pristine pharmaceuticals, without any adsorbed foreign molecules, typically range between 5 and 50 mN/m¹⁵¹. Therefore, the calculated value of γ is deemed reasonable and is used to determine $C_{s,r}$ in Eq. (2.1).

Using the lower limit of the nanoplex size, i.e. $r = 200$ nm, $C_{s,r}$ is calculated from Eq. (2.1) to be equal to $1.02 \times C_{s,\infty}$. In other words, the theoretical increase in the CIP saturation solubility due to the nanosizing of CIP crystals is only $\approx 2\%$, which does not explain for the doubling of the saturation solubility measured in the experiment. Even when the upper limit value of γ (i.e. 50 mN/m) is used in Eq. (2.1), only $\approx 5\%$ increase

in the saturation solubility is expected theoretically. These calculated results are in accordance with the experimental observations of Van Eerdenbrugh *et al.*¹⁵³, which demonstrated that the saturation solubility increase attributed to nanosizing is at best 15% for drug nanoparticles having sizes in the few hundred nanometer range. The increase in the saturation solubility of CIP nanoplex can therefore be attributed to the amorphous state of the nanoplex.

Amorphous forms of API are known to revert into their corresponding crystalline forms upon exposure to a dissolution medium, thus nullifying the solubility advantages afforded by the amorphous state¹⁵⁴. However, the generation of the supersaturated solution of CIP upon CIP nanoplex dissolution indicates that the amorphous CIP nanoplex do not transform into crystalline state upon exposure to PBS. If such instantaneous amorphous-to-crystal transition occurs, the supersaturated solution will not be generated, and the saturation solubility of amorphous CIP nanoplex will be identical to that of the raw CIP crystals¹⁵⁴. Instead, CIP nanoplex dissolution results in a high supersaturation level, though only over a short period (≈ 5 min), before it gradually decreases as CIP in the supersaturated solution crystallizes. While the crystallization of CIP from the supersaturated solution translates to a lowering of CIP concentration in solution, the final equilibrium concentration achieved upon cessation of CIP crystallization remains twice higher than that obtained from the dissolution of the raw CIP crystals. In other words, the use of nanoplex doubles the saturation solubility as compared to that afforded by the raw CIP crystals.

Lastly, MIC of the CIP nanoplex against *P. aeruginosa* bacteria is found to be equal to 0.25 $\mu\text{g/mL}$ for both the suspension and dry powder forms, which is comparable to that of the native CIP at 0.13 $\mu\text{g/mL}$. The complexation therefore does not jeopardize the antimicrobial activity of CIP released from the CIP nanoplex.

It must be pointed out that the fast crystallization of CIP from the supersaturated solution can erode the solubility advantage offered by the amorphous CIP nanoplex. Inclusion of crystallization inhibitors, such as hydroxypropylmethylcellulose (HPMC) and polyvinylpyrrolidone (PVP), in the dry powder formulation is therefore needed to prolong the supersaturation period. The adsorption of such crystallization inhibitors onto the surfaces of precipitated crystal nuclei, prevents further crystal growth hence allowing for a higher supersaturation period and sufficient time for absorption

following oral ingestion¹⁵⁴. The use of HPMC as crystallization inhibitor is presented in the next chapter.

In perspective, as with other preparation methods, the current technique does have its limitations, for example, the sparingly soluble drug needs to be soluble in either acidic or alkaline solutions, and the dissolved drug must carry some charge to interact with the polyelectrolyte. In addition, whether the absence of protons in the CIP nanoplex formulation (as compared to the salt formulations) affects the stability of the charged form of CIP has yet to be ascertained. Our preliminary work using κ -carrageenan as the polyelectrolyte, in place of DXT, nonetheless has demonstrated that CIP nanoplex with similar physical characteristics can be produced (result not shown), which suggests the promising universality potential of the current nanoplex preparation technique.

9.4 Chapter Summary

The findings discussed in this chapter have been published as:

W.S. Cheow and K. Hadinoto. Self-assembled amorphous drug-polyelectrolyte nanoparticle complex with enhanced dissolution rate and saturation solubility. *J Colloids Interface Sci* 367:518–526 (2011).

Stable amorphous nanoparticle powders of a poorly-soluble drug have been successfully prepared via a self-assembly drug-polyelectrolyte complexation process in the presence of salt. CIP and DXT are used as the models for poorly-soluble drug and polyelectrolyte, respectively. The advantages conferred by the amorphous nanoplex are evident in the (i) faster dissolution rate, (ii) enhanced saturation solubility, and (iii) higher achievable supersaturation level compared to those of the raw crystalline drug. The complexation process, which only involves a simple mixing of the drug and polyelectrolyte solutions, is entirely aqueous based, energy-minimal, and fast, with high production yield and drug loading. The drug concentrations and its ratio to the salt concentration have been identified as the two parameters governing the nanoplex formation. The faster dissolution rate of the nanoplex can be attributed to both the nanoscale size and amorphous state of the nanoplex, whereas the improvements in the saturation solubility and supersaturation level are owed more to the amorphous formulation.

Chapter 10

Spray-Freeze-Drying Production of Thermally-Sensitive Polymeric Nanoparticle Aggregates for Pulmonary Delivery

10.1 Introduction

Dry powder inhaler (DPI) represents an effective pulmonary delivery platform for direct dosing of therapeutic agents to the lungs because of its portability, long shelf-life, and high delivery efficiency. These characteristics of DPI are in contrast to the relative inconvenience (e.g. lengthy treatment time, non-portability) and low delivery efficiency associated with the more conventional nebulization delivery platform. The use of nanoparticles as therapeutic carriers in DPI delivery has recently gained significant interest as nano-scale formulations enable the therapeutic agents to evade the lung phagocytic clearance and enhances the dissolution rate of poorly water-soluble drugs⁹. Importantly, antibiotic-encapsulated nanoparticles can be used to deliver antibiotics to the lungs for inhaled anti-biofilm therapy.

The use of spray drying to prepare dry powder inhalable nano-aggregates of PLGA nanoparticles has been widely demonstrated^{93, 155-156}. Due to the relatively higher thermostability of PLGA, the transformation of PLGA nanoparticle suspensions into nano-aggregates by spray drying is considered straightforward. On the other hand, the dry powder transformation of lipid-PLGA hybrid nanoparticles poses a challenge as a result of the presence of the lipid layer on the surface of the nanoparticles. Nevertheless, the hybrid nanoparticles have been successfully formulated into inhalable nano-aggregates using the spray drying technique (Section 5.3.4). In addition,

the systematic comparison of two different techniques—spray drying and spray-freeze-drying—in dry powder transformation have been investigated and published as:

Y. Wang, K. Kho, W.S. Cheow, and K. Hadinoto. A comparison between spray drying and spray-freeze-drying for dry powder inhaler formulation of drug loaded lipid–polymer hybrid nanoparticles. *Int J Pharm* 424:98–106 (2012).

In contrast to the relatively thermostable PLGA, the transformation of the thermosensitive PCL nanoparticles (low melting point of 62°C) into inhalable dry powder nano-aggregates by using the spray drying technique involves a more intricate formulation process. In a study published as:

K. Kho, W.S. Cheow, R. H. Lie, and K. Hadinoto. Aqueous re-dispersibility of spray dried poly-caprolactone nanoparticle aggregates for inhaled anti-biofilm therapy. *Powder Technol* 203:432–439 (2010),

in which PCL nanoparticles were spray dried using mannitol, lactose, and leucine as drying adjuvants, the detrimental effects of the high temperature in a spray-drying process have been demonstrated. Even though the nano-aggregates produced exhibited d_A in the range ideal for inhaled delivery, less than half of the nanoparticles were recovered from the nano-aggregates upon re-dispersion. The poor aqueous re-dispersibility of the spray-dried PCL nano-aggregates was caused by irreversible nanoparticle coalescences, where PCL nanoparticles softened and fused together upon exposure to temperature near or above their melting point.

In this regard, the use of spray-freeze-drying (SFD) to transform aqueous suspensions of thermosensitive biodegradable polymeric nanoparticles (such as PCL) into their inhalable dry powder form remains unexamined. Therefore, the objective of the present chapter is to examine the effects of freeze drying adjuvant formulation on the morphology, aerodynamic diameter, aqueous re-dispersibility, flowability, and production yield of PCL nano-aggregates produced by SFD. In addition to transforming aqueous suspensions of thermosensitive polymeric nanoparticles into dry powder, the SFD technique is also applicable to encapsulated materials that are thermosensitive, for example antimicrobial peptide encapsulated in nanoparticles.

Four generally-recognized-as-safe (GRAS) excipients (i.e. mannitol, lactose, poly(vinyl alcohol) (PVA), and leucine), which have been widely studied for inhaled

dosage form formulations, are used. The thermosensitive PCL nanoparticles are loaded with levofloxacin to represent a model of aerosol antibiotic formulation intended for lung biofilm infection therapy. Leucine is a hydrophobic compound, whose inclusion results in particles having corrugated surfaces, hence better particle entrainment off the inhaler¹⁵⁷. PVA, a biodegradable and non-cytotoxic surfactant, has been found to be a suitable lyoprotectant for PCL nanocapsules¹⁵⁸, while mannitol and lactose have often been used as coarse carrier particles in DPI¹⁵⁹.

10.2 Methodology

10.2.1 Preparation and characterization of PCL nanoparticles

LEV-loaded PCL nanoparticles were prepared by the ESE method (Section 3.2.1.2), and characterized in terms of initial nanoparticle size (S_i), encapsulation efficiency, and drug loading according to the methods presented in Section 3.2.2.

10.2.2 Spray-freeze-drying of PCL nanoparticle suspension

The feed suspension was prepared by adding drug-loaded PCL nanoparticles into aqueous solutions of the adjuvants. SFD was performed in a modified BÜCHI B-290 mini spray-dryer (BÜCHI, Switzerland) as illustrated in Fig. 10.1, where the drying chamber of the spray dryer was removed and replaced with a polypropylene vessel containing 500 mL of liquid nitrogen under constant stirring. As preliminary experiments showed that the use of ultrasonic atomizer (Vibracell VC 5040, Sonics and Materials, USA) resulted in frozen feed in the nozzle, a two-fluid atomizer with 1.5 mm nozzle diameter was used instead. The feed was atomized into liquid nitrogen with the nozzle positioned 10 cm above the liquid nitrogen level. The following three parameters were kept constant i.e. 0.24 L/hour feed rate, 240 L/hour atomizing air flow rate, and 500 RPM liquid nitrogen stirring speed.

Following atomization, the slurry containing the frozen droplets and the remaining liquid nitrogen was transferred to a lyophilization container, and the excess liquid nitrogen was evaporated. The freezing was continued at -80°C for 16 hours after which lyophilization was carried out for 24 hours in a Alpha 1-2 LD Plus freeze dryer (Martin Christ, Germany) at a temperature of -52°C and a vacuum level of 0.05 mbar resulting in dry powder production. The production yield was determined from

the ratio of the nano-aggregate mass recovered after SFD to the initial mass added in the feed (i.e. PCL and adjuvant).

Figure 10.1: A schematic of the SFD set-up.

10.2.3 Physical characterizations of PCL nano-aggregates

10.2.3.1 Particle morphology

The nano-aggregate morphology was characterized using a scanning electron microscope (SEM) model JSM-6700F (JEOL, USA) equipped with energy dispersive x-ray spectroscopy (EDX) to verify the presence of adjuvant in the spray-freeze-dried particles. As the nano-aggregates were specifically designed to re-disperse into primary nanoparticles in an aqueous medium, d_G of the nano-aggregates is determined by image analysis instead of by laser diffraction-based sizing method, which employs high-intensity wet dispersion methods to break particle agglomerates prior to measurements. The wet dispersion method is unfavourable for sizing the nano-aggregates because it would lead to inaccurate d_G measurements due to premature nano-aggregates re-dispersions. In the image analysis, SEM images of the nano-aggregates were analysed using ImageJ software (NIH, USA), where sixplicates of 1000 particles were measured for each sample. The number-based size distribution

from the image analysis was converted to the volume-based distribution yielding the reported d_G values. Experimental uncertainties in the d_G values are $\pm 7\%$.

10.2.3.2 Particle density, aerodynamic diameter, and flowability

ρ_e of the nano-aggregates was determined from the tap density (ρ_{tap}) values obtained from two replicates of 1 mL particle volume each, where each sample was subjected to 2000 taps in a tap densitometer (Quantachrome, USA). The d_G and ρ_e values were subsequently used to calculate theoretical d_A using Eq. (2.2). Experimental uncertainties in the d_A values are $\approx 8\%$ based on the experimental errors in the d_G and ρ_{tap} measurements. The particle flowability was characterized by Carr's compressibility index (CI) defined in Eq. 10.1 using the bulk (ρ_{bulk}) and ρ_{tap} values, where weighed amount of powders was loosely filled into a 5 mL measuring cylinder to determine ρ_{bulk} . $CI \leq 25$ and $CI \geq 40$ are indicative of free-flowing and poorly-flowable particles, respectively¹⁶⁰.

$$CI = \left(1 - \frac{\rho_{bulk}}{\rho_{tap}} \right) \times 100\% \quad (10.1)$$

Experimental uncertainties in the calculated CI values are $\approx 7\%$ based on the experimental errors in the ρ_{bulk} and ρ_{tap} measurements.

In addition, particle flowability upon aerosolization was examined in terms of the emitted dose (ED) and fine particle fraction (FPF) using Next Generation Pharmaceutical Impactor (NGI) (Copley Scientific, UK) equipped with an induction port and a pre-separator. ED is defined as the percentage mass of powders that are emitted off the inhaler, whereas FPF is defined as the percentage mass of powders recovered in the impactor that possess $d_A \leq 5 \mu\text{m}$. Standardized powder entrainment tube (SPET) detailed in Louey *et al.*¹⁶¹ was adopted in the aerosolization characterization study in place of an inhaler. In this regard, aerosolization characteristics of powders delivered by SPET have been shown to be comparable to those delivered by two commercial inhaler devices (i.e. Rotahaler and Inhalator) as manifested by their similar ED, FPF, and mass median aerodynamic diameter (MMAD) values¹⁶¹.

The inhalation flow rate needed to achieve the standard 4 kPa pressure drop in SPET was 85 L/min measured using a critical flow controller (Copley Scientific, UK).

The air flow duration was set to 2.8 s to simulate a typical inhalation volume of 4 L. The effective cut-off diameters at 85 L/min are 6.7, 3.7, 2.4, 1.4, and 0.3 μm for stages 1 to 5, respectively. The impactor plates were coated with silicone grease (Dow Corning®) to prevent particle re-entrainment. The amount of powder recovered in each stage was determined from the amount of adjuvants recovered by means of colorimetric assays¹⁶²⁻¹⁶³ using UV-Vis spectrophotometer. ED and FPF measurements were performed in triplicates using 5 mg of powders each time. Experimental uncertainties in the ED and FPF values are $\approx 8\%$ and 5% , respectively.

10.2.3.3 Aqueous re-dispersibility

Conventional characterization methods for aqueous re-dispersions, which involve homogenization or sonication, were not employed in the present work as high intensity dispersion forces do not represent the actual re-dispersion mechanism in the epithelial lining fluid of the lungs. Instead, spontaneous aqueous re-dispersion characterization method was employed. Briefly, 10 mg of the dry powder nano-aggregates were added to 1 mL of DI water and the suspension was allowed to sit for 10 min. The size of the particles present in the suspension (S_f) was measured afterwards by PCS and the ratio of S_f/S_i was calculated to determine the extent of the re-dispersion, where ratio of $S_f/S_i \approx 1$ signifies complete aggregate re-dispersions into primary nanoparticles.

To determine the mass fraction of nano-aggregates that have re-dispersed during the 10 min period (i.e. % re-dispersed), the suspension was centrifuged at $5000\times g$ for 1 min after which 1.5 mL of the supernatant, which contains the dissolved adjuvants and the re-dispersed nanoparticles, was removed and replaced with 1.5 mL of DI water without stirring. The resulting suspension was centrifuged for the second time. The dilution and centrifugation were repeated two more times to ensure the removal of all the re-dispersed nanoparticles and the dissolved adjuvants. After the fourth centrifugation, 1.5 mL of the supernatant was discarded and the remaining 0.5 mL sedimented pellets were lyophilized for 24 h. The lyophilized pellets, which contain the nano-aggregates that have not re-dispersed, were weighed. As the three cycles of centrifugation and re-suspension ensures the removal of most of the water-soluble adjuvants, the % re-dispersed was determined by calculating the ratio of the lyophilized pellet weight to the initial PCL nanoparticle weight in the feed.

10.3 Results and Discussion

10.3.1 Drug-loaded PCL nanoparticle characteristics

Drug-loaded PCL nanoparticles are produced with diameter (S_i) of 290 ± 40 nm and the % encapsulation is low at $\approx 8\%$ due to the high aqueous solubility of the model drug (≈ 100 mg/mL), which causes a majority of the drug to diffuse out into the aqueous phase during the emulsification and solvent evaporation steps. In this regard, % encapsulation of water-soluble drugs in hydrophobic polymeric nanoparticles can be enhanced by modifying the nanoparticle preparation method (e.g. using alternative solvents and surfactants, w/o/w double emulsification)¹⁶⁴⁻¹⁶⁶. Improving the % encapsulation, however, is not pursued in the present work as the emphasis is on examining the effects of adjuvant formulation on the physical characteristics of spray-freeze-dried nano-aggregates.

10.3.2 Adjuvant screening

In the absence of adjuvants, PCL nanoparticle suspension at 5.0% (w/v) concentration can be transformed into dry powder nano-aggregates using SFD. Fig. 10.2A shows the large and spherical morphologies of the spray-freeze-dried nano-aggregates ($d_G \gg 10$ μm). Importantly, the large d_G is still smaller than the secondary bronchial diameter that is in the half-millimeter range. A closer look at the particle surface in Fig. 10.2B reveals porous structures manifested in the low particle density ($\rho_{tap} \approx 0.05$ g/cm³). The particle geometric size distribution shown in Fig. 10.2C indicates a rather wide distribution between 10 and 60 μm with mean $d_G \approx 18$ μm . Despite the large d_G , d_A of the nano-aggregates remains in the $\approx 4\text{--}5$ μm range owed to the low particle density hence they are suitable for lung deposition.

In terms of the aqueous re-dispersibility shown in Fig. 10.2D, however, only approximately 20% of the nano-aggregates are recovered as primary nanoparticles upon aqueous re-dispersion. A majority of the nano-aggregates are recovered as aggregate fragments, whose sizes are in the 1500–2500 nm range, indicating poor aqueous re-dispersibility manifested in the high ratio of S_f/S_i (≈ 5). The results of the adjuvant-free formulation indicate that adjuvants must be added to facilitate aqueous re-dispersions of the nano-aggregates. To assess the suitability of lactose, leucine, mannitol, and PVA as SFD adjuvants, their aqueous solutions are spray-freeze-dried in

the absence of PCL nanoparticles. Figs. 10.3A, B, and C show the morphology of particles produced from SFD of 7.0% (w/v) aqueous solutions of lactose, mannitol, and PVA, respectively. SFD of leucine in Fig. 10.3D is conducted at a lower concentration of 1.5% (w/v) due to its lower aqueous solubility limit.

Figure 10.2: (A) Large and spherical morphologies of PCL nano-aggregates, (B) PCL nanoparticles on the surface, (C) geometric particle size distribution, and (D) size distribution of PCL nanoparticles before SFD and after re-dispersion in an aqueous medium.

The results of the PCL-free formulation indicate that the high hygroscopicity of lactose leads to the production of highly cohesive particles (Fig. 10.3A) that leads to poor flowability. The use of lactose as the adjuvant is therefore ruled out. On the contrary, SFD of both mannitol and PVA solutions lead to the production of non-cohesive spherical particles shown in Fig. 10.3B and 10.3C, respectively, where PVA particles exhibit smooth surfaces and mannitol particles exhibit highly porous structures. Not unexpectedly, PVA particles exhibit a higher density ($\rho_{tap} = 0.09 \text{ g/cm}^3$) than that of the mannitol particles ($\rho_{tap} = 0.07 \text{ g/cm}^3$). In contrast, SFD of the leucine solution produces irregularly shaped and fragile fragments (Fig. 10.3D), where intact leucine particles are rarely observed. The results of the adjuvant screening therefore

suggest that mannitol and PVA are the two most promising SFD adjuvant candidates. The effects of mannitol and PVA inclusions on the morphology, aqueous re-dispersibility, flowability, and production yield of the PCL nano-aggregates are investigated next.

Figure 10.3: Morphologies of spray-freeze-dried (A) lactose, (B) mannitol, (C) PVA, and (D) leucine.

10.3.3 Mannitol as adjuvant

The effects of adjuvant formulation are investigated by varying the nanoparticle to mannitol concentration ratio (i.e. N/M ratio) at two total solid concentrations (i.e. 5.0% and 2.5% w/w) in the feed. The results of experimental runs at 5.0% (w/w) (i.e. Runs A1–A4) and at 2.5% (w/w) (i.e. Runs B1–B4) are presented in Table 10.1. The N/M ratios are varied by varying mannitol concentration in the feed between 20% and 70% (w/w). Using mannitol as adjuvant, SFD at feed concentrations lower than 2.5% (w/w) produces fragile particles that are not likely to withstand the aerosolization force. On the other hand, SFD at higher solid concentrations than 5.0% (w/w) produces large but non-porous particles, such that they do not satisfy the $d_A \approx 1\text{--}5\text{ }\mu\text{m}$ requirement.

10.3.3.1 Morphology, density, aerodynamic diameter, flowability, and production yield

In the absence of mannitol, the results in Table 10.1 indicate that nano-aggregates produced at 5.0% (w/w) feed concentration (i.e. Run A1) exhibit higher ρ_{tap} and slightly larger d_G than Run B1 at 2.5% (w/w) feed concentration. This observation is not unexpected as similar trends have been observed in SFD of aqueous solutions¹⁶⁷. In the presence of mannitol, a similar trend in d_G is observed in which nano-aggregates produced at a higher feed concentration exhibit larger d_G (i.e. 25–40 μm in Runs A2–A4 versus 20–25 μm in Runs B2–B4). Significantly, nano-aggregates produced from the mannitol-free formulations (i.e. Runs A1 and B1) exhibit higher ρ_{tap} and smaller d_G compared to those produced with mannitol at the same feed concentration. These results indicate that more porous and larger nano-aggregates are produced with the mannitol inclusion. In addition, the production yields are reduced from ≈ 60 –70% in the mannitol-free formulations to ≈ 15 –40% in the presence of mannitol depending on the mannitol concentration.

Table 10.1: Summary of PCL–Mannitol nano-aggregate experiments.

Run	Feed Conc. (w/w)	% Mannitol (w/w)	ρ_e (g/cm ³)	d_G (μm)	Yield (%)
A1	5.0%	0	0.076	18	58
A2		20	0.042	25	41
A3		40	0.018	35	15
A4		70	0.021	41	26
B1	2.5%	0	0.056	16	70
B2		20	0.022	20	16
B3		40	0.017	23	13
B4		70	0.025	25	40

SEM image of the PCL–Mannitol nano-aggregates in Fig. 10.4A reveals their large and highly porous morphologies. A close-up view of the particle surface in Fig. 10.4B indicates that PCL nanoparticles are physically dispersed in porous mannitol matrix, such that the nanoparticles are not in direct contacts with each other. High nano-aggregate aqueous re-dispersibility is therefore expected. In the presence of mannitol, the d_G values increase with increasing mannitol concentrations in the feed (i.e. lower N/M ratio) as larger porous mannitol particles are produced. The trends in ρ_{tap} and production yield as a function of the N/M ratio are less predictable.

Importantly, despite the variations in the d_G and ρ_{tap} values, the d_A values in Runs A2–A4 and B2–B4 remain relatively constant as a function of the mannitol concentration as shown in Fig. 10.5A.

Figure 10.4: (A) Large, spherical and porous morphologies of PCL–Mannitol nano-aggregates produced in Run A4, (B) PCL nanoparticles are physically dispersed in porous mannitol matrix.

Figure 10.5: (A) d_A , (B) CI as a function of mannitol concentration, (C) d_G distributions at 2.5% (w/w) feed concentration, and (D) d_G distributions at 5.0% (w/w) feed concentration.

Owed to their larger d_G , nano-aggregates produced at 5.0% (w/w) feed concentration consistently exhibit larger d_A compared to those produced at 2.5% (w/w) feed concentration. Specifically, the former attains d_A of ≈ 4.0 – $5.0\ \mu\text{m}$ and the latter attains slightly smaller d_A at ≈ 2.5 – $3.5\ \mu\text{m}$. Both of them therefore satisfy the d_A requirement for effective lung depositions. In terms of the flowability, CI values of the nano-aggregates produced in Runs A1–A4 and B1–B4 remain below 20 independent of the mannitol concentration as shown in Fig. 10.5B. The low CI values denote good particle flowability consistent with the discrete particle morphology observed in Fig. 10.4A. Importantly, the nano-aggregates can be effectively aerosolized off the entrainment tube as manifested in the high ED values of $\approx 94\%$. The FPF values, however, are rather low at 10–14% as a majority of the aerosolized nano-aggregates (40%) deposit in the pre-separator as shown in Fig. 10.6. The low FPF values indicate that the nano-aggregates tend to agglomerate upon aerosolization.

Figure 10.6: Particle deposition profile of PCL–mannitol nano-aggregates in NGI (SPET = standardized powder entrainment tube; IP = induction port; PS = pre-separator).

Particle size distributions of the nano-aggregates produced at 5.0 and 2.5% (w/w) feed concentrations are presented in Figs. 10.5C and 10.5D, respectively. At 5.0% (w/w) feed concentration, the d_G values are in the range of 10–120 μm , whereas at 2.5% (w/w) feed concentration, the d_G values vary within a smaller range of 10–60 μm . Therefore, the particle size distribution is less uniform at higher feed concentrations rendering formulations at low feed concentrations (i.e. Runs B2–B4) more attractive as

particle size distributions significantly impact aerosolization, which in turn influence the emitted dose and dosing uniformity. In this regard, Run B2 represents the most ideal formulation as it produces a highly monodisperse distribution with mean d_G of 20 μm . The production yield of Run B2, however, is rather low at 16% making it a less than ideal formulation. A similarly low production yield is also observed in Run B3.

On the other hand, Run B4 produces a relatively similar mean d_G value as that of Run B2, though with a less uniform distribution, but with a significantly higher production yield of 40%. In summary, with the exception of Run A4 in which excessive d_A ($\approx 5.5 \mu\text{m}$) is observed, the use of mannitol as the adjuvant results in nano-aggregates having (i) large d_G and low ρ_{tap} values resulting in $d_A \approx 3\text{--}5 \mu\text{m}$, (ii) good flowability as demonstrated by $CI \leq 20$ and effective aerosolization off the entrainment tube, and (iii) reasonably high production yields ($\approx 40\%$) and uniform particle size distributions can be achieved at 2.5% (w/w) feed concentration and at N/M ratio of 3:7.

10.3.3.2 Aqueous re-dispersibility

The ratio of S_f/S_i and the % re-dispersed of the PCL–Mannitol nano-aggregates are presented in Figs. 10.7A and 10.7B, respectively. The ratio of S_f/S_i and % re-dispersed and their variations as a function of the mannitol concentration in the feed are found to be independent of the feed concentration. The ratio of S_f/S_i values are reduced from about 4.5 to approximately 1.5 when mannitol concentration in the feed is increased from 0 to 70% (w/w). The decrease in the ratio of S_f/S_i with increasing mannitol concentration in Fig. 10.7A corresponds to an increased fraction of the nano-aggregates being re-dispersed into primary nanoparticles.

The improved re-dispersions at higher mannitol concentrations are confirmed by the increasing % re-dispersed values with increasing mannitol concentration observed in Fig. 10.7B. Specifically, the % re-dispersed is increased from 30% in the absence of mannitol in Runs A1 and B1 to $\approx 85\%$ at 70% (w/w) mannitol concentration in Runs A4 and B4. Importantly, the % re-dispersed of the nano-aggregates produced by SFD represents a significant improvement compared to the PCL–Mannitol nano-aggregates produced by spray drying in Kho *et al.*¹⁶⁸, which are not at all re-dispersible even at N/M ratio of 1:6. The improved re-dispersion signifies the SFD ability to prevent the occurrence of irreversible nanoparticle coalescence.

Figure 10.7: Aqueous re-dispersibility of PCL-Mannitol nano-aggregates (A) ratio of S_f/S_i , (B) % re-dispersed, and (C) size distributions of re-dispersed nano-aggregates.

Particle size distributions of re-dispersed nanoparticles from Runs B2 and B4 are presented in Fig. 10.7C. Particle size distribution of re-dispersed nanoparticles from Runs A1–A4 exhibit nearly identical profiles hence they are not presented here for brevity. In Run B2 at 20% (w/w) mannitol concentration, micro-scale fragments (> 1500 nm) of the nano-aggregates are present in a significant amount denoting

incomplete re-dispersions. As the mannitol concentration is increased to 70% (w/w) in Run B4, the amount of particles recovered in the micro-scale range, though still present, is considerably reduced. A larger fraction of the nano-aggregates are recovered as primary nanoparticles having the same size as the original nanoparticles (≈ 300 nm). The results indicate that a high mannitol concentration (i.e. 70% w/w) is needed to achieve complete aggregate re-dispersions.

10.3.4 PVA as adjuvant

The effects of the nanoparticle to PVA concentration ratio (i.e. N/P ratio) are investigated at two total solid concentrations (i.e. 2.5 and 1.5% w/w) in the feed. The results of experimental runs at 2.5% (w/w) (i.e. Runs C1–C4) and 1.5% (w/w) (i.e. Runs D1–D4) are presented in Table 10.2. The N/P ratios are varied by varying the PVA concentration in the feed between 20% and 70% (w/w). Using PVA as adjuvant, SFD at solid concentrations higher than 2.5% (w/w) produces large non-porous particles resulting in $d_A \gg 5$ μm .

Table 10.2: Summary of PCL—PVA nano-aggregate experiments.

Run	Feed Conc. (w/w)	% PVA (w/w)	ρ_e (g/cm ³)	d_G (μm)	Yield (%)
C1	2.5%	0	0.056	16	70
C2		20	0.084	18	29
C3		40	0.078	20	27
C4		70	0.084	14	36
D1	1.5%	0	0.060	22	72
D2		20	0.063	23	82
D3		40	0.049	18	50
D4		70	0.056	21	45

10.3.4.1 Morphology, density, aerodynamic diameter, flowability, and production yield

In the absence of PVA, nano-aggregates produced at the two feed concentrations are relatively similar in terms of their ρ_{tap} and d_G values, which fall in the range of ≈ 0.06 g/cm³ and 16–22 μm , respectively. The production yields of the PVA-free formulations are high at $\approx 70\%$, however, they are reduced to ≈ 30 –50% with PVA inclusion. One exception is Run D2 in which the yield is increased to 82%. The production yields are at least 10% higher at 1.5% (w/w) feed concentration than at 2.5%

(w/w) in the presence of PVA. In contrast to the results of the PCL–Mannitol formulation, the effects of PVA inclusion on the ρ_{tap} and d_G values do not exhibit general trends that are independent of the feed concentration.

At 2.5% (w/w) feed concentration, the ρ_{tap} values are increased from 0.04 to $\approx 0.06 \text{ g/cm}^3$ with PVA inclusion, whereas the variations in the d_G values are relatively insignificant. On the other hand, at 1.5% (w/w) feed concentration, the PVA inclusion does not have any significant impacts on both the ρ_{tap} and d_G values. On the whole, the d_G values in Runs C2–C4 and D2–D4 are basically unchanged at $\approx 20 \text{ }\mu\text{m}$. Overall, variations in the ρ_{tap} and d_G values as a function of the N/P ratio are relatively insignificant at these low feed concentrations.

SEM image of the PCL–PVA nano-aggregates in Fig. 10.8A indicates that PCL–PVA particles are less porous than their PCL–Mannitol counterparts shown in Fig. 10.4A. A close-up view of the particle surface in Fig. 10.8B indicates that PCL nanoparticles are distributed more closely when PVA is used as the adjuvant, instead of mannitol. Importantly, the porous adjuvant matrix observed in the PCL–Mannitol nano-aggregates (Fig. 10.4B) is not observed in Fig. 10.8B, which explains for the lower apparent porosity of the PCL–PVA nano-aggregates. The lower porosity is manifested in the overall higher ρ_{tap} values for the PCL–PVA nano-aggregates (i.e. $\approx 0.04\text{--}0.07 \text{ g/cm}^3$ for PCL–PVA versus $\approx 0.01\text{--}0.03 \text{ g/cm}^3$ for PCL–Mannitol). Significantly, at feed concentration of 1.5% (w/w), the ρ_{tap} values of the PCL–PVA nano-aggregates ($\approx 0.06 \text{ g/cm}^3$) are still three-fold higher than those of the PCL–Mannitol nano-aggregates produced at higher feed concentrations.

Figure 10.8: (A) Large and spherical morphologies of PCL–PVA nano-aggregates produced in Run C3, (B) closely-packed PCL nanoparticles exhibiting larger size due to PVA coatings.

In this regard, PCL nanoparticles visible on the aggregate surface in Fig. 10.8B closely resemble the nanoparticles in the adjuvant-free formulation shown in Fig. 10.2B, except for the larger size (≈ 500 nm) compared to their original size prior to SFD (≈ 300 nm). The presence of PVA in the spray-freeze-dried particles has been verified using EDX by examining the carbon to oxygen ratio. As PVA matrix formation is not evident, instead of forming porous matrix like mannitol, PVA is postulated to coat the nanoparticle surface upon lyophilization. The PVA coating provides an explanation for the apparent almost two-fold increase in the nanoparticle size after SFD. In this regard, formation of PVA coating on PCL nanoparticle surface upon lyophilization has previously been reported by Abdelwahed *et al.*¹⁵⁸.

Figure 10.9: (A) d_A , (B) CI as a function of PVA concentration, (C) d_G distributions at 1.5% (w/w) feed concentration, and (D) d_G distributions at 2.5% (w/w) feed concentration.

As a result of the difference in their nanoparticle-adjuvant structures, PCL–PVA nano-aggregates exhibit slightly larger d_A values than their PCL–Mannitol counterparts

despite their relatively similar d_G values. The d_A values of Runs C2–C4 and Runs D2–D4 shown in Fig. 10.9A are found to vary in the range of 3.5–5.5 μm depending on the feed concentration and the N/P ratio. Certain formulations (e.g. Runs C3 and D2) produce d_A values that are too large for effective lung depositions. In terms of the flowability, PCL–PVA nano-aggregates possess CI values in the range of 15–25 as shown in Fig. 10.9B, which signifies good flowability similar to the PCL–Mannitol nano-aggregates. Not unexpectedly, the PCL–PVA nano-aggregates exhibit similar aerosolization characteristics as their PCL–Mannitol counterparts.

With regard to the particle size distribution, nano-aggregates produced at 1.5% and 2.5% (w/w) feed concentrations exhibit wide variations depending on the N/P ratio as shown in Figs. 10.9C and 10.9D, respectively. Nevertheless, highly monodisperse distributions can be obtained at (i) 70% PVA at 2.5% (w/w) feed concentration in Run C4 and (ii) 40% PVA at 1.5% (w/w) feed concentration in Run D3. Importantly, the monodisperse distributions are accompanied by reasonably high production yields ($\approx$ 40–50%). In summary, similar to the PCL–Mannitol formulation, a majority of the PCL–PVA formulations can produce large spherical nano-aggregates having appropriate d_A , good flowability, and reasonable production yields.

10.3.4.2 Aqueous re-dispersibility

The ratio of S_f/S_i and the % re-dispersed of the PCL–PVA nano-aggregates are presented in Figs. 10.10A and 10.10B, respectively. Similar to the results of the PCL–Mannitol formulation, the variations in the ratio of S_f/S_i and % re-dispersed are independent of the feed concentration. The PVA inclusion reduces the ratio of S_f/S_i from $\approx$ 5–6 in its absence to $\approx$ 1–3 depending on the N/P ratio. The ratio of S_f/S_i decreases and % re-dispersed increases with increasing PVA concentration (i.e. lower N/P ratio), which are again similar to the trends observed in the PCL–Mannitol formulations. At 70% PVA concentration in Runs C4 and D4, close to 90% of the nano-aggregates are re-dispersed resulting in the ratio of $S_f/S_i \approx$ 1.

Particle size distributions of the re-dispersed nanoparticles from Runs D2–D3 are presented in Fig. 10.10C. Particle size distributions of Runs C2–C4 are nearly identical to those of Runs D2–D4 hence they are not presented here for brevity. In Run D2 at 20% PVA concentration, a small amount of micro-scale fragments of the nano-aggregates ($>$ 1000 nm) are recovered upon re-dispersion. As the PVA concentration in the feed is

increased to 40%, the micro-scale fragments are no longer observed and the size of the re-dispersed nanoparticles all falls within the $\approx 300\text{--}500\text{ nm}$ range. Removal of the micro-scale fragments at 40% adjuvant concentration is not observed in the PCL-Mannitol formulation, where fragments in the size range of $\approx 1000\text{--}1500\text{ nm}$ are still present even when 70% of mannitol is used (Fig. 10.7C).

Figure 10.10: Aqueous re-dispersibility of PCL-PVA nano-aggregates (a) ratio of S_f/S_i , (b) % re-dispersed, and (c) size distributions of re-dispersed nano-aggregates.

In this regard, the high crystallization propensity of mannitol may contribute to the poorer aqueous re-dispersibility of the PCL-Mannitol formulation. Mechanical

stresses exerted by the growing mannitol crystals upon freezing can promote irreversible nanoparticle fusions¹⁶⁹. Furthermore, crystallized mannitol may undergo phase separation in the cryo-concentrated portion of the frozen nanoparticle suspension¹⁶⁹, which consequently eliminates the colloidal stabilizing functions of the adjuvant. At low N/M ratios, fewer PCL nanoparticles are dispersed in the vast mannitol matrix, hence keeping inter-nanoparticle contacts at a minimum resulting in reasonably effective re-dispersion of the nano-aggregates.

In contrast, PVA exists in its glassy state during lyophilization and it has been found to be capable of inhibiting ice nucleation, thereby preventing the formation of ice crystals that exert mechanical stresses on the nanoparticles¹⁵⁸. Moreover, the presence of PVA coating on the PCL nanoparticle surface can act as stabilizers that minimize inter-nanoparticle contacts during lyophilization. As a result, PCL–PVA nano-aggregates can fully re-disperse into primary nanoparticles more readily (i.e. at lower adjuvant concentration) than the PCL–Mannitol nano-aggregates.

As a majority of the formulations can satisfy the d_G and d_A requirements, as well as good flowability, using either mannitol or PVA as adjuvants, the determining nano-aggregate characteristics in selecting the optimal formulation are (i) production yield, (ii) particle size distribution, and (iii) aqueous re-dispersibility. In this regard, the PCL–Mannitol formulation in general results in lower production yields (i.e. 15–40%) compared to the PCL–PVA formulation (i.e. 30–80%). Both mannitol and PVA formulations are capable of producing monodisperse particle size distributions (i.e. Runs B2, C4, and D3). However, unlike the PCL–PVA formulation, uniform particle size distributions for the PCL–Mannitol formulation are not always accompanied by reasonable production yields and vice versa. Taking all the three characteristics into consideration, the PCL–PVA formulation on the whole is considered to be superior to the PCL–Mannitol formulation.

In this regard, Run D3 of the PCL–PVA formulation represents an optimal formulation, where spherical nano-aggregates having mean $d_G \approx 18 \mu\text{m}$, $d_A \approx 4 \mu\text{m}$, CI ≈ 15 , production yield $\approx 50\%$, and monomodal size distribution (d_G mode $\approx 30 \mu\text{m}$) are produced. Furthermore, the nano-aggregates produced can be effectively re-dispersed into primary nanoparticles having a monomodal size distribution at ratio of $S_f/S_i \approx 1.3$ and % re-dispersed $\approx 80\%$. Importantly, compared to the optimal formulation for PCL

nano-aggregate productions by spray drying using lactose and leucine as adjuvants presented in Kho *et al.*¹⁶⁸, the PCL–PVA formulation in Run D3 is superior in terms of its (i) higher production yield ($\approx 50\%$ vs. 25%), (ii) higher % re-dispersed ($\approx 80\%$ vs. 50%) and lower S_f/S_i ratio (≈ 1.3 vs. 1.5), and lastly (iii) lower amount of adjuvant needed to achieve effective re-dispersions ($\approx 40\%$ vs. 88% of feed concentration). Therefore, SFD represents a better technique than spray drying to produce inhalable dry powder aggregates of thermally-sensitive polymeric nanoparticles.

10.4 Chapter Summary

The results and discussion presented in this chapter has been published as:

W.S. Cheow, M. L. L. Ng, K. Kho, and K. Hadinoto. Spray-freeze-drying production of thermally sensitive polymeric nanoparticles aggregates for inhaled drug delivery: Effect of freeze drying adjuvants. *Int J Pharm* 404:289–300 (2011).

Dry powder aggregates of drug-loaded PCL nanoparticles having a large geometric size while remaining in the inhalable size range have been successfully produced using SFD. Mannitol and PVA have been identified in the screening experiments as the two most suitable SFD adjuvants, over lactose and leucine, to serve as cryoprotective agents and to facilitate aqueous re-dispersion of the nanoparticle aggregates. The effects of feed concentration and its adjuvant content on the particle morphology, aerodynamic diameter, aqueous re-dispersibility, flowability, and production yield have been examined. The results indicate that the adjuvant selection dictates the resulting nanoparticle-adjuvant structures, which in turn influence the aerodynamic diameter and aqueous re-dispersibility of the dry powder aggregates.

Using mannitol as adjuvant, PCL nanoparticles are physically dispersed in porous mannitol matrix, whereas using PVA as adjuvant leads to PVA coating the nanoparticle surface. Both adjuvants nevertheless enhance the aqueous re-dispersibility and produce particles in the inhalable size range having the desired morphology, good flowability, and effective aerosolization off the inhaler. However, the fine particle fraction of the aerosolized nano-aggregates still leaves much room for improvement. The incorporation of the hydrophobic amino acid leucine in the SFD formulation might be beneficial to prevent powder agglomeration and hence improve the fine particle fraction of the aerosolized nano-aggregates. On the whole, PCL–PVA

formulation results in better aqueous re-dispersibility and higher production yield compared to the PCL–Mannitol formulation. Taking all the particle characteristics into consideration, PCL–PVA nano-aggregates produced at 1.5% (w/w) feed concentration and 40% (w/w) PVA content have been identified as the optimal formulation.

Chapter 11

Conclusions and Recommendations

11.1 Conclusions

In the present thesis, the development and applications of (i) antibiotic-loaded nanoparticles for inhaled anti-biofilm therapy, and (ii) drug-polyelectrolyte nanoplexation to prepare amorphous antibiotic nanoparticle have been discussed. The delivery of dry powder aerosol of antibiotic-loaded nanoparticles to the lungs is an attractive anti-biofilm therapy targeted to individuals with impaired mucocilliary clearance mechanism in their lungs. Towards this aim, the optimum antibiotic release profile for maximum biofilm eradication by LEV-loaded nanoparticles has been identified in **Chapter 4** as a biphasic extended release profile. A high initial antibiotic concentration ensures maximum biofilm cells are killed, while the second phase, a slower extended release phase, maintains an antibiotic concentration high enough to arrest biofilm proliferation. The maximum killing of biofilm cells initially is crucial, as surviving biofilm cells are less susceptible to antibiotics.

The requirement for a high initial antibiotic concentration (discussed in Chapter 4) necessitates a high loading of the antibiotic in the nanoparticles, forming the focus of **Chapter 5**, in which the standard nanoparticle preparation methods (NPC, ESE, DESE) are modified in order to achieve a higher drug loading. In the case of DESE, the experimental results indicate that doubling of % encapsulation when two single-phase modifications are performed separately does not translate to quadrupled % encapsulation when the two modifications are performed simultaneously. PC addition in the ESE method represents the optimal formulation in terms of the nanoparticle size, % encapsulation, drug loading, *in vitro* drug release profile, and production yield. In the LEV-loaded PC-modified PLGA nanoparticles, the drug loading is doubled

compared to the standard method. The nanoparticles can also be straightforwardly transformed into the inhalable solid dosage form by spray-drying with the inclusion of drying adjuvants, demonstrating the feasibility of delivering dry powder aerosols of nanoparticle aggregates into the lungs.

In **Chapter 6**, the PLGA–PC lipid–polymer hybrid nanoparticles have been found to exhibit higher antibacterial efficacy against 1 day-old *P. aeruginosa* biofilm cells than their non-hybrid PLGA nanoparticle counterparts, after 24 h of antibiotic exposure. The higher anti-biofilm efficacy is observed across a wide range of antibiotic concentrations and for nanoparticles in both their aqueous suspension and dry powder forms. However, the higher efficacy is not due to higher affinity of the hybrid nanoparticles towards biofilm cells, nor antibiotic release rate. The mechanisms responsible for the higher anti-biofilm efficacy of the hybrid nanoparticles remain to be investigated.

In addition to LEV, other antibiotics such as OFX, CIP, and TOB have also been encapsulated in PLGA–PC lipid–polymer hybrid nanoparticles (**Chapter 7**). It was found that (i) the ionic interactions between oppositely charged drug and lipid can prevent nanoparticle formation, which can be remedied by reversing the charge of the lipid through counterionic surfactant (e.g. SA) addition, (ii) drug loading of the hybrid nanoparticles is influenced by both the aqueous solubility and lipophilicity of the drug, where compared to the non-hybrid nanoparticles, the drug loading of the hybrid nanoparticles are twice as high, with the exception of CIP-encapsulated nanoparticles due to CIP being less lipophilic, and (iii) the impact of the lipid coat on the *in vitro* drug release profile highly depends on the drug lipophilicity.

Chapter 8 of this dissertation saw the development of a drug-polyelectrolyte nanoplexation method to prepare antibiotic nanoparticle with high drug loading, which only involves the mixing of aqueous solutions of the drug and PE at ambient conditions. The method represents a significant improvement over existing drug nanoparticles preparation methods in terms of its simplicity. Employing OFX and LEV as the model drugs, nanoplex with high drug loadings of up to 80% has been successfully prepared by careful examination of the preparation conditions (i.e. drug to PE charge ratio, pH, drug, and salt concentrations). At the optimal preparation conditions, nearly 100% of the drug molecules are transformed into the nanoplex

hence minimizing drug wastage. In addition, the nanoplex size is below 400 nm hence are ideal for anti-biofilm therapy. Importantly, the antimicrobial activities of the antibiotics are preserved upon complexation. Both nanoplexes are also highly charged owed to the DXT presence on the surface making them readily functionalized for diverse therapeutic functions.

In **Chapter 9**, the nanoplexation method is applied to the sparingly soluble antibiotic, CIP. The resultant nanoplex exhibits (i) faster dissolution rate, (ii) enhanced saturation solubility, and (iii) higher achievable supersaturation level compared to those of the raw crystalline drug. The faster dissolution rate of the nanoplex is attributed to both the nanoscale size and amorphous state of the nanoplex, whereas the improvements in the saturation solubility and supersaturation level are owed more to the amorphous formulation. The nanoplexation technique is thus viewed as an attractive method to prepare amorphous antibiotic nanoparticles for to enhance the bioavailability of poorly soluble antibiotics.

In **Chapter 10**, dry powder aggregates of drug-loaded PCL nanoparticles having a large geometric size while remaining in the inhalable size range have been successfully produced using SFD. The results indicate that the adjuvant selection dictates the resulting nanoparticle-adjuvant structures, which in turn influence the aerodynamic diameter and aqueous re-dispersibility of the dry powder aggregates. Both PVA and mannitol adjuvants enhance the aqueous re-dispersibility and produce particles in the inhalable size range having the desired morphology, good flowability, and effective aerosolization off the inhaler. Nevertheless, the fine particle fraction of the aerosolized nano-aggregates still leaves much room for improvement.

In summary, the conclusions reached in the first parts of the thesis contributes to future work on (i) developing an optimal anti-biofilm therapy based on drug release profile, (ii) designing drug-loaded lipid-polymer hybrid nanoparticles, and (iii) transforming nanoparticle suspensions into inhalable dry powder nano-aggregates by spray drying and spray-freeze-drying. On the other hand, the later part of this thesis offers a solution to the preparation of antibiotic nanoparticle in a sustainable manner, for both water-soluble and sparingly soluble drugs. Significantly, the conversion of water-soluble drugs into drug nanoparticles offers the possibility of further functionalization of the antibiotic nanoparticle, while the nanoplexation of sparingly

soluble drugs represents a means towards the bioavailability enhancement of said drugs.

11.2 Recommendations

11.2.1 Triggered-release antibiotic nanoparticles for inhaled anti-biofilm therapy

The encapsulation of antibiotic in nanoparticles alone does not guarantee a maximized antibiotic concentration in close proximity to bacterial biofilm colonies. Upon deposition in the lungs, the encapsulated antibiotics will inadvertently be released, before coming into close contact with biofilm colonies, hence resulting in the wastage of the high initial antibiotic concentration. To this end, polymeric nanoparticles encapsulating antibiotic should be engineered to exhibit (i) a triggered-release profile where the encapsulated antibiotic will only be released upon stimulation by biofilm-secreted molecules and (ii) functionalized surface with an affinity for adsorption onto biofilm cells or EPS.

The triggered-release characteristic is significant to ensure that the encapsulated antibiotics are not released while nanoparticles are penetrating the depth of the sputum. This is an important feature considering a majority of biofilm colonies are often located deep in the sputum, away from the airway surface, and within 20 μm from the epithelial layer¹⁷⁰. In addition, the surface modification of the polymeric nanoparticles enhances their interaction with biofilm colonies, thus permitting the encapsulated antibiotic to be released in the local vicinity of biofilm colonies. It is envisioned that the inhaled polymeric nanoparticles will first penetrate the sputum, followed by the adsorption of the nanoparticles onto biofilm colonies, after which the targeting moiety will detach allowing the nanoparticles to penetrate the biofilm EPS, and finally the encapsulated antibiotic is released upon triggering event.

Thus far, the triggered-release of antibiotic-loaded liposomes represents the only delivery system similar to that suggested²¹. However, the relative instability of the liposomal delivery system suggests that lipid-polymer hybrid nanoparticles that retain the benefit of liposomes as well as the structural integrity of polymeric nanoparticles may be more ideal for this purpose. Towards this aim, our preliminary studies demonstrating the suggested triggered-release mechanism for inhaled anti-biofilm

therapy has been studied using dye-loaded lipid–polymer hybrid nanoparticles, while the actual triggered-release of antibiotics remains to be investigated. The preliminary study has been published as:

W.S. Cheow and K. Hadinoto. Lipid–polymer hybrid nanoparticles with rhamnolipid-triggered release capability as anti-biofilm drug delivery vehicles. *Particuology*. 2012. In press

The triggered-release feature should ideally be applied to antibiotic nanoplex as well, due to the higher drug loading (and thus higher initial antibiotic concentration for maximum initial biofilm killing) afforded by the nanoplex. However, the sustained antibiotic release characteristic of antibiotic-loaded nanoparticles remain attractive, in that they can be relied upon to maintain a high antibiotic concentration to limit the proliferation of surviving biofilm cells. Therefore, the nanoplex and antibiotic-loaded nanoparticles will result in maximum benefit when delivered simultaneously in a single dry powder inhalation dosage, where the initial high antibiotic concentration is provided by the nanoplex, and the subsequent sustained release is afforded by the antibiotic-loaded nanoparticles.

Thus far, the efficacy of the antibiotic-loaded nanoparticles has only been tested against biofilms of *E. coli* grown in the peg-plate system. The design of an optimal antibiotic-loaded nanoparticle for *in vivo* anti-biofilm therapy, however, should ideally be tested against biofilms under conditions which highly resembles that of the disease conditions. As such, the presence of clinical sputum samples during nanoparticle efficacy testing will increase in the relevance of the experimental results in terms of clinical applicability.

11.2.2 Supersaturating drug delivery systems by nanoplexation

While Chapters 8–9 saw the application of the nanoplexation technique to prepare amorphous nanoparticles of antibiotics with different characteristics, the applicability of the nanoplexation technique can definitely be expanded with the use of other sparingly soluble drugs. In particular, the nanoplexation method should be extended to poorly-soluble drugs that are not freely soluble in either acid or base (e.g. tamoxifen and itraconazole). Furthermore, the antibiotic nanoparticles of the currently

developed nanoplex (LEV, OFX, and CIP) can be further functionalised to enhance their relevance to the targeted therapy/delivery mode.

The coating of the surface of the antibiotic nanoparticles with polyelectrolyte shells by using the layer-by-layer technique represent a viable means towards tailoring the antibiotic release profile for inhaled anti-biofilm therapy. Specifically, biocompatible and biodegradable polyelectrolytes such as chitosan, alginate, and carrageenan can be employed as the antibiotic release modulator, while the polyelectrolyte shell thickness can be varied to obtain the desired antibiotic release rate.

The effect of various preparation parameters on the size of the nanoplex prepared can also be studied, to tailor the size of the nanoplex to its potential application. For example, the (i) incubation time allowed for nanoplexation, (ii) order of addition—drug solution to PE solution or the opposite, and (iii) effect of mixing during incubation can be investigated for their roles in the final nanoplex size. In addition, alternative cationic PEs with higher pK_a for complexation with acidic drugs, such that complete ionization in basic solution can be achieved can be used, hence reducing the amount of PE required to form nanoplex resulting in a higher yield. Furthermore, the effect of the PE molecular weight on complexation efficiency, nanoplex size, and drug loading can also be investigated. The supersaturation behavior of the nanoplex in acidic environment mimicking that of the stomach similarly warrants further investigations.

While the dry powder transformation of the nanoplex has been demonstrated in Chapter 9, whether the dry powder nanoplex prepared are suitable for inhalation remains to be investigated. In contrast to the dry powder transformation of polymeric and hybrid nanoparticles, the dry powder nanoplex will not require substantial adjuvant formulation for re-dispersion, since the nanoplex exhibits near instantaneous dissolution. In such case, the nanoplex re-dispersion is not a concern as nanoplex dissolution occurs before re-dispersion, hence adjuvant formulations are only necessary for improvement of the aerosol characteristics of the dry powder nanoplex.

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