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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

**DEVELOPMENT OF INHALABLE ANTIMICROBIAL
NANOPARTICLE COMPLEX**

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SCHOOL OF CHEMICAL AND BIOMEDICAL ENGINEERING

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**DEVELOPMENT OF INHALABLE ANTIMICROBIAL
NANOPARTICLE COMPLEX**

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SUMMARY

As a novel developed delivery method for treatment of lung infections, dry powder inhaler (DPI) delivery attracts more and more attention in studies about inhalation therapy. Conventional dry powder inhaler delivery form is used for administration of nanoparticles (liposomes, polymeric nanoparticles, etc.). However, DPI form for these nanoparticles possesses some drawbacks such as lower payload, lack of long term storage stability. The emergence of nanoparticle complex (nanoplex) is able to improve this situation greatly considering its many merits like higher payload, better long term storage stability, better supersaturation generation ability and so on. Thus here we studied the feasibility of application of nanoplex in DPI form for inhalation therapy of lung infections.

Firstly we investigated the effects of dextran chain length on characteristics of ciprofloxacin nanoplex. Characterization of nanoplex showed that chain length can only affect payload and yield of nanoplex in which longer dextran chain resulted in lower payload and higher yield. Afterwards we compared effects of different drying methods (spray drying and spray freeze drying) on aerosolization efficiency of ciprofloxacin nanoplex DPI form powders and effects of different adjuvants in mitigating adverse effects of drying process on supersaturation generation ability of ciprofloxacin nanoplex spray dried powders. In these studies, Carr's Index, Fine Particle Fraction and other aerodynamic properties were tested and compared in

determining whether dried powders possessed capability to deliver nanoplex into infection sites in pulmonary system via inhalation. Supersaturation profiles and dissolution rates were determined to evaluate whether dried powders were able to generate supersaturated drug solution upon reaching the pulmonary system. Data proved the superiority of spray freeze dried powders of ciprofloxacin nanoplex in terms of aerosolization efficiency and importance of hydroxypropyl methylcellulose (HPMC) as adjuvant in maintaining higher level of supersaturated drug solution within longer time period.

In this thesis, we also investigated feasibility in developing dual drug nanoplex by using ciprofloxacin and itraconazole as drug models. It was reported that a combination therapy with two antibiotics was able to effectively eradicate *Pseudomonas aeruginosa* [1] and this inspired us with the idea about developing dual drug nanoplex system. The potential of dual drug nanoplex system in inhalation therapy is promising especially when multi-drug application is needed. The dosage, administration frequency and patient's compliance will be greatly improved. In this study, different preparation conditions such as acetic acid concentration and mass ratio of drugs were studied for condition optimization of dual drug nanoplex formulation. We can manipulate proportion of payload for each individual drug in dual drug nanoplex by simply changing the mass ratio of drugs during preparation process.

In the end, we studied the curcumin (CUR) nanoplex in DPI form and feasibility of curcumin nanoplex formation by complexation with bovine serum albumin (BSA). Curcumin solubility was improved in DPI form regardless of slower dissolution rate resulted from drying process. The superiority of curcumin nanoplex with BSA was proved in this thesis. As a type of protein, BSA has higher bioavailability and be able to render nanoplex better colloidal stability. Denaturation of BSA could affect nanoplex structure which resulted in influences on drug release. CUR-BSA nanoplex had superiority in terms of size, colloidal stability as well as supersaturation generation ability. More BSA in nanoplex meant higher susceptibility of CUR-BSA nanoplex to heat treatment which led to increase in release of curcumin from nanoplex.

LIST OF ABBREVIATIONS

Abbreviation	Description
DPI	Dry Powder Inhaler
Nanoplex	Nanoparticle Complex
CIP	Ciprofloxacin
CUR	Curcumin
ITZ	Itraconazole
LMW DXT	Low Molecular Weight Dextran Sulfate
HMW DXT	High Molecular Weight Dextran Sulfate
BSA	Bovine Serum Albumin
CHI	Chitosan
Leu	Leucine
HPMC	Hydroxypropyl Methylcellulose
SD	Spray Drying
SFD	Spray Freeze Drying
CE	Complexation Efficiency
d_G	Geometric Diameter
d_A	Aerodynamic Diameter
ED	Emitted Dose
FPF	Fine Particle Fraction
MMAD	Mass Median Aerodynamic Diameter

Abbreviation	Description
MIC	Minimum Inhibitory Concentration

CHAPTER 1. INTRODUCTION

1.1 Clinical Significances of Chronic Lung Infections

As the sites for gas interchange in our body, lung and the whole respiratory system are susceptible to exterior pathogens like bacteria due to massive airflow during gas exchange between environment and human body which leads to a more complex and non-aseptic environment compared to other *in vivo* systems [2]. This makes respiratory system more vulnerable to many kinds of bacteria or other microorganisms and this will be easier to cause diseases [3]. In fact, lower respiratory infections have ranked top 10 causes of death worldwide according to data from the World Health Organization (WHO) (Fig. 1-1) and lung infections have been reported as one of leading reasons that is responsible for human morbidity [2]. In patients suffering from lung infections, dehydration of airways, accumulations of mucus and dysfunctional clearance of airways in respiratory system provide an ideal living environment for growth of bacteria and make it harder to remove them. This leads to requirements for a more effective and efficient treatment such as faster mucus penetration of drugs, higher drug concentration at the sites and slower clearance by airways.

Figure 1-1 Top 10 causes of death globally (Year 2015)

<http://www.who.int/mediacentre/factsheets/fs310/en/>

1.2 Nano-sized Drug Delivery System (nano-DDS) for Lung Infection Therapy

In order to meet above requirements for therapy of lung infections, nano-sized drug delivery system (nano-DDS) has been studied and applied to the treatment. It has been proved that nano-DDS is able to confer drugs many characteristics that cannot exist in conventional forms [2] like increased solubility, slower degradation, prolonged circulation time in human bodies and targeted delivery [4]–[6]. The application of nano-DDS in inhalation therapy for pulmonary diseases has also been investigated. Preliminary data have proven the feasibility of nano-DDS in inhalation therapy and it is very promising [2], [4]–[8]. As discussed above, nano-sized drugs have a higher solubility and can accumulate in pulmonary system, thus there will be more drugs penetrate and be dissolved in mucus layer where bacteria grow. This can generate a higher concentration of drugs at infection sites

with less dose resulting in improvement of efficiency. Besides, dose, administration frequency as well as possible side effects can be reduced contributed to higher site concentration of drugs [7], [8]. Furthermore, as has been proved in many studies, mucus which overlies on the epithelial cells has a porous structure with 100 – 1000 nm of meshes size that only nano-sized particles are able to penetrate through it and reach the bacteria and biofilms beneath it [9], [10]. In the work of Nafee et al, solid lipid nanoparticles modified with PVA (hydrophilic surfactant) as a nano-DDS can easily penetrate mucus with size < 100 nm and negative charged surface [11]. Schuster et al found that PEG (hydrophilic surfactant) coated polymeric nanoparticles with size < 500 nm (around 200 nm is better) can penetrate mucus successfully [12]. These works are also evidences that support the feasibility of nano-DDS as an ideal treatment for lung infections.

Since the discovery and application of penicillin in clinical treatment in 20th century, antibiotics have been widely used as a way to cure infections [13] including lung infections. In study of nano-DDS application in antimicrobial therapy for lung infections, three conventional drug carriers received the most attention from researchers. They are liposome, polymeric nanoparticles and lipid-polymer hybrid nanoparticles. However, regardless of their advantages like better bioavailability and numerous studies about drug delivery based on these carriers, these nanoparticles are not so ideal for pulmonary delivery due to higher cost and higher frequency needed for administration resulting from lower

encapsulation efficiency or payload [14]. These carriers have a lower payload especially for water soluble antibiotics. For example, polymeric nanoparticles have lower payload because of fast drug leakage during high-energy emulsification in preparation while liposomes lack structural integrity due to higher biocompatibility which leads to drug leakage, too. Data have shown that the percentage of loading can be less than 20% while the waste of drug can be higher than 50% [15]. Lower payload is main reason for limited clinical potentials of polymeric nanoparticles and liposomes. In the treatment of lung infections, in order to eradicate bacteria and biofilms which lie below mucus, higher dose of antibiotics is necessary due to drug resistance of bacteria [3], [14]. Below Table 1-1 showed the resistance of *Pseudomonas aeruginosa* isolates. The drug resistant strains of *Pseudomonas aeruginosa* increased from year 2001 to year 2011 (fluoroquinolone' resistance > 30%) [16]. Clearly, conventional nanoparticle carriers cannot meet the requirement for the treatments with higher drug dosage.

	Normal phenotype			Mucoid phenotype		
	2001	2011	P value	2001	2011	P value
	(N = 136)	(N = 209)		(N = 48)	(N = 118)	
Amikacin	12.7	79.8	< 0.001	8.3	72.2	< 0.001
Tobramycin	8.1	52.6	< 0.001	4.2	36.4	< 0.001
Ciprofloxacin	4.4	23.1	< 0.001	0	19.1	< 0.001

Table 1-1 Resistance of *Pseudomonas aeruginosa* isolates (comparison between year 2001 and year 2011) [16]

In order to deal with drawbacks of conventional nanoparticles, a novel form for drug delivery is advanced. Nanoparticle complex, referred to as nanoplex, uses interactions between amphiphilic drug molecules and opposite charged polyelectrolyte to self-assemble into amorphous complex [14]. This process greatly improves the payload and encapsulation efficiency. Data showed that they can be increased to 80% and more than 80%, respectively which means a lower cost and less waste [17]. Some ongoing studies also have revealed the fact that nanoplex has a size range from 300 to 500 nm which is suitable for mucus penetration and it has a better ability for long term storage. Moreover, amorphous drug nanoplex possesses higher saturated solubility than its corresponding crystalline form. This can greatly increase the solubility of water-insoluble drugs which are the majority of drugs in market (several times the solubility of crystalline form) [14], [17], [18]. Amorphous drug nanoplex is also able to generate a supersaturation drug solution upon dissolution which improves the bioavailability of water-insoluble drugs. The supersaturation profiles of nanoplex showed that the drug concentration reached the highest level which is times more than saturated solubility of native drug (4 times the saturated solubility for ciprofloxacin nanoplex) indicating a much higher concentration at the site of airways and this meets the needs of higher dose of drug at site due to drug tolerance of bacteria [18].

Figure 1-2 Formation of nanoplex

There are two conventional approaches, top-down approach and bottom-up approach, which are widely applied to prepare nano-sized particles. However, both of them have difficulties to prepare stable nanoparticles [19]. For top-down approach like wet milling, higher energy is needed and it is time consuming. Also consistency cannot be guaranteed from batches [20]. Bottom-up approach such as nano-crystallization requires a large amount of organic solvent and needs many steps for purification which will produce many toxic wastes. On the contrast, nanoplex preparation method is easier which makes it more suitable for commercial production. The process for preparation is simpler, more cost-effective and friendlier to environment due to solvent-free complexation process (Fig. 1-2) [14]. In sum, the numerous merits and simpler, faster, energy-saving preparation method make nanoplex promising for therapy of lung infections.

1.3 Dry Powder Inhaler (DPI) Delivery

In pulmonary delivery for lung infection therapy, dry powder inhaler (DPI), as a novel developed device to deliver drugs into pulmonary system, attracts more and more attention due to its portability and convenience for usage. Drug powder particles are commonly prepared via two methods, spray drying and spray freeze drying. For spray drying, it gains a lot of attention due to lower cost, faster powder preparation while spray freeze drying is more suitable for thermo-sensitive drug nanoparticles [21], [22]. Researches about antimicrobial therapy using DPI form are widely carried out throughout the world.

For dried powder of nanoparticles, they have much lower payload as discussed above that a large amount of dose is needed for administration to ensure that drug concentration at site is enough for eradication of bacteria. The conventional nanoparticles used for DPI delivery are liposomes, polymeric nanoparticles and lipid-polymer hybrid nanoparticles (LPNs). Liposomes are not ideal for aerosolization because they lack long term storage stability, easy to degrade causing drug leakage during nebulization as well as higher cost [23]–[25] and there exists disruption in integrity of dried powder form for polymeric nanoparticles during drying process [26]. Wang et al compared aerodynamic characteristics of lipid-polymer hybrid nanoparticles in the forms of spray drying (SD) and spray freeze drying (SFD) powder particles [27]. Most SD powder particles containing LPNs had high Carr's Indexes (poor flowability) and poor re-dispersibility.

Furthermore, SD powder particles did not have ideal fine particle fractions (FPF) while SFD form of LPNs had even lower FPF. All these proved that LPNs cannot meet the requirements to prepare ideal SD or SFD powder particles for inhalation drug delivery to lungs.

Dried powder form of microparticles consist of nanoparticles which is called nano-embedded microparticles (NEM) have been proven the feasibility of releasing NPs after reaching infection sites in pulmonary systems [28]. However, there still exist some issues such as the percentage of drug in powders. This must be regulated to ensure effective and efficient delivery of drugs. Moreover, addition of adjuvants (also called excipients, polymers that form dried powder particles together with nanoparticles) will decrease the percentage of NPs in powders thus will certainly further reduce the payload of drugs in powder particles [26]. However, the inclusion of adjuvants is necessary and has been proved to be better for improving performance of dried powders [10], [29], [30].

There are still some other improvements need to be achieved for inhaled delivery of nanoparticles in dried powder form to enhance therapeutic efficiency. For example, it is needed to get higher FPF to ensure more drugs be delivered to deeper parts of pulmonary system. A better reconstitution ability for dried powder particles is expected so that nanoparticles can go back to single particles after drying process, transferring to lungs and dissociating from dried powder particles

when reaching mucus layer. A suitable size of nanoparticles is also wanted so that it can penetrate mucus layer.

Based on above limitations of nanoparticles like lower payload (< 20%) and non-ideal aerodynamic performance in dried powder forms, it is worth a try to combine both advantages of nanoplex and DPI form in the study of inhaled drug delivery in pulmonary systems. The higher payload of nanoplex will improve the reduced payload of drugs in dried powder particles due to addition of excipients.

1.4 Dual Drug Delivery System in Lung Infection Therapy

In airways of patients suffering from lung infections, there are more than one kind of pathogen live at the infection sites. Actually dehydration of airways and accumulation of mucus make airways ideal for growth of many kinds of pathogens including bacteria, fungus and virus [2]. Sometimes multi-drug application is needed for the treatments of lung infections. However, this may lead to higher frequency of administration and larger dose applied which will increase burdens to patients whose respiratory system has already functioned badly results in poor compliance. In order to avoid this potential exacerbation of situation for patients with lung infections, multi-drug application simultaneously is considered to be promising for the therapy. In work of Ibrahim et al, it has been pointed out that therapy combining two antibiotics at the same time will eradicate *P. aeruginosa* effectively [1]. In patients with lung infections, not only bacteria but also other microorganisms lie in airways. Some fungi such as *Sporothrix schenckii* have been

reported to be found in airways too [2]. In this thesis we used itraconazole, an anti-fungal drug, to develop dual drug nanoplex system together with antibiotic. By encapsulating both anti-fungal drug and antibiotic into nanoplex simultaneously, hopefully we can achieve lower frequency and lower dose of administration during the therapy for lung infections leading to improvements for patients' compliance.

1.5 Ciprofloxacin and Curcumin Application in Lung Infection Therapy

In this thesis, two antimicrobial chemicals were studied for inhalation therapy of lung infections, ciprofloxacin (CIP) and curcumin (CUR). For CIP, it is a kind of antibiotic which belongs to fluoroquinolone group and has been proven to have less possibility to cause resistance of bacteria [31]–[34]. There are studies which prove that CIP has antibacterial efficacy against many pathogens in pulmonary systems [35] but conventional applications are via oral or ocular pathways [36]. Existing results have shown that the highest concentration of CIP in lungs can be as high as 3.84 mg/kg which exceed the minimum inhibitory concentration (MIC) of most pathogens in lungs [37], [38]. Thus, CIP is promising for treatments of lung infections.

However, CIP has a poor solubility in aqueous phase and its aqueous solubility is highly pH dependent. At lower pH situation, CIP is freely soluble in cationic form [17]. Hence it is one of the challenges for researchers to develop a method to improve the solubility of CIP in neutral pH which is a similar pH as it is in lung

system. As mentioned above, nano-sized drug delivery system confers drugs many promising characteristics including increased solubility. CIP in nano-DDS form greatly improve the further application of CIP in therapy of lung infections. Traditional formation of CIP in nano-DDS is CIP encapsulated liposomes [2] but also cannot avoid the problems that CIP loaded liposomes have lower payload [14]. The emergence of nanoplex concept significantly enhanced the payload and some studies about CIP nanoplex have already been carried out.

In Cheow and Hadinoto's work [17], low molecular weight of dextran sulfate (DXT) was used as polyelectrolyte and interacted with opposite charged CIP molecules via electrostatic interaction to form CIP-DXT nanoplex. The payload of CIP has been enhanced up to 80%. Furthermore, as discussed above, the method of CIP-DXT nanoplex preparation is easier, solvent free and fast. The formed nanoplexes possess relatively uniform size distribution which is ideal for mucus penetration after reaching the sites of lung infections and have higher dissolution rate as well as saturation solubility. This means higher CIP concentration at sites of infections will be achieved leading to better antimicrobial efficacy especially for those drug-resistant pathogens. Even though CIP nanoplex has so many merits, only one study in related to inhaled CIP nanoplex form were carried out before. Kho and Hadinoto studied effects of lactose carrier particle shape and size for CIP nanoplex spray dried powders [39]. The highest FPF could reached 18% only in the studies which left room for improvements. Based on all these conditions, CIP

nanoplex was studied in this thesis for inhalation therapy application to ensure an ideal delivery of CIP nanoplex into lung systems via inhalation.

For CUR, as a natural extract from turmeric plants, has been used in the therapy of inflammations since ancient time. It has been proven the efficacy of CUR in the treatments of infections and inflammation [40], [41]. Since inflammation of airways is one of the symptoms among many kinds of lung diseases, the studies of CUR therapeutic application for lung diseases have been carried out a lot [42] and the efficacy of CUR in relieving inflammation of airways has been widely accepted [43]–[45]. Even though CUR is promising for lung infection therapy, its aqueous solubility in native form is quite low ($4.15\text{ }\mu\text{g/mL}$ in PBS at $37\text{ }^{\circ}\text{C}$). In this regard, the form of nanoplex for CUR will be able to enhance the solubility obviously. Nguyen et al has successfully developed CUR nanoplex using chitosan (CHI) as polyelectrolyte [46]. In his work, CUR supersaturation can reach nearly 5-fold of saturation solubility even after 3h and the payload can be as high as 80%. The conventional administration of CUR is via oral delivery. However, the effectiveness of CUR will be affected via oral delivery because of the rapid metabolism of CUR in digestive systems [47]. In order to improve the systemic bioavailability of CUR, pulmonary delivery is an ideal way for CUR administration. Thus, it is feasible to study the potential of CUR nanoplex in dried powder forms in order to achieve effective localized delivery of CUR to the lungs for lung infection therapy. In Nguyen et al's work, it was also pointed out that

CUR-CHI nanoplex tended to agglomerate in suspension form [46]. Hence we also studied the possibility to use bovine serum albumin (BSA) as complexation partner for CUR in this thesis because BSA was reported to have the ability to maintain colloidal stability of suspensions [48]–[50].

1.6 Objectives

The objectives of this dissertation are

- To investigate the effect of dextran sulfate chain length on CIP nanoplex formation. Optimize ideal conditions for CIP nanoplex with longer chain of dextran sulfate (high molecular weight). Study the effects of dextran sulfate's chain length on characteristics of CIP nanoplex by comparing to CIP nanoplex with low molecular weight dextran sulfate in terms of supersaturation and dissolution profiles.
- To Develop DPI formulations of CIP nanoplex by SD and SFD in which we determined the optimal parameters and excipient formulation of SD and SFD in terms of the resultant aerosolization properties. In addition, we investigated the effect of the dry powder transformation on the supersaturation profiles of CIP SD powders.
- To develop dual drug nanoplex system using ciprofloxacin (antibiotic) and itraconazole (antifungal drug). Optimize ideal conditions for dual drug

nanoplex formation and study its *in vitro* behaviors in terms of dissolution and supersaturation profiles.

- To develop DPI of curcumin (CUR) nanoplex. Explore ideal parameter for spray freeze drying curcumin nanoplex and compare performances of powders with different parameters in terms of aerodynamic characteristics. Compare supersaturation and dissolution profiles, payload and stability of curcumin nanoplex spray freeze dried powders prepared with different parameters.
- To develop a new class of antimicrobial nanoplex by complexation with proteins. Develop curcumin nanoplex using bovine serum albumin (BSA) and optimize the ideal conditions for CUR-BSA nanoplex. Modify the release of nanoplex by using thermo-denaturation of BSA.

CHAPTER 2. LITERATURE REVIEW

2.1 Background

Lung and the whole respiratory system are sites for gas interchange within our body [2]. The massive airflow makes lung exposed to a more complex and non-aseptic environment compared to other in vivo systems. This makes respiratory system more vulnerable to many kinds of bacteria or other microorganisms such as fungi and cause diseases. It has been reported that infections of lower parts in pulmonary system ranked top three main reasons for the death all over the world [51]. Majority of patients with infections suffer a lot due to the growth of bacteria and biofilm produced by these microorganisms. Actually in patients suffering from lung infections, the dehydration of airways, accumulation of mucus and dysfunctional clearance of airways in respiratory system provide an ideal living environment for growth of bacteria and other microorganisms which make it harder to remove them. Furthermore, the abuse of drugs resulted in emergence of anti-drug strains which makes it more difficult to cure patients by conventional therapy [2].

Normally the conventional and most applied treatment for these disease is using oral administration which needs higher dose of drugs and may have side effects on patients [52]. Recent years, more and more attention have been put onto the inhalation therapy for lung infections considering smaller dose used, less side

effects and other advantages [53], [54]. In spite of all the advances for inhaled drug therapy, there are still challenges that remain to be addressed in this area.

First of all, there should be enough amount of drugs inhaled and deposited at the infection site of respiratory system to ensure optimal therapeutic efficacy. Many factors will affect deposition of drugs like the formulation of drugs, size of drugs, lung anatomy, etc. [54]. There are many bronchia in respiratory system. The size of inhaled drug particles is crucial in determining distribution of drugs in lungs. Inhaled particles with less than 5 μm of aerodynamic size are able to reach deep inside lungs even alveolar parts to achieve systematic delivery while too small particles (less than 1 μm) will be exhaled [26].

Secondly, unlike healthy people, patients suffer from infections have declining water amount in mucus which covers epithelia of respiratory system. Furthermore, presence of mucin, DNA and actin also contribute to the crosslinking structure of mucus to increase viscoelasticity and form a barrier for drug delivered to site [9], [55], [56]. This porous structure can entrap drug molecules or particles. Some other components can attract drugs or carriers via hydrophobic, electrostatic bonding, etc. [57] and the negative charged mucin and DNA molecules can also interact with drug particles with positive charges [58], [59]. All these characteristics make mucus layer a barrier for successful drug delivery. Moreover, these characteristics of mucus in patients' lungs also make it an ideal environment

for growth of bacteria especially for some anaerobic bacteria. These anaerobic bacteria reside in mucus layer which provides an ideal living environment for growth [60].

Lastly, there are also physiological-barriers for successful delivery. One is biofilm that formed by bacteria living within the mucus lying on epithelial cells in airways. This is the biggest challenge for successful treatment. Biofilms are extracellular matrix secreted by bacteria and surround the colonies. It is composed of DNA, protein and polysaccharides [61]–[63]. These components impair the penetration of drugs when they go through biofilm layer and try to reach the bacteria. Thus this is the main reason for drug resistance of bacteria. The other is the clearance of airways. Lung phagocytic clearance will clear those drug particles that do not go into mucus layer and penetrate through biofilm. This happens when drug particles are too big to fit in pores of mucus structures and is another evidence proves that drug particle size is crucial for successful inhaled delivery.

2.2 Inhaled Nanoparticles and Nanoparticle Complex (Nanoplex)

2.2.1 Inhalation Therapy

Inhalation therapy as a type of treatment for diseases can be traced back to around 2000 BC when ancient Chinese and Indian people burned herbs and inhaled the smoke to cure asthma [64], [65]. Nowadays, more and more researchers have found inhalation an ideal platform for drug delivery. As discussed above, the

airways of respiratory system with thinner epithelial tissue, massive blood supply and gas interchange, larger surface area are susceptible to pathogens [2]. Despite its susceptibility, these anatomical properties make it possible to be a better portal or route for drug delivery as well.

There are many advantages for administration of drugs through inhalation. First of all, drug concentration in airways is higher than it is via other administration methods such as oral administration. This leads to lower dose needed for therapy. Besides, inhalation of drugs can also decrease systematic toxicity due to lower dose used. The direct application of drugs on sites also results in immediate therapeutic efficacy. Drugs are applied directly onto airways and pathogens rather than carried and reach the sites via other routes (i.e. via blood circulation when drugs are oral applied). The time period to achieve therapeutic efficacy will be shortened.

2.2.2 Nanoparticles and Nanoparticle Complex (Nanoplex)

Inhalation therapy using antibiotics in the form of nebulized solution has been widely used for bronchiectasis therapy due to higher local exposure of drugs and lower risk of adverse systemic effect compared to oral delivery route [66], [67]. Antibiotic delivery via dry powder inhaler (DPI) also received plenty attention due to its superior delivery efficiency. Inhaled delivery of native antibiotic forms has been proven to effectively assuage lung infections symptoms and exacerbation.

However, it is reported that the effectiveness of antibiotics will be reduced after repetitive applications [68]. This phenomenon occurs because of appearance of sub-inhibitory antibiotic exposure which leads to emergence of drug resistant microorganisms [69]. There are two main factors which are responsible for the sub-inhibitory antibiotic exposure. One is thick stationary mucus layer lying over the infection sites where bacteria grow. This barrier contains many anionic molecules like mucin, actin and DNA which can bind to drug molecules via electrostatic or hydrophobic interactions thus prevent antibiotic molecules reaching infection sites [70]. The other is phagocytic clearance towards drug molecules which cannot penetrate mucus layer fast [71].

This issue about long term efficacy can be improved by introducing inhaled antibiotic nanoparticles to the lung infection therapy. As discussed above, drug particle size is one of the crucial factors in successful drug delivery during inhalation therapy for lung infections. Antibiotic nanoparticles attract a lot of attention from researchers in terms of its nano-size. It has been demonstrated that inhaled antibiotic nanoparticles possess superior antimicrobial efficacy than inhaled native ones [72]–[74]. This is contributed to the ability of nanoparticles that can penetrate the mucus layer and create higher exposure in the vicinity of the infection sites.

Antibiotic encapsulated in polymeric or lipid nanoparticle carriers are conventional

form for inhaled antibiotic nanoparticles [75]. Despite some promising characteristics of these nanoparticles such as mucus-inert surface which can minimize binding with anionic contents in mucus [70], smaller size which is ideal for diffusion in mucus mesh and can avoid lung clearance [76] and sustained release of encapsulated antibiotics which prolongs retention time of drugs in lungs, there still some drawbacks such as costly preparation method and lower antibiotic payload which is less than 30% [75], [77].

On the contrary, nanoparticle complex or called nanoplex has a more cost-effective preparation method and much higher antibiotic payload. By self-assembly complexation between antibiotic and polyelectrolyte, the payload of antibiotic nanoplex can reach as high as 80% [17]. This is because that unlike conventional polymeric and lipid nanoparticles loaded with antibiotic, antibiotic itself is the component and core of the nanoplex structure and each antibiotic molecule interacts with polyelectrolyte molecule via electrostatic interaction. Another important advantage of nanoplex is its amorphous form. Nowadays nearly 80% of marketed drugs have poor aqueous solubility due to increasing intricate chemistry. This leads to lower bioavailability after administration [78]. It is reported that amorphous form of such nanoplex which is attributed to amorphous form stabilization by outer polyelectrolyte chain can have a much better bioavailability for poorly aqueous soluble drugs compared to their crystalline counterparts [79]–[82]. This enhancement in bioavailability of amorphous nanoplex is due to two

aspects. One is the fact that metastable state of amorphous form is able to generate a highly supersaturated drug solution when dissolved in aqueous phase which leads to an apparent improvement in solubility. Cheow and Hadinoto's work pointed out solubility of curcumin nanoplex which is hardly soluble in aqueous phase as crystalline form can be improved to 9 folds of its original solubility as crystals [18]. The other one is that faster dissolution rate due to nano-sized drug particles will diminish solution-mediated crystallization tendency for the rest solid drugs which can ensure creation of supersaturation solution [83], [84]. It is reported that dissolution rate for nanoplex can be as high as twice of its crystalline counterparts [17]. Also the higher glass transition temperature of nanoplex results in long term stability which is suitable for long-term storage [17]. These aspects determine a more promising application of antibiotic nanoplex for future studies about inhalation therapy of lung infections.

2.3 Drying Method for Inhalation Products (DPI preparation method)

Dry powder inhalers (DPI) is a delivery platform and device that helps to transfer dry powders to lungs for treatments of pulmonary diseases like cystic fibrosis or asthma. It has many advantages compared to conventional nebulizer like better portability and longer shelf-life [21]. The dose of drugs can also be metered before inhalation which is another merit of DPI. Most DPIs work on the basis of pressure drop caused by inhalation of patients. This breath actuation of DPI is better than other pressurized metered-dose inhalers (pMDIs) [85] but on the other hand, less

flow rate due to lacking inhalation of patients can result in decreased dissociation of dry powders and less drugs delivered to airways leading to a bad performance of DPI.

Increasing studies have put attention on antimicrobial therapy of pulmonary diseases like CF in dried powder form considering the fact that it can easily go to deeper sites of respiratory system compared to liquid phase leading to higher efficiency and less administration. In order to make sure most dried powders can reach the sites, these powders must possess proper geometric diameter so that they can reach deeper in respiratory system and cannot be exhaled out [26]. The designs of methods and composition to prepare suitable powders for DPI delivery are crucial in studies about therapy for pulmonary diseases using DPI platform. There are two major methods for preparing dried powders containing antibiotic nanoparticles, spray drying and spray freeze drying which are usually referred to as SD and SFD, respectively.

2.3.1 Spray Drying

As reflected in the name, spray drying is a process to prepare dried powders from liquid form which is sprayed from atomizer and rapidly dried with hot gas. During the drying process, liquid droplets turn to dried powder form with a porous or hollow structure and the size distribution of powder particles is quite homogeneous because of spraying process.

Figure 2-1 Structure of spray dryer: 1) feed solution or suspension containing drugs; 2) nozzle for spraying; 3) drying chamber; 4) separation flask for collection of waste; 5) cyclone; 6) chamber containing final SD powders

There is considerable amount of studies focused on spray dried powders for inhaled drug delivery due to easier preparation of powders, less time needed and lower cost compared to spray freeze drying method. Sham et al [86] spray dried gelatin and polybutylcyanoacrylate nanoparticles respectively and investigated their structures, mean sizes, fine particle fractions (FPF) and mass median aerodynamic diameter (MMAD). According to the results, both particles were able to maintain nano-size after spray drying and both FPF and MMAD of spray dried powders did not change a lot due to inclusion of nanoparticles. It proved that nanoparticles can be applied to drug delivery system using DPI method. Pilcer et al [52] co-spray dried tobramycin-clarithromycin nanoparticles and compared its characteristics with spray dried physical mixture of drug and clarithromycin. The

results showed that compared to physical mixture, SD nanoparticles have a better fine particle fraction which means more drug particles are able to reach the deeper site of respiratory tract. They also characterized the spray dried tobramycin nanoparticles with different surfactants like Na glycocholate [87]. In their studies, they combined both high-pressure homogenizations (HPH) which aims to prepare nano-sized particles and spray drying method to produce inhaled powders. They concluded that this formulation of spray dried powders can meet the needs of higher drug dose at the site for CF patients and reduce the frequency of administration result in enhancement of CF patients' compliance.

In some studies, researchers have successfully made spray dried solid lipid particles using lipid like phospholipid to encapsulate antibiotics like tobramycin [88], [89]. Though rare experiments were carried out for solid lipid nanoparticles (SLNs) using DPI, a kind of SLN loaded with amikacin was developed for therapy of CF. Both minimum inhibitory concentration (MIC) and minimum biofilm inhibitory concentration (MBIC) of amikacin loaded SLN exhibited higher antimicrobial efficiency with a 2-fold lower values [90]. Recently, a dried powder form of microparticle which is composed of nanoparticles was studied and applied to polymeric nanoparticles. This form combined both advantages of polymeric nanoparticles like long circulation time as well as release period and microparticles like better flowability and other aerodynamic properties [26].

During the process of spray drying, nanoparticles tend to form aggregates in micro size when water is evaporating. In order to achieve ideal aerodynamic properties and good re-dispersibility after reaching the mucus via inhalation, the spray dried (SD) powders should have large, hollow or porous and spherical structure, thus the spray drying parameters and usage of excipients or adjuvants are necessary. Indeed, numerous studies are focused on this. Varshosaz et al [91] made doxorubicin encapsulated albumin nanoparticles and used the optimal run for spraying drying with three different excipients (trehalose, leucine, mannitol) and characterized their aerodynamic properties. In their studies, they concluded that spray dried nanoparticles using mannitol as excipient and 80°C of inlet temperature during spray drying performed the best among all groups. The optimal powder has 4.58 µm of aerodynamic diameter and fine particle fraction (FPF) could reach as high as 66%.

For inert excipients, lactose and mannitol are mainly applied in DPI. Especially for mannitol, its higher solubility has gained a lot of attention [88], [92], [93]. Indeed, some studies have found that water-soluble excipients can act as “bridges” in the form of spray dried powders to connect nanoparticles together and contribute to ideal re-dispersibility, and clearly the physicochemical properties of excipients can affect re-dispersibility a lot like hydrophilicity [94]. The introduction of hydrophobic excipients like leucine can improve aerodynamic properties. Furthermore, the concentration of feed suspension for spray drying and ratio of

different compositions in feed can also affect re-dispersibility [95].

Spray drying method is also applicable to lipid-polymer hybrid nanoparticles (LPNs) which are a novel promising drug carrier for DPI delivery. In Wang et al.'s work [27], they used PLGA-LC(soy-bean lecithin) as hybrid nanoparticle and PVA as excipient to evaluate effects of ratio of nanoparticles over excipient on aerodynamic properties like Carr's Index, geometric diameter, aerodynamic diameter, effective density of powders and re-dispersibility, etc.. According to the data, they concluded that spray dried powders are more likely to have a dimpled spherical morphology and PVA is superior in terms of re-dispersibility than mannitol. And inclusion of hydrophobic substances like leucine is helpful in improving aerodynamic characteristics which has proven the above assumption about application of hydrophobic excipients in enhancing aerodynamic properties of dried powders.

Till now, manipulate the ratio of drug or nanoparticles in powder and spray drying parameters to achieve optimal spray dried powders with ideal aerodynamic properties are still crucial in spray drying studies. Moreover, the selection of excipients and ratio of hydrophilic excipients and hydrophobic excipients need to be investigated for spray drying of different nanoparticles.

2.3.2 Spray Freeze Drying

Even though spray drying can achieve powders with better aerodynamic properties using less cost, it has to be performed at a relatively higher temperature. When it comes to thermally-sensitive nanoparticles, spray freeze drying is an ideal option and be widely applied because it does not need high temperature [21], [22]. Furthermore, since spray freeze drying will produce porous particles due to sublimation of ice in frozen particles, this will result in lower effective density with larger geometric diameter (d_G) [21]. During spray freeze drying, feed solution containing drug loaded nanoparticles is atomized into liquid nitrogen under constant stirring instead of drying chamber with higher temperature. Then the frozen droplets are forwarded for lyophilization under lower pressure. This will take longer time to get the final product but on the other hand, product yield is higher than it of spray drying.

Figure 2-2 Overview of spray freeze drying: 1) feed solution or suspension; 2) nozzle for spraying; 3) liquid nitrogen under constant stirring; 4) stirrer bar

There are not so many studies were carried out which are related to spray freeze dried powders containing antibiotic nanoparticles for inhalation. Azarmi et al [96] evaluated the cytotoxicity of doxorubicin encapsulated nanoparticles in spray freeze dried powder form. Spray freeze dried nanoparticles exhibited not only a better deposition pattern, but also a higher cytotoxicity to cancer cells than blank nanoparticles and free doxorubicin indicating a promising application in lung diseases treatments.

Same like spray drying, some studies are focused on effects of excipients on spray freeze dried powders like polycaprolactone (PCL), a polymer with lower melting temperature (62 °C) and be regarded as a potential drug delivery vehicle considering the fact that it has superior permeability and physical stability [97]. Some existed results have shown that compared to a hollow structure of spray dried powder, spray freeze dried powder tends to have a larger and more porous structure [67] and the selection of excipients can affect final structures [29]. Addition of hydrophobic excipients like leucine can be helpful in improving aerodynamic properties of spray freeze dried powders [27], [95] without affecting other aerodynamic characteristics [21]. Work of Cheow et al also discussed the effects of different excipients on spray freeze dried powders [29]. By comparing spray freeze dried PCL nanoparticles using mannitol and PVA as adjuvants respectively, it showed that both spray freeze dried powders have a porous and large structure with a good flowability. For SFD powders using mannitol as

excipient, PCL nanoparticles tend to distribute in mannitol matrix while PVA usually covers onto PCL nanoparticles. Regardless of this difference in structure, both powders have better re-dispersibility than it of spray dried powders. Furthermore, results about aerodynamic characterization of spray freeze dried powders showed that they have higher value of fine particle fraction (FPF) and emitted dose (ED) which means they perform better in inhalation therapy [27].

In terms of spray freeze dried lipid-polymer hybrid nanoparticles, the results are similar to those of spray dried hybrid nanoparticles except for one difference. Spray freeze dried hybrid nanoparticles tend to have some changes in aspect of size after re-dispersed in aqueous phase and this is considered to be the results of lyophilization. [27]. Besides, it should be brought to attention that spray freeze dried powders are fragile due to higher porosity [98].

2.4 Dual Drug Nanoparticle Delivery System

Pulmonary system takes the role for gas exchange between body and environment. This function determines that lung takes higher risk than other organs or systems in body to be exposed to many kinds of pathogens that will lead to infectious diseases. These pathogens live under mucus in lower respiratory tract of lung system and result in dysfunction of pulmonary system. At the same time, the thicken of mucus in airways, increased viscoelasticity of mucus due to more DNA and actin in sputum and poor mucociliary clearance provide an ideal environment for growth of

these pathogens [1]. Furthermore, the abuse of antibiotic results in emergence of drug-resistant bacteria which further increase the difficulties to eradicate pathogens from lung systems.

In order to eradicate multi-types of microorganisms and drug-resistant strains in lung systems, sometimes multi-drug administration is required during the antiinfection therapy. It is reported that a combined therapy using two antibiotics is able to effectively eradicate *Pseudomonas aeruginosa* in lungs [99]. There are already some studies focus on the co-deliver of multi-drugs in curing lung infections nowadays. Pilcer et al. used a co-spray-drying method to prepare inhaled powders containing tobramycin nanoparticles and native clarithromycin for treatment of lung infections [52]. They proved that this combination exhibited a better lung deposition form which is promising for improving inhaled antibiotic administration. In Li et al. work, daptomycin and clarithromycin loaded liposomes were able to effectively cure methicillin-resistant *Staphylococcus aureus* (MRSA) infections [100]. However, the payload for both drugs were lower which was only 8.45% for clarithromycin and 0.29% for daptomycin. There is an accepted opinion that multiple drugs administration in a combinational therapy can contribute to synergistic effects so that achieves higher potency in future treatment of diseases [101], [102]. Also the abilities of suppression of drug resistance and modification of payload for each drug in the level of single nanoparticle make dual drug nanoparticle delivery system an ideal candidate as an advanced therapy for lung

infections [103].

Despite all these advantages of dual drug nanoparticle delivery system, the study of it is mainly focused on the possible application in anticancer therapies nowadays. In fact, in the airways of patients suffering from kinds of lung infection, there are more than one type of pathogens. The ideal environment of dysfunctional airways is the seedbed for growth of kinds of microorganisms including bacteria, fungi and viruses [2]. Multifunctional nanoparticles also have a great possibility for application in treatment of pulmonary diseases. In inhalation therapy of lung infections, dual drug nanoparticle delivery system has its specific merit. For patients with pulmonary diseases whose airways have already functioned badly. Multiple inhaled administration of different drugs will increase burdens to airways hence cause worse compliance of patients. Dual drug nanoparticle delivery system will be able to alleviate this situation by delivering different drugs simultaneously so that will reduce frequency of inhaled administration.

As discussed above, the payloads of dual drug encapsulated nanoparticles are very low which means more times and dosages of inhaled administration may be needed in the inhalation therapy especially for eradication of drug-resistant strains. This will lead to poor patients' compliance due to increased burdens. On the other hand, more inhaled administration means more nano-carriers which have no therapeutic efficacy will be inhaled into body. In Li et al [104] and Weber et al

[105] studies, they both mentioned the potential nanotoxicity caused by nanoparticles inhaled. For nanoparticles with lower payload, even higher dose of nanoparticles should be inhaled into airway sites to ensure enough drugs for therapeutic efficacy. The simultaneously increased amount of nanoparticles may exceed the ability of lung phagocytic clearance. This will be harmful towards pulmonary system and exacerbate the situation of patients with lung infections [106].

In this thesis, we introduce the nanoparticle complex (nanoplex) as a new dual drug nanoparticle delivery system. With the self-assembly between drug molecules and polyelectrolyte molecule chains via electrostatic interaction, the total payload of this new dual drug nanoplex delivery system can be achieved as high as 75% which is a great improvement compared to it of conventional nanoparticles such as aforementioned liposomes. This will also bring more possibility in studies about application of inhaled dual drug delivery system in therapy of lung infections with different types of pathogens and will enhance therapeutic efficacy and efficiency.

2.5 Terminology and Related Concepts

In this thesis, we used some parameters to characterize properties of inhaled dried particles as well as nanoparticle complex we prepared. These parameters helped to determine whether the dried powder particles have ideal aerodynamic behaviors as we expected or whether the nanoparticle complex has the suitable particle size and other properties we want. These characterizations involved some terminologies,

definitions and concepts and will be explained in this part.

Complexation Efficiency (CE)

Complexation Efficiency (CE) characterizes drug utilization rate during nanoparticle complex formation and as defined in Eq. (2-1). Mass of drug that formed the complex is determined from the difference between the initial mass of drug added and the mass of drug recovered in the supernatant after centrifugation of the complex suspension.

$$\text{CE (\%)} = \frac{\text{Mass of drug that formed nanoparticle complex}}{\text{Initial mass of drug added}} \times 100 \quad (2-1)$$

Yield

Yield was defined as the percentage of final product (nanoparticle complex or dried powders) we get from all materials initially added. Yield of nanoparticle complex is shown in Eq. (2-2).

$$\text{Yield (\%)} = \frac{\text{Mass of drug nanoparticle complex produced}}{\text{Initial masses of drug and polyelectrolyte added}} \times 100 \quad (2-2)$$

For yield of nanoparticle complex dried powders (spray dried or spray freeze dried powders), the equation is described in Eq. (2-3).

$$\text{Yield (\%)} = \frac{\text{Mass of dried powder produced}}{\text{Initial masses of nanoparticle complex and excipients added}} \times 100 \quad (2-3)$$

Payload

Payload means the percentage of drug in formed nanoparticle complex or dried powders which can be calculated from below equation.

$$\text{Payload (\%)} = \frac{\text{Mass of drug measured in weighed nanoparticle complex or dried powder}}{\text{Mass of formed nanoparticle complex or dried powder weighed}} \times 100 \quad (2-4)$$

In the studies of nanoparticle complex in inhaled dried powder forms, there are some parameters to characterize aerodynamic properties of dried powders. These parameters reflect aerodynamic behaviors of dried powder particles after inhalation and very important in this thesis.

Geometric Diameter (d_G) and Aerodynamic Diameter (d_A) and Mass Median Aerodynamic Diameter (MMAD)

Geometric diameter is a measured value for a powder particle based on images of an actual powder particle. When it comes to aerodynamic diameter, if an actual powder particle has the same settling velocity as a hypothesized solid spherical particle (1000 kg/m^3 of density), the diameter of this hypothesized solid spherical particle is aerodynamic diameter of this actual powder particle. Aerodynamic diameter is used to evaluate powder particle deposition tendency in pulmonary

system and calculated from a function of d_G (theoretical value, Eq. (2-5)). MMAD is measured aerodynamic diameter of powder particles after aerosolization in experiments and difference between d_A and MMAD indicates agglomeration of powder particles upon aerosolization.

$$d_A = d_G \sqrt{\frac{\rho_{\text{tap}}}{1\text{g/cm}^3}} \quad (2-5)$$

Carr's Index

In order to evaluate movement of powder particles in airways, flowability is introduced in this thesis. Flowability is the ability of object that moves by the flow (fluid flow or air flow). In inhaled delivery, it refers to the ability of powder particles move evenly by the air flow in lungs generated by inhalation. It is affected by a set of conditions that combines both inherent physical properties like density and environmental properties like air surrounding the particles.

Carr's Index (or called Carr's Compressibility Index) is used to evaluate compressibility of powders which can reflect the flowability. In Eq. (2-6), bulk density (ρ_{bulk}) was determined by measuring the volume of a known mass of the powders without tapping, whereas the tap density (ρ_{tap}) was powder density after 2000 taps. Big difference between tap density and bulk density means big change of volume after tapping indicating bigger space between powder particles due to agglomeration. Thus higher value of Carr's Index means greater interparticle

interaction. The interparticle interaction means agglomeration of powder particles leading to poor flowability.

$$\text{Carr's Index} = \left(1 - \frac{\rho_{\text{bulk}}}{\rho_{\text{tap}}} \right) \times 100\% \quad (2-6)$$

The aerosolization efficiency and deposition pattern of inhaled dried powder particles are usually characterized by Next Generation Impactor (NGI). In this thesis, a seven-stage NGI equipped with induction port (IP) and pre-separator (PS) (Fig. 2-3) was employed for the determination in terms of %Recovery, Emitted Dose (ED), Fine Particle Fraction (FPF), etc..

Figure 2-3. Next Generation Impactor with induction port and pre-separator

%Recovery and Emitted Dose (ED)

As shown in Fig. 2-3, %Recovery is defined as percentage of powders that is recovered (including powders that remain in inhaler) during the characterization. Among recovered powders, the percentage of powders that emitted from inhaler is defined as Emitted Dose (ED).

Fine Particle Fraction (FPF)

Fine Particle Fraction is defined as the percentage of powder particles with the aerodynamic diameter less than 5 μm among all powder particles emitted from the inhaler. As mentioned above, particles with d_A between 1 and 5 μm are able to deposit in lungs hence FPF indicates effective percentage of powder particles that can reach deeper parts of pulmonary system through inhalation.

Supersaturation and Dissolution Rate

For those highly water-insoluble drugs such as ciprofloxacin and curcumin used in this thesis, amorphous form (nanoparticle complex) is able to generate higher saturation solution upon dissolution [107]. Furthermore, amorphous form possesses the ability to form supersaturated solution which is times higher than that of its crystalline form. This phenomenon is called supersaturation and dissolution rate is used to evaluate how fast amorphous drug form is able to generate supersaturated solution. The generation of supersaturation drug solution improves therapeutic efficiency especially for drug resistant microorganisms.

Sink Condition

Dissolution rate test is usually performed in sink condition. In dissolution rate test, a sheer volume of solvent is used for the test. The volume is usually 5 to 10 times of it is used in saturated solution for tested chemicals. The curve of results shows percentage of drug released from nanoparticles and the slope of curve indicates dissolution rate.

CHAPTER 3. EFFECTS OF DEXTRAN SULFATE'S CHAIN LENGTH ON THE CHARACTERISTICS OF CIP-DXT NANOPLEX

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3.1 Introduction

Self-assembled colloidal complexes formed via complexation between polysaccharides and therapeutic small molecules have recently emerged as an effective formulation strategy to enhance the delivery efficacy of small-molecule drugs, for example, (i) to achieve their controlled release [109], [110] and targeted delivery [14], (ii) to preserve their therapeutic activities [111], and (iii) to enhance the dissolution for poorly soluble drugs [46], [112], [113]. Various polysaccharides (e.g. methylcellulose, chitosan, carrageenan, dextran sulfate) have been studied to form colloidal complexes ($< 1\ \mu\text{m}$) with a wide range of natural and synthetic therapeutic molecules [14], [46], [109]–[114].

In particular, dextran sulfate (DXT) has been one of the most widely studied polysaccharides to form colloidal drug complex attributed to its strong polyelectrolyte properties [115], which enable DXT to readily undergo electrostatically driven complexation with oppositely charged drug molecules [14], [109], [112], [114]. The widespread use of DXT is also attributed to the fact that successful complexation between DXT and amphiphiles, which most drugs are [116], has been well established. The effects of different variables (e.g. charge density, charge ratio of amphiphiles to DXT, amphiphiles hydrophobicity) on the DXT-amphiphiles binding have been elucidated [117]–[119].

Similar to the mechanisms in the DXT-amphiphiles complexation, the formation of the drug-DXT colloidal complex begins with the electrostatic binding between anionic DXT chains and oppositely charged drug molecules, resulting in the formation of soluble drug-DXT complex. Subsequently, aggregates of the soluble complex are formed as a result of the hydrophobic interaction among the bound drug molecules. Upon reaching a critical aggregate mass, the complex precipitates out of the solution to form the drug-DXT colloidal complex in which DXT acts as both steric and charge stabilizers of the colloidal complex. The colloidal complex predominantly exists in an amorphous solid state as the strong electrostatic binding with DXT prevents the drug molecules from arranging into highly organized crystalline structures upon their precipitation [17].

While the effects of various variables (e.g. drug concentration, drug to DXT charge ratio, pH, salt) on the drug-DXT colloidal complex formation have been investigated [14], [109], [112], [120], the previous studies were limited to the use of short-chain DXT with average molecular weight (MW) of < 10 kDa. In this regard, the use of longer polyelectrolyte (PE) chains in complexation between PE and amphiphiles has been shown to cause the soluble complex to undergo earlier phase transition to insoluble colloidal complex (i.e. at a lower critical aggregate mass) [121], [122]. The earlier phase transition was caused by the increased occurrence of inter-PE chain interactions, as opposed to predominantly intra-PE chain interactions when short-chain PE was used [121], [122]. The same studies also showed that the use of longer PE chains resulted in colloidal complexes exhibiting different morphology and higher product recovery (i.e. increased insoluble complex formation).

From the perspectives of the drug-DXT colloidal complex preparation, the earlier phase transition suggested by the previous studies when longer PE chains were used could result in a smaller number of drug molecules forming complex with DXT prior to the phase transition. As a result, the colloidal complex produced could potentially exhibit a lower drug payload and a lower drug utilization rate (hence increased drug wastage), which both are undesirable. On the other hand, the use of different DXT chain lengths would enable us to manipulate the morphology

(e.g. size, shape) of the resultant colloidal complex depending on the desired therapeutic applications.

Herein we investigated the effect of using long-chain DXT (average MW of ≈ 500 kDa) on the physical characteristics (i.e. size, shape, zeta potential, drug payload, and colloidal stability) of the drug-DXT colloidal complex produced. In addition, the effects of using long-chain DXT on the preparation efficiency (i.e. drug utilization rate, overall production yield) and amorphous state of the colloidal complex were also examined.

Ciprofloxacin (CIP) – a fluoroquinolone antibiotic with broad-spectrum antimicrobial activities – was used as the amphiphilic drug model [123], [124]. The CIP-DXT colloidal complex is useful as a bioavailability enhancement strategy of CIP, which has a notoriously low aqueous solubility at neutral pH (< 0.1 mg/mL) [125]. The enhanced CIP bioavailability was by virtue of the dissolution enhancement and supersaturation generation capabilities of the CIP-DXT colloidal complex attributed to its amorphous and nanoscale size [112].

As colloidal complex of CIP and high MW DXT (or HMW DXT in short) had not been prepared previously, we first carried out an investigation to determine the optimal condition to prepare CIP-HMW DXT colloidal complex. For this purpose,

the effects of the two key parameters in drug-polysaccharide complexation (i.e. pH and charge ratio of CIP to HMW DXT) on the physical characteristics and preparation efficiency of CIP-HMW DXT colloidal complex were examined. Subsequently, we compared the CIP-DXT colloidal complexes prepared by the long and short-chain DXTs in terms of their (1) physical characteristics, (2) preparation efficiency, and (3) dissolution characteristics (i.e. under sink and non-sink conditions).

3.2 Materials and Methods

3.2.1 Materials

Low MW DXT (LMW DXT, ≈ 5 kDa) and HMW DXT (≈ 500 kDa) were purchased from Wako Pure Chemical (Japan). CIP, glacial acetic acid (AA), Pluronic F68, phosphate buffered saline (PBS, pH 7.4), methanol, acetonitrile, and potassium dihydrogen phosphate (KH_2PO_4) were purchased from Sigma-Aldrich (Singapore).

3.2.2 Methods

3.2.2.1 Preparation of CIP-DXT Colloidal Complex

To investigate the effect of the preparation pH, CIP was dissolved at 10 mg/mL in 1 mL of aqueous AA solution with varying concentrations (i.e. AA = 0.3 to 1.0% (v/v) corresponding to pH in the range of 3.0 and 2.7). CIP with pK_a values of 6.1 and 8.6 [126] was fully protonated in this pH range to form cationic CIP molecules

with a charge density of 3.2×10^{-6} mol-charge/mg. Separately, HMW DXT, whose $pK_a \leq 2$ [127], was dissolved in 1 mL of deionized water at neutral pH to form anionic DXT chains with a charge density of 4.8×10^{-6} mol-charge/mg. Surfactant Pluronic F68 was added at 0.3% (w/v) to the aqueous HMW DXT solution. The HMW DXT solution was prepared at different concentrations to investigate the effect of the charge ratio of CIP to HMW DXT ($R_{CIP/DXT}$) (Table 3-1).

Concentration of CIP (mg/mL)	10	10	10	10	10	10	10	10	10	10
Concentration of DXT (mg/mL)	1.5	2.0	2.5	3.0	3.5	4.0	4.5	8.0	10.0	12.0
$R_{CIP/DXT}$	4.2	3.1	2.5	2.1	1.8	1.6	1.4	0.8	0.6	0.5

Table 3-1 A summary of $R_{CIP/DXT}$ and corresponding concentrations of CIP and DXT solutions

Subsequently, the cationic CIP solution was mixed with the anionic DXT solution at equal volumes (1 mL), followed by 10 s vortexing. The resultant CIP-HMW DXT colloidal complex suspension then underwent three cycles of centrifugation ($14,000 \times g$ for 10 min) and washing with deionized water to remove the excess CIP and HMW DXT (i.e. those that did not form complex). Finally, the washed colloidal complex suspension was re-dispersed in aqueous solution of 0.3% (w/v) Pluronic F68 for characterizations. In the case where the CIP-HMW DXT colloidal

complex was ultrasonicated to break up its agglomerates, the ultrasonication was performed thrice for 5 s each using a probe sonicator (CV24, Sonics, USA). The CIP-LMW DXT was prepared by the same procedures described in the above at the optimal condition determined previously in Cheow and Hadinoto [17] (i.e. AA = 0.2% (v/v) and $R_{\text{CIP/DXT}} = 1.3$).

3.2.2.2 Physical Characterizations of CIP-DXT Colloidal Complex

For both the HMW DXT and LMW DXT, the size and zeta potential of the CIP-DXT colloidal complexes produced were determined by dynamic light scattering (DLS) using Brookhaven 90 Plus Nanoparticle Size Analyzer (Brookhaven Instruments Corporation, USA). The morphology of the CIP-DXT colloidal complex prepared at the optimal condition was examined by field emission scanning electron microscope (FESEM) image (JSM-6700F, JEOL, USA). The FESEM was carried out using freeze-dried powders of the colloidal complex after sputter coating them with platinum. The CIP payload, which was defined as the amount of CIP present per unit mass of the CIP-DXT colloidal complex, was determined by dissolving a known mass of the colloidal complex in 10 mL PBS after which the amount of CIP was quantified by UV-vis spectrophotometry (UV Mini 1240, Shimadzu, Japan) at absorbance wavelength of 324 nm. The size, zeta potential, and CIP payload were reported from a minimum of three independent replicates.

The amorphous state of the CIP-HMW DXT colloidal complex prepared at the optimal condition was verified by Powder X-ray Diffraction (PXRD) analysis carried out between 10° and 70° (2 θ) with a step size of 0.02°/s using D8 Advance X-ray Diffractometer (Bruker, Germany). The PXRD analysis was also performed for the native CIP, native HMW DXT, and native Pluronic F68 for comparison. The colloidal stability of the aqueous suspension of the CIP-DXT colloidal complex was examined in triplicates by monitoring the variations in its size and zeta potential by DLS during ambient storage for 30 h, where minimal variations denoted high colloidal stability.

3.2.2.3 Preparation Efficiency

The efficiency of the CIP-DXT colloidal complex preparation was characterized in triplicates by the CIP utilization rate and the overall production yield. The CIP utilization rate was characterized by the complexation efficiency (CE) defined in Eq. (2-1), where the mass of CIP that formed the colloidal complex was determined from the difference between the initial mass of CIP added and the mass of CIP recovered in the supernatant after centrifugation of the colloidal complex suspension. The amount of CIP in the supernatant was quantified by UV–vis spectrophotometry at 324 nm. The yield was defined in Eq. (2-2), where the mass of the CIP-DXT colloidal complex produced in Eq. (2-2) was determined from the freeze-dried mass of a known volume of the washed colloidal complex suspension.

3.2.2.4 Dissolution Characteristics

The dissolution rate of the CIP-DXT colloidal complex under a sink condition was determined in triplicates using the dialysis bag method. Briefly, 0.124 mg of the colloidal complex was placed in a dialysis bag containing 2 mL PBS after which the dialysis bag was immersed in 18 mL PBS in a shaking incubator at 37 °C. At specific time points over 3 h, 2.0 mL aliquot was withdrawn and 2.0 mL fresh PBS was added back to the dissolution vessel. The amount of CIP in the aliquot was quantified by high performance liquid chromatography (HPLC) at detection wavelength of 324 nm. The HPLC was carried out in Agilent 1100 (Agilent Technologies, Singapore) using ZORBAX Eclipse Plus C18 column (250 × 4.6 mm and 5-μm particle size). Aqueous solution of 0.1 M KH_2PO_4 : acetonitrile : methanol at 60 : 25 : 15 (% v/v) was used as the mobile phase at flow rate of 1 mL/min, resulting in the CIP's retention time of approximately 5 min.

The dissolution rate of the CIP-DXT colloidal complex under a non-sink condition, which characterized the supersaturation generation afforded by the amorphous solid state, was determined by adding the colloidal complex in excess at $10 \times C_{\text{Sat}}$ to 8.5 mL PBS in a shaking incubator at 37 °C. Herein C_{Sat} represented the thermodynamic saturation solubility of CIP determined previously to be equal to approximately 0.19 mg/mL [110]. Next, 200 μL aliquot was withdrawn and syringe filtered (0.22-μm pore size) at specific time points over 24 h. The aliquot was immediately diluted nine folds with fresh PBS to prevent precipitation of CIP

from the supersaturated solution. The amount of CIP in the aliquot was subsequently determined by HPLC as previously described. The supersaturation generation was reported based on three replicates in term of the ratio of the supersaturated CIP concentration generated by the amorphous colloidal complex (C_{Supersat}) to C_{Sat} .

3.3 Results and Discussion

3.3.1 Optimal Preparation Condition for the CIP-HMW DXT Colloidal Complex

3.3.1.1 Effects of Preparation Condition on Physical Characteristics

The effects of the preparation pH (expressed in terms of the AA concentration used to dissolve CIP) on the size and zeta potential of the resultant CIP-HMW DXT colloidal complex were presented in Fig. 3-1A. The study was conducted at a fixed CIP concentration of 10 mg/mL and a fixed $R_{\text{CIP/DXT}} = 1.4$, where the $R_{\text{CIP/DXT}}$ value was selected based on the optimal $R_{\text{CIP/DXT}}$ (≈ 1.3) for CIP-LMW DXT colloidal complex determined previously in Cheow and Hadinoto [17]. The results showed that the size exhibited minimal fluctuations in the narrow range of $\approx 500 - 600$ nm upon varying the AA concentration between 0.4% and 1.0% (v/v). One exception was at AA = 0.3% in which the size was considerably larger at 755 ± 50 nm.

The results also showed the insignificant impact of pH on the zeta potential as

reflected by the minimal variations of the zeta potential in the narrow range of ≈ -30 to -35 mV upon the changes in AA concentration (Fig. 3-1A). In this regard, the negative zeta potential of the colloidal complex indicated the predominant presence of the anionic DXT on its surface. Recognizing the minimal influence of pH, the CIP-HMW DXT colloidal complex was prepared in the subsequent studies at a fixed AA = 0.7% at which the variations in the size (590 ± 29 nm) and zeta potential (-33 ± 1.5 mV) were at the minimum.

Figure 3-1 Effects of (A) preparation pH expressed in terms of AA concentration and (B) $R_{CIP/DXT}$ on the size and zeta potential of the CIP-HMW DXT colloidal complexes produced.

Next, the effects of $R_{CIP/DXT}$ on the size and zeta potential of the CIP-HMW DXT colloidal complex were investigated for $1.4 \leq R_{CIP/DXT} \leq 4.2$. Higher $R_{CIP/DXT}$ values above unity signified a decreasing amount of charged DXT molecules available for complexation with the fixed amount of CIP molecules (i.e.

10 mg/mL). Similar to the trend observed for the pH variation, the results in Fig. 3-1B showed that the size was minimally affected by the $R_{CIP/DXT}$ variation. On the other hand, the zeta potential was found to experience a slight decrease from ≈ -33 mV for $R_{CIP/DXT} \leq 2.1$ to ≈ -29 mV at higher $R_{CIP/DXT}$ (Fig. 3-1B). The CIP payload was also found to experience a slight gradual decrease from ≈ 51 – 53% (w/w) for $R_{CIP/DXT} \leq 1.8$ to ≈ 46 – 47% for $1.8 < R_{CIP/DXT} < 3.1$, before the payload settled at $\approx 41\%$ for $R_{CIP/DXT} \geq 3.1$ (Fig. 3-2A).

Figure 3-2 Effects of $R_{CIP/DXT}$ above unity on (A) CIP payload; (B) CE and yield of the CIP-HMW DXT colloidal complex.

3.3.1.2 Effects of Preparation Condition on Preparation Efficiency

Significantly, the CE was also found to gradually decrease with increasing $R_{CIP/DXT}$ from $81 \pm 1.5\%$ at $R_{CIP/DXT} = 1.4$ to $57 \pm 1\%$ and $30 \pm 1.5\%$ at $R_{CIP/DXT} = 2.1$ and 4.2 , respectively (Fig. 3-2B). The gradual decrease of CE with

increasing $R_{CIP/DXT}$ translated to a similar gradual decrease in the production yield, which decreased from $80 \pm 3\%$ (w/w) at $R_{CIP/DXT} = 1.4$ to $68 \pm 3\%$ and $48 \pm 3\%$ at $R_{CIP/DXT} = 2.1$ and 4.2 , respectively (Fig. 3-2B). As the study was carried out at a fixed CIP concentration, the lower CE at higher $R_{CIP/DXT}$ indicated that a lower percentage of CIP in the feed was transformed to the colloidal complex when there was a smaller presence of DXT available for complexation. The lower CE at higher $R_{CIP/DXT}$ also explained the lower CIP payload observed at higher $R_{CIP/DXT}$.

The trend in CE as a function of $R_{CIP/DXT}$ was not unexpected because one of the roles of the PE in amphiphiles-PE complexation was to bring the amphiphiles closer together by essentially gathering the amphiphiles together by binding them electrostatically. This in turn promoted the cooperative binding of the free amphiphiles to the bound ones, resulting in higher complexation efficiency of the amphiphiles. Without the presence of PE, there would be minimal interactions among the amphiphiles due to their repulsive electrostatic forces [117].

Recognizing the importance of CE, we subsequently investigated the possibility of increasing the CE further by means of providing excess charges of DXT in the feed, in other words having $R_{CIP/DXT} < 1$. For this purpose, the effects of decreasing $R_{CIP/DXT}$ to values below unity (i.e. 0.8, 0.6, and 0.5) on the CE, yield, and payload were examined. The results in Fig. 3-3A showed that having excess DXT did not lead to any improvement in the CE suggesting that the same amount of CIP was

transformed to the colloidal complex. As a result, the CIP payload was not improved either upon decreasing $R_{\text{CIP/DXT}}$ below unity (Fig. 3-3B). Not unexpectedly, the yield was also found to decrease with decreasing $R_{\text{CIP/DXT}}$ from $80 \pm 3\%$ at $R_{\text{CIP/DXT}} = 1.4$ to $56 \pm 1\%$ at $R_{\text{CIP/DXT}} = 0.5$ (Fig. 3-3A).

Figure 3-3 Effects of $R_{\text{CIP/DXT}}$ below unity on (A) CE and yield; (B) CIP payload of the CIP-HMW DXT colloidal complex.

Based on the above results, the optimal preparation condition for the CIP-HMW DXT colloidal complex was determined to be at AA = 0.7% and $R_{\text{CIP/DXT}} = 1.4$ attributed to the highest preparation efficiency and payload observed at this condition. In the next section, we examined the morphology (i.e. size, shape) of the colloidal complex prepared at the optimal condition more closely, as well as its amorphous state and colloidal stability.

3.3.2 Physical Characteristics of the Optimal CIP-HMW DXT Colloidal Complex

The FESEM image of the CIP-HMW DXT colloidal complex prepared at the optimal condition showed that the complex exhibited a roughly spherical or globular shape with individual sizes of slightly less than 100 nm (Fig. 3-4A). Hence, the size when viewed under FESEM was significantly smaller than the size reported earlier by DLS ($\approx 500\text{--}600\text{ nm}$), which suggested the occurrence of agglomeration of the colloidal complex. To investigate whether the agglomeration could be reduced by brief ultrasonication, we repeated the DLS measurement of the colloidal complexes prepared at different $R_{\text{CIP/DXT}}$ after ultrasonication. The results showed that the sizes after ultrasonication were reduced considerably to $\approx 140\text{--}230\text{ nm}$ (Fig. 3-4B) indicating successful dispersion of the agglomerates.

Figure 3-4 (A) FESEM image of the CIP-HMW DXT colloidal complex prepared at the optimal condition; (B) the size and zeta potential of the CIP-HMW DXT colloidal complexes prepared at different $R_{\text{CIP/DXT}}$ after ultrasonication.

The reduced size after ultrasonication did not adversely affect the zeta potential, which remained in the range of $\approx -33 - -38$ mV. Specifically, for the colloidal complex prepared at the optimal condition, the size was reduced to 212 ± 28 nm with -33 ± 1.5 mV. Importantly, as reflected by the DLS measurements at different times, the ultrasonicated CIP-HMW DXT colloidal complex did not experience a size increase for at least 30 h during ambient storage, which signified the absence of re-agglomeration (Fig. 3-5A). In fact, smaller particles in the range of ≈ 110 – 160 nm were reported during storage suggesting the dynamic nature of the agglomeration and de-agglomeration events (Fig. 3-5B).

Figure 3-5 Colloidal stability of the CIP-HMW DXT and CIP-LMW DXT colloidal complexes prepared at their respective optimal conditions characterized in terms of the variations in their (A) size and (B) zeta potential as a function of time.

The amorphous state of the CIP-HMW DXT colloidal complex was verified by its

PXRD pattern, which showed the low-intensity broad halos characteristic of an amorphous solid, in contrast to the sharp crystalline peaks exhibited by the native CIP (Fig. 3-6). In this regard, the two low-intensity peaks appearing in the PXRD pattern of the colloidal complex at $2\theta \approx 18$ and 23 were contributed by the presence of Pluronic F68 used as an additional surfactant stabilizer of the complex. The same two peaks appeared in the PXRD pattern of the native Pluronic F68, but not in the patterns of the native HMW DXT or the native CIP (Fig. 3-6).

Figure 3-6 PXRD patterns of the CIP-HMW DXT colloidal complex prepared at the optimal condition, native CIP, native HMW DXT, and native Pluronic F68.

3.3.3 Comparison Between the CIP-LMW DXT and CIP-HMW DXT Colloidal Complexes

In this section, we compared the CIP-HMW DXT and CIP-LMW DXT colloidal complexes prepared at their respective optimal conditions in terms of the (1)

physical characteristics (i.e. size, shape, zeta potential, colloidal stability, and payload), (2) preparation efficiency, and (3) dissolution characteristics.

3.3.3.1 Physical Characteristics and Preparation Efficiency

While the DLS measurement indicated that the CIP-HMW DXT colloidal complex was larger than its CIP-LMW DXT counterpart before ultrasonication (Table 3-2), their FESEM images suggested otherwise. The FESEM image of the CIP-LMW DXT colloidal complex presented in our earlier work (i.e. Fig. 4 in Cheow and Hadinoto [17]) showed individual colloidal particles in the range of $\approx 300\text{--}400\text{ nm}$, which were in agreement with the size reported by DLS. Thus, the FESEM image of the CIP-HMW DXT colloidal complex shown earlier in Fig. 3-4A indicated that the CIP-HMW DXT colloidal complex was in fact smaller ($< 100\text{ nm}$) than its CIP-LMW DXT counterpart. The large size of the CIP-HMW DXT colloidal complex reported by DLS was caused by agglomeration, which as shown earlier could be reduced by ultrasonication.

Optimal characteristics	CIP-HMW DXT	CIP-LMW DXT
Size by DLS (nm)	590 ± 29 (w/o sonication) 212 ± 28 (w/sonication)	187 ± 13 (w/o sonication)
Shape	roughly spherical	roughly spherical
Zeta potential (mV)	-33 ± 1.5	-31 ± 1.9
CIP payload (% w/w)	51 ± 2	71 ± 9
CE (% w/w)	81 ± 1	85 ± 4
Yield (% w/w)	80 ± 3	65 ± 2

Table 3-2 A summary of comparisons between the physical characteristics of the optimal CIP-HMW DXT and CIP-LMW DXT colloidal complexes.

Hence, the use of HMW DXT resulted in smaller individual colloidal complex, which, nevertheless, not stable enough that it had a tendency to agglomerate to form clusters that were about six times larger than its actual individual size. Despite their different size, both colloidal complexes shared similar shapes and sufficiently large negative zeta potentials (Table 3-2). As a result of the large zeta potential, both complexes exhibited good colloidal stability during 30-h ambient storage, where further agglomeration did not take place (Fig. 3-5).

The CIP-HMW DXT colloidal complex, however, exhibited a significantly lower payload at $51 \pm 2\%$ compared to $71 \pm 9\%$ for its CIP-LMW DXT counterpart (Table 3-2). This result confirmed our hypothesis that the use of longer DXT chain could result in lower CIP payload due to the aforementioned earlier phase transition of the soluble complex to insoluble complex when longer PE chains were used. The earlier phase transition when longer DXT chain was used, however, did not lead to a lower CIP utilization rate, where the CEs of both colloidal complexes were comparable at around 80 – 85% (Table 3-2).

Despite their comparable CEs, the production yield of the CIP-HMW DXT colloidal complex was considerably higher at $80 \pm 3\%$ compared to $65 \pm 2\%$ for the CIP-LMW DXT colloidal complex (Table 3-2). As both complexes were prepared at similar $R_{\text{CIP/DXT}}$ values (i.e. ≈ 1.3 – 1.4) and identical CIP concentrations (i.e. 10 mg/mL), the higher yield obtained at the similar CE exhibited by the

CIP-HMW DXT colloidal complex indicated that a higher percentage of DXT in the feed participated in the complexation when longer DXT chain was used. The higher participation rate of the HMW DXT was likely attributed to the aforementioned increase in the inter-PE chain interactions when longer PE chains were used. The higher participation rate of the HMW DXT led to the earlier phase transition of the CIP-HMW DXT complex to insoluble complex, resulting in not only its lower CIP payload, but also its smaller individual size.

3.3.3.2 Dissolution Characteristics

Under the sink condition, both CIP-HMW DXT and CIP-LMW DXT colloidal complexes exhibited similar dissolution rates, despite the differences in their size and payload. Specifically, approximately 60% of the CIP payload was dissolved after 0.5 h and nearly 100% dissolution was achieved after 1.5 h, which signified fairly fast dissolution rates attributed to the amorphous state and small size (Fig. 3-7A). Significantly, the difference in the dissolution rates between the CIP-HMW DXT colloidal complex that was (≈ 200 nm) and was not (≈ 600 nm) ultrasonicated was insignificant. Hence, the larger particle size due to agglomeration did not adversely affect the dissolution characteristics of the CIP-HMW DXT colloidal complex. Thus, the amorphous state had a greater influence than the particle size and CIP payload on the resultant dissolution rates.

Under the non-sink condition, both CIP-HMW DXT and CIP-LMW DXT colloidal

complexes also exhibited comparable supersaturation generation, where both exhibited the typical “spring and parachute” supersaturation time-profiles of amorphous drug formulations [128]. Specifically, the supersaturation level rapidly reached a peak (“spring”) upon dissolution at $\approx 8 \times C_{\text{Sat}}$ after 10 min, as a result of the fast dissolution rate of the amorphous state (Fig. 3-7B). Subsequently, the supersaturation level gradually decreased (“parachute”) to $\approx 4\text{--}5 \times C_{\text{Sat}}$ and $\approx 2 \times C_{\text{Sat}}$ after 1 h and 4 h, respectively, due to the precipitation of the supersaturated solution. The supersaturation level eventually dropped back to $\approx 1 \times C_{\text{Sat}}$ after 24 h (data not shown).

Figure 3-7 (A) Dissolution rates under sink condition and (B) supersaturation generation of the CIP-HMW DXT and CIP-LMW DXT colloidal complexes prepared at their respective optimal conditions.

At the intermediate time points (i.e. 0.5–1.5 h), the supersaturation level of the CIP-HMW DXT colloidal complex was slightly higher than that of the CIP-LMW

DXT counterpart (Fig. 3-7B). This signified the lower precipitation propensity of the supersaturated CIP solution when the HMW DXT was used. These results hence suggested that the presence of long DXT chains in the supersaturated solution inhibited the CIP nuclei formation and its subsequent crystal growth.

3.4 Conclusion

First, we successfully prepared amorphous colloidal complex of CIP and HMW DXT having individual size, zeta potential, and CIP payload of ≈ 100 nm (as indicated by its FESEM image), ≈ -33 mV, and $\approx 50\%$, respectively. The CIP-HMW DXT colloidal complex, however, had a tendency to agglomerate in its aqueous suspension form, resulting in its effective size of ≈ 500 – 600 nm as measured by DLS. The agglomeration could be reduced by brief ultrasonication to produce an effective size of ≈ 200 nm, which was maintained at this value during prolonged ambient storage indicating the absence of re-agglomeration, hence good stability of the colloidal complex after ultrasonication. Significantly, the larger size of the CIP-HMW DXT colloidal complex due to the agglomeration was not found to adversely affect its fast dissolution characteristics.

Second, the CIP-HMW DXT colloidal complex was prepared at $\approx 80\%$ CIP utilization rate (CE) and $\approx 80\%$ overall production yield denoting its high preparation efficiency. The optimal preparation condition was determined to be at $AA = 0.7\%$ and $R_{CIP/DXT} = 1.4$. While the size and zeta potential were minimally

affected by the preparation condition, the same could not be said about the preparation efficiency and CIP payload. Beyond the optimal value, the CE, yield, and CIP payload were found to decrease with increasing $R_{\text{CIP/DXT}}$ above unity (i.e. less DXT) signifying the importance of having sufficient DXT. On the other hand, having $R_{\text{CIP/DXT}}$ below unity (i.e. excess DXT) resulted in lower production yield with no improvement in the CE and payload.

Third, in comparison with its CIP-LMW DXT counterpart, the CIP-HMW DXT colloidal complex exhibited (1) smaller size with similar zeta potential, (2) lower CIP payload, (3) higher yield at similar CE, and (4) relatively similar dissolution and supersaturation generation characteristics. In addition, both colloidal complexes shared fairly similar optimal preparation conditions. The higher yield and the lower payload obtained at similar CE indicated that a higher percentage of DXT participated in the complexation when the HMW DXT was used. This was attributed to the supposed increase in the inter-PE chain interactions in amphiphiles-PE complexation when long-chain PE was used, which in turn was believed to make the CIP-DXT soluble complex undergo earlier phase transition to its insoluble form, resulting in its smaller size and lower payload.

To conclude, the use of different DXT chain lengths did result in the formation of CIP-DXT colloidal complexes having unique characteristics in certain aspects. However, the similarity in their dissolution profiles, which represented one of the

most important characteristics in any drug formulations, rendered the impact of DXT chain length less significant from the practical point of view. In the short term, the future research direction will be to evaluate the effect of the DXT's chain length (if any) on the amorphous state stability of the CIP-DXT colloidal complex during long-term storage. In this regard, the amorphous state of the amphiphiles-polysaccharide complex was stabilized by mobility restrictions of the amphiphiles by the binding with the polysaccharide chains. Hence, the DXT's chain length may have an effect on minimizing the crystallization propensity of the amorphous CIP during storage, which is critical for maintaining the dissolution characteristics of the colloidal complex. In the longer term, the future research direction will be to carry out a similar study using different amphiphilic drug-polysaccharides systems to evaluate whether the same observations on the impact of polysaccharide's chain length are made.

CHAPTER 4. DRY POWDER INHLAER FORMULATION OF CIPROFLOXACIN NANOPLEX: SPRAY DRYING VERSUS SPRAY FREEZE DRYING

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4.1 Introduction

Most of the people diagnosed with lung infections exhibit bronchiectasis symptoms characterized by irreversible dilation of the bronchial trees [3]. Individuals with impaired mucociliary clearance mechanisms caused by either congenital (e.g. cystic fibrosis) or acquired (e.g. tobacco smoking) factors are particularly susceptible to bronchiectasis. The impaired mucociliary clearance causes a vicious cycle of infection and inflammation in bronchiectasis lungs due to the mucus buildup that provides a nutrient-rich environment ideal for the growth of bacterial pathogens [129].

Inhaled delivery of antibiotics in the form of nebulized solution (e.g. TOBI1,

CAYSTON[®]) has been the mainstay of bronchiectasis treatment owed to the higher localized antibiotic exposure and lower risk of adverse systemic effect compared to the oral delivery route [66]. Antibiotic delivery by dry powder inhaler (DPI) has also been actively investigated for potential clinical applications attributed to its higher delivery efficiency compared to nebulization [130]–[132]. While the inhaled delivery of antibiotics in their native form has been effective in alleviating bronchiectasis symptoms and suppressing disease exacerbation, its effectiveness has been shown to diminish after repeated uses [68].

As discussed in Chapter 2 of this thesis, the diminished effectiveness of inhaled native antibiotics in long-term lung infections therapy is primarily caused by the production of sub-inhibitory antibiotic exposure in the infection sites, which in turn gives rise to the emergence of antibiotic resistant strains [69]. The sub-inhibitory antibiotic exposure is caused by two factors: one is a physical barrier provided by a thick stationary mucus layer where bacterial colonies live beneath it as well as polyanionic contents of the mucus (e.g. mucin, actin, DNA) physically binding to antibiotic molecules by both electrostatic and hydrophobic interactions [70]. Hence limiting the amount of antibiotic molecules that can reach the bacterial colonies. The other is the hindered diffusion of the antibiotic molecules due to the presence of the mucus barrier makes them highly vulnerable to the lung phagocytic clearance, causing their short retention time in the lung [71].

The long-term efficacy of the antibiotic therapy can be improved by developing antibiotic formulations that can overcome the mucus barrier and prolong the lung retention time. Inhaled antibiotic nanoparticles (or nano-antibiotics in short) have been widely demonstrated to exhibit superior antimicrobial efficacy than inhaled native antibiotics in both in vivo and clinical settings [72]–[74]. The superior antimicrobial efficacy of nano-antibiotics is attributed to the higher antibiotic exposure generated in the vicinity of the bacterial colonies when considering smaller size to penetrate mucus layer, modified surface to reduce interactions with components in mucus and higher aqueous solubility. In this regard, the nano-antibiotics are typically prepared by encapsulating antibiotic molecules in lipid or polymer-based nanoparticle carriers [75].

The higher antibiotic exposure generated by inhaled nano-antibiotics has been attributed to several factors. First, the mucus-inert surface of the nano-antibiotics minimizes their physical binding with the polyanionic contents of the mucus. At the same time, the nanoscale size enables the nano-antibiotics to diffuse through the water channels of the mucus, which are typically in the size range of 200 to 500 nm for infectious lungs [70]. Second, nanoparticles in general have been found to be less vulnerable to the lung phagocytic clearance owed to their small size [76]. Third, the antibiotic encapsulation enables a sustained antibiotic release that prolongs its retention time in the lung. Extensive clinical applications of inhaled nano-antibiotics, however, face a major challenge due to their costly preparation

method and low antibiotic payload (< 30%), both of which are typical consequences of drug nano-encapsulation process [75], [77].

For this reason, we developed in a previous study a cost-effective nano-antibiotic preparation method by self-assembly drug-polysaccharide complexation to produce ciprofloxacin-dextran sulfate nanoparticle complex (or nanoplex in short) exhibiting high antibiotic payload (around 80%) [17]. Ciprofloxacin has been widely used in bronchiectasis therapy owed to its prolonged lung retention time and high antimicrobial activity towards *Pseudomonas aeruginosa*—the main bacterial pathogen in bronchiectasis lungs [133][134]. Also it has been proven that ciprofloxacin have less possibility to cause resistance of bacteria [31]–[34]. Importantly, the antibiotic nanoplex also possesses mucus-inert characteristics (i.e. anionic and hydrophilic) ideal for mucus penetration attributed to the presence of dextran sulfate on its surface.

Herein we developed coarse-carrier-free DPI formulations of antibiotic nanoplex by directly transforming the nanoplex to its inhalable form using the two most commonly used DPI formulation techniques for drug nanoparticles, i.e. spray drying (SD) and spray freeze drying (SFD) [135], [136]. We opted for the carrier-free DPI formulation approach (1) to overcome issues associated with the use of coarse carriers (e.g. blend uniformity, drug-carrier detachment) and (2) to enable antibiotic delivery at a higher dose by limiting the amount of excipient used

[137]. The novelty of the present work lies in the fact that previous studies on DPI of nano-antibiotics were limited to polymer nanoparticles encapsulating antibiotics [28], [138], whose clinical potential is limited due to the low antibiotic payload of the nanoparticles

The objective of this chapter was to evaluate the DPI formulations of the antibiotic nanoplex prepared by SD and SFD in terms of their (1) aerosolization efficiency, (2) aqueous reconstitution, (3) antibiotic release profile, and (4) antimicrobial activity against clinically derived *P. aeruginosa* isolate. From this evaluation, the optimal DPI formulation technique was determined. D-mannitol was used as the thermal and cryogenic protectants for SD and SFD, respectively, because of the well-known mucolytic property of mannitol [139].

4.2 Materials and Methods

4.2.1 Materials

Ciprofloxacin (CIP), D-mannitol, L-leucine, phosphate buffered saline (PBS, pH 7.4), glacial acetic acid, and Pluronic F68 were purchased from Sigma–Aldrich (Singapore). Dextran sulfate (DXT) with molecular weight of 5 kDa was purchased from Wako Pure Chemical (Japan). Mueller Hinton broth (MHB) was purchased from BD Diagnostics (USA). Clinically derived isolate of *P. aeruginosa* was obtained from the National University Hospital (Singapore).

4.2.2 Methods

4.2.2.1 Preparation of CIP Nanoplex

Briefly, 10 mg of CIP was dissolved in 1.0 mL of 0.2% (v/v) aqueous acetic acid solution and 4.5 mg DXT was dissolved separately in 1.0 mL deionized water in the presence of 0.2% (w/v) Pluronic F68. Subsequently, the CIP solution was added dropwise to the DXT solution under gentle stirring upon which self-assembled CIP-DXT complex formed attributed to the electro- static binding between the cationic CIP and anionic DXT molecules. The complex solution was let equilibrate for 0.5 h during which the CIP-DXT complex precipitated upon reaching a critical concentration to form the CIP nanoplex. Afterwards, the CIP nanoplex was recovered by three cycles of centrifugation and washing at $13,000 \times g$ for 5 min before being resuspended in deionized water for characterizations.

4.2.2.2 Spray Drying of CIP Nanoplex

SD of the CIP nanoplex was carried out in a Büchi B-290 mini spray dryer (Büchi, Switzerland) equipped with a two-fluid atomizer having a nozzle diameter of 1.5 mm. D-mannitol, L-leucine were used as excipients to be spray dried together with CIP nanoplex. The SD was performed at a constant solid concentration in the feed at 1.0% (w/v), where the ratio of the CIP nanoplex to D-mannitol in the feed was maintained at unity. The feed flowrate, atomizing air flowrate, and drying temperature were investigated in the range of 0.12 to 0.32 L/h, 300 to 330 L/h, and 100 to 140 °C, respectively.

4.2.2.3 Spray Freeze Drying of CIP Nanoplex

SFD of the CIP nanoplex was performed in a modified Büchi B-290 mini spray dryer in which the drying chamber was removed. D-mannitol, L-leucine were also used as excipients during spray freeze drying process. A polypropylene vessel containing 400 mL of liquid nitrogen under constant stirring at 500 RPM was placed beneath the atomizer. The distance between the atomizer and the liquid nitrogen vessel was fixed at 10 cm as this distance was found in our previous study to result in the highest production yield [29]. The sprayed droplets were instantaneously frozen upon entering the liquid nitrogen after which they were freeze dried at -52°C and 0.05 mbar for 24 h in Alpha 1-2 LD Plus freeze dryer (Martin Christ, Germany). The SFD was performed at a constant feed rate of 0.25 L/ h and atomizing air flowrate of 300 L/h. The feed concentration was investigated in the range of 1.0 to 6.0% (w/v) at the ratio of CIP nanoplex to D-mannitol equal to unity.

4.2.2.4 Aerosolization Efficiency

Aerosolization efficiency of the CIP nanoplex powders prepared by SD and SFD was characterized using a seven-stage Next Generation Impactor (NGI) (Copley Scientific, UK) equipped with an induction port (IP) and a pre-separator (PS). A standardized powder entrainment tube (PET) described in Louey et al. [140] was used as the inhaler model. The PET has been demonstrated to produce similar aerosolization profiles as two widely-used commercial inhalers in Rotahaler1 and

Inhalator1. The inhalation flow rate was set at 85 L/min to achieve the recommended 4 kPa pressure drop across the PET. The airflow duration was maintained for 2.8 s to simulate the volume of air (i.e. 4 L) drawn in a normal human inhalation process. The aerosolization efficiency was characterized in terms of their (1) emitted dose (ED), (2) fine particle fraction (FPF), (3) mass median aerodynamic diameter (MMAD), and (4) dispersibility index (DI). The reported aerosolization efficiency was based on two independent NGI experiments using two batches of powders prepared on different days for a total of four replicates. At 85 L/min inhalation flowrate, the cut-off d_A values of the NGI were 6.7, 3.7, 2.4, 1.4, 0.8, 0.5 and 0.3 μm for stages 1 to 7, respectively. The mass of powders recovered in each section of the NGI was determined by dissolving the powders in 5 mL PBS after which the CIP concentration was measured by UV-vis spectrophotometer (UV Mini-1240, Shimadzu, Japan) at the maximum absorbance wavelength for CIP i.e. 324 nm [141]. First, ED was determined from the ratio of the mass of powders recovered in the IP, PS, and stages 1–7 of the NGI to the initial mass of powders placed in the PET (i.e. 20 mg). Second, FPF defined as the mass percentage of the emitted dose that possessed $d_A \leq 5.0 \mu\text{m}$ was determined from interpolation of the cumulative d_A distribution curve between stage 1 ($d_A = 6.7 \mu\text{m}$) and stage 2 ($d_A = 3.7 \mu\text{m}$). Third, MMAD defined as the median d_A of the powders recovered in stages 1 to 7 was read from the cumulative d_A distribution curve at the 50% point. Lastly, DI was determined from the ratio of the MMAD to the theoretical aerodynamic diameters (d_A) calculated from Eq. (2-5), where a high

DI value signified poor midair dispersion of the aerosols upon exiting the inhaler.

The geometric diameter (d_G) in Eq. (2-5) was determined by size analysis of the scanning electron microscope (SEM) image of the powders (JSM-6700F, JEOL, USA) using ImageJ software (NIH, USA) with a minimum of 300 particle counts. The results of d_G were presented as the volume-averaged value $\pm$ the standard deviation of the particle size distribution ($n \geq 300$). In addition to d_G , the SEM was also used to visually examine the morphology (e.g. shape, porosity, surface rugosity) of the CIP nanoplex powders. The tap density (ρ_{tap}) was determined in triplicates using a tap densitometer at 2000 taps (Quantachrome, USA).

4.2.2.5 Aqueous Reconstitution

Aqueous reconstitution was defined as the ability of the CIP nanoplex powders prepared by SD and SFD to reconstitute back to individual nanoparticles upon exposure to an aqueous environment [142], thereby they could perform their intended therapeutic function. The aqueous reconstitution was characterized in triplicates as the ratio of the CIP nanoplex size after reconstitution (S_{final}) to the initial CIP nanoplex size prior to SD and SFD (S_{initial}), where a high $S_{\text{final}}/S_{\text{initial}}$ ratio ($\gg 1.0$) signified poor aqueous reconstitution. S_{final} and S_{initial} were determined by photon correlation spectroscopy (PCS) using Brookhaven 90Plus Zeta Sizer (Brookhaven Instruments Corporation, USA), where S_{final} was measured after dispersing 2 mg of the CIP nanoplex powders in 1.5 mL deionized water.

4.2.2.6 Antibiotic Release Profile

The in vitro antibiotic release profile was characterized using two replicates under a sink condition. Briefly, 2 mg of the CIP nanoplex powders were placed inside a dialysis bag filled with 2 mL PBS. The dialysis bag was then immersed in 18 mL PBS held at 37 °C in a shaking incubator. At fixed time intervals over a 4 h-period, 0.5 mL aliquots were withdrawn and fresh PBS of the same volume was added to the incubator. The CIP concentration in the aliquot was subsequently quantified by UV–vis spectrophotometer at 324 nm. The antibiotic release profile of the aqueous CIP nanoplex suspension was also characterized for comparison.

4.2.2.7 Antimicrobial Activity

Antimicrobial activities of the CIP nanoplex after SD and SFD were evaluated from its minimum inhibitory concentration (MIC) against clinically derived isolate of *P. aeruginosa*. The MICs were determined by microbroth dilution method according to Clinical and Laboratory Standards Institute guidelines (CLSI, M100-S17, 2007). MIC was defined as the lowest antibiotic concentration capable of suppressing visible bacterial growth after overnight incubation at 37 °C. The reported MIC was based on two biological replicates (i.e. two batches of cell cultures prepared on different days) and three technical replicates for each batch. Optical density at 600 nm (OD₆₀₀) was used to quantify the extent of bacterial growth in which OD₆₀₀ < 0.1 characterized no visible growth. MICs were determined for the native CIP, CIP nanoplex suspension, and CIP nanoplex

powders prepared by SD and SFD.

Briefly, 20 mL of the CIP nanoplex suspension was added to 80 mL MHB followed by serial twofold dilutions to produce CIP concentrations in the range of 0.025 and 11.8 mg/mL. Overnight cultures of *P. aeruginosa* were adjusted to 0.5 McFarland standard in Mueller Hinton broth (MHB) and subsequently diluted 100-fold to produce cell suspension having 0.5×10^6 colony forming units (CFU)/mL. Afterwards, 100 μ L of the cell suspension was added to 96-well microplate wells containing CIP and incubated at 37 °C for 24 h. Wells without CIP and without the cells were used as the positive and negative controls, respectively. Next, OD₆₀₀ of the cell suspension in each microplate well was measured using a microplate reader (Synergy HT, Biotek, USA).

4.2.2.8 Statistical Analysis

The results of the aerosolization efficiency (ED, FPF, and MMAD), aqueous reconstitution ($S_{\text{final}}/S_{\text{initial}}$), and antibiotic release rate of the CIP nanoplex powders were presented as the mean $\pm$ standard deviation of the experimental replicates. The statistical significance between the treatment groups and control was analyzed by unpaired two-tailed Student's t-test at significance level of 0.05.

4.3 Results and Discussion

4.3.1 Aerosolization Efficiency

The carrier-free DPI formulation strategy requires the preparation of aerosols that can be readily emitted off the inhaler, while at the same time possesses aerodynamic diameters (d_A) between 1 and 5 μm for effective deposition in the lower respiratory tract [143]. In theory, this can be achieved by having aerosols with large geometric size (d_G) and low particle density (i.e. porous or hollow structures) as can be seen from Eq. (2-5) [144]. The large d_G reduces interparticle cohesiveness making the particles readily aerosolized, whereas the low density ensures d_A to remain below 5 μm despite the large d_G .

Using SFD, large and low-density particles can be readily produced as the instantaneous freezing afforded by the cryogen preserves the size of the sprayed droplet, hence ensuring the formation of large particles. Subsequently, sublimation of the ice crystals in the frozen droplets occurs interstitially, resulting in the formation of porous particles [29]. In contrast, SD preparation of large and low density nanostructured aerosols is highly intricate because it requires precise manipulations of multiple SD variables as demonstrated in our previous study [145]. For this reason, we incorporated L-leucine in the DPI formulation as L-leucine has been widely used as aerosol dispersion enhancer in DPI delivery [146]. The addition of L-leucine enabled us to work around the requirement of producing large and low-density particles in SD.

4.3.1.1 DPI of CIP Nanoplex Prepared by SD

The effects of formulation variable and process variable, i.e. L-leucine to D-mannitol ratio (Leu/Man) and drying temperature, on the aerosolization efficiency of the SD powders were investigated. The effect of Leu/Man on the aerosolization efficiency was investigated using SD powders prepared at drying temperature of 100 °C and feed and air flowrates of 0.12 L/h and 330 L/h respectively (i.e. SD Condition A in Fig. 4-1). This SD condition was determined from our preliminary study, whose objective was to prepare aerosols of the CIP nanoplex having $1 \leq d_A \leq 5 \mu\text{m}$.

In the absence of L-leucine, d_G was in the range of $4.1 \pm 2.0 \mu\text{m}$ with $\rho_{\text{tap}} = 0.4 \pm 0.007 \text{ g/cm}^3$, resulting in d_A of $\approx 3.0 \mu\text{m}$ (Fig. 4-1A). The addition of L-leucine at Leu/Man = 1:40 and 1:30 increased d_G to $5.0 \pm 2.5 \mu\text{m}$ and $6.1 \pm 3.0 \mu\text{m}$, respectively, with minimal impact on ρ_{tap} , resulting in powders having larger d_A of $\approx 3.5 \mu\text{m}$ and $4.3 \mu\text{m}$, respectively (Fig. 4-1A). As L-leucine was significantly less soluble in water than D-mannitol, L-leucine precipitated earlier during droplet evaporation leading to the accumulation of L-leucine precipitates at the liquid-air interface due to its poor diffusive mobility [146]. The significant presence of solid L-leucine on the droplet surface led to the formation of solid shells, which in turn reduced the droplet shrinkage rate during liquid evaporation, resulting in the formation of powders having larger d_G .

In terms of the aerosolization efficiency, ED increased slightly from $45.0 \pm 1.6\%$ in the absence of L-leucine to approximately 50% for both Leu/Man = 1:40 and 1:30 (Fig. 4-1B). The FPF also increased from $12.5 \pm 2.5\%$ in the absence of L-leucine to $18.6 \pm 3.0\%$ and $20.9 \pm 2.0\%$ at Leu/Man = 1:40 and 1:30, respectively (Fig. 4-1B). These results suggested that the addition of L-leucine at Leu/Man = 1:40 and 1:30 improved the aerosol dispersion, where a larger number of the CIP nanoplex powders were successfully entrained from the inhaler (i.e. higher ED) and fewer of them were deposited in the IP and PS sections of the NGI (i.e. higher FPF). Thus, the aerosol dispersion enhancing capability of L-leucine was also demonstrated for the CIP nanoplex powders, which was attributed to the surface rugosity generated in its presence [146].

Figure 4-1 The effects of L-leucine to D-mannitol ratio (Leu/Man) on (A) d_G , d_A , and MMAD; (B) ED and FPF of spray-dried CIP nanoplex powders. Note: A and B refer to the spray drying conditions used. The data for ED, FPF, and MMAD were presented as the mean $\pm$ standard deviation ($n = 4$).

To investigate whether the improved ED and FPF were attributed to the larger d_G produced in the presence of L-leucine, we re-examined the effect of Leu/Man in powders having larger d_G using Leu/Man = 1:40. To increase d_G , SD was performed at higher feed rate (i.e. 0.16 L/h) and lower air flowrate (i.e. 300 L/h) while the drying temperature was maintained at 100 °C (i.e. SD Condition B in Fig. 4-1). At this SD condition, larger CIP nanoplex powders having d_G in the range of $8.1 \pm 3.0 \mu\text{m}$ and d_A of $\approx 5.7 \mu\text{m}$ were successfully produced (Fig. 4-1A). The ED and FPF of the larger powders (around $8.0 \pm 3.5 \mu\text{m}$ of d_G), however, were equal to $54.1 \pm 2.0\%$ and $17.1 \pm 0.7\%$, respectively. Hence, they did not vary significantly from the ED and FPF of the smaller powders prepared earlier at Leu/Man = 1:40, whose d_G was in the range of $5.0 \pm 2.5 \mu\text{m}$ (Fig. 4-1B). Thus, we concluded that the improved ED and FPF in the presence of L-leucine were not attributed to the larger d_G .

Despite the increase in ED and FPF afforded by the L-leucine inclusion, the MMAD remained above the 5- μm threshold at ≈ 6.0 - $6.7 \mu\text{m}$ independent of Leu/Man values (Fig. 4-1A). As a result, DI was considerably larger than unity (Table 4-1) with the exception for the powders prepared under SD Condition B due to their larger starting d_A . The large MMAD indicated that a majority of the powders entering the NGI were deposited in stage 1. Hence, the improved aerosol dispersion at Leu/Man = 1:40 and 1:30 was insufficient for a higher percentage of the aerosols to be deposited in the lower respiratory tract, which typically required

MMAD to be $\leq 5 \mu\text{m}$ [144]. This result prompted us to investigate at a higher Leu/Man of 1:10.

SD condition	Leu/Man	DI	SD condition	Leu/Man	T (°C)	DI
A	0	2.05 ± 0.11	C	1:10	100	1.63 ± 0.04
A	1:40	1.83 ± 0.07			120	1.33 ± 0.03
A	1:30	1.39 ± 0.08			140	1.31 ± 0.02
B	1:40	1.17 ± 0.02	-			

Table 4-1 Dispersibility index (DI) of spray-dried CIP nanoplex powders.

A: Feed = 0.12 L/h, air = 330 L/h; B: feed = 0.16 L/h, air = 300 L/h; C: feed = 0.32 L/h, air = 300 L/h. The data were presented as the mean $\pm$ standard deviation (n=4).

At Leu/Man = 1:10, however, the SD feed flowrate had to be increased from 0.12 to 0.32 L/h to produce spherical powders, because the addition of L-leucine at higher concentrations resulted in the production of crumpled particles under the SD Condition A. This phenomenon was likely caused by the buckling of the L-leucine shell at the liquid-air interface during droplet evaporation [136]. At Leu/Man = 1:10, d_G was in the range of $4.7 \pm 3.0 \mu\text{m}$ with ρ_{tap} of $0.45 \pm 0.02 \text{ g/cm}^3$, resulting in d_A of $\approx 3.6 \mu\text{m}$ (Fig. 4-2A). Hence, they were not significantly different from the powders prepared earlier at Leu/Man of 1:40 and 1:30. Despite their similar d_G and d_A , a considerably higher ED at $61.7 \pm 2.0\%$ was observed at Leu/Man of 1:10 (Fig. 4-2B). The SEM image in Fig. 4-3 showed that the surface of the CIP nanoplex powders prepared at Leu/Man = 1:10 was significantly rougher compared to those prepared without L-leucine. The high surface rugosity increased the powders' interstitial distance resulting in less cohesive powders that

were more easily entrained from the inhaler [147]. The FPF, however, was only improved slightly to $23.6 \pm 1.2\%$ (Fig. 4-2B) suggesting the minimal impact of the surface rugosity on the midair dispersion of the entrained aerosols. Not unexpectedly, the MMAD remained high at $\approx 5.9 \mu\text{m}$ (Fig. 4-2A) with DI of 1.63 ± 0.04 (Table 4-1) denoting their predominant deposition in stage 1 of the NGI.

Figure 4-2 The effects of drying temperature on (A) d_G , d_A , and MMAD; (B) ED and FPF of spray-dried CIP nanoplex powders prepared at Leu/Man = 1:10. The data for ED, FPF, and MMAD were presented as the mean $\pm$ standard deviation ($n = 4$).

Next, the effect of increasing the drying temperature at Leu/Man of 1:10 was examined motivated by the following factors. First, the higher drying temperature would enhance the drying rate, which in turn would expedite the formation of the L-leucine shell causing the droplet evaporation to occur at a larger droplet size. Thus, particles having larger d_G that could lead to better ED were anticipated to be

produced. Second, the higher drying temperature would reduce the moisture content of the particles, hence lowering their degree of cohesiveness, which in turn would positively impact the aerosolization efficiency.

Figure 4-3 SEM images of the spray-dried CIP nanoplex powders prepared at Leu/Man = 1:10 exhibiting higher surface rugosity compared to those prepared without L-leucine

Increasing the drying temperature to 120 and 140 °C as expected resulted in powders having larger d_G of 6.4 ± 3.3 and 6.9 ± 3.4 µm, respectively (Fig. 4-2A). Despite their larger d_G , d_A of the powders remained below the 5-µm threshold. The higher drying temperatures increased ED slightly from $61.7 \pm 2.0\%$ at 100 °C to 66.5 ± 1.3 and $66.1 \pm 5.3\%$ at 120 and 140 °C (Fig. 4-2B), respectively.

Improvements in the FPF and MMAD at the higher drying temperature, however, were not evident (Fig. 4-2). Thus, the effects of increasing the drying temperature on the FPF and MMAD were found to be statistically insignificant. To conclude, the optimal SD formulation exhibited ED of $\approx 66\%$ and FPF of $\approx 23\%$ with MMAD of $\approx 6\ \mu\text{m}$ obtained at Leu/Man of 1:10 and drying temperature of $120\ ^\circ\text{C}$.

4.3.1.2 DPI of CIP Nanoplex Prepared by SFD

The effect of the feed concentration on the feasibility of forming physically robust powders by SFD was investigated prior to studying the effect of Leu/Man. From this investigation, the minimum feed concentration to obtain physically robust powders was determined to be equal to 5.0% (w/v). SFD at 1.0% (w/v) feed, which was equal to the feed concentration used in SD, resulted in flake-like powders as shown in Fig. 4-4A. The flakes were likely caused by the collapse of the frozen droplets during lyophilization due to the high porosity generated at low feed concentrations. In contrast, at 5.0% (w/v) feed, the powders were highly spherical in the size range of $60 \pm 28\ \mu\text{m}$ and exhibited a high degree of porosity with ρ_{tap} of $0.025 \pm 0.001\ \text{g/cm}^3$ (Fig. 4-4B). The porous morphology was also evident from a close-up view of the powder surface in Fig. 4-4C. Spherical powders of similar d_G and ρ_{tap} were produced at 6.0% (w/v) feed.

Owing to their large d_G and low ρ_{tap} , the spherical SFD powders were easily emitted off the inhaler as reflected by the high ED (Fig. 4-4D). Specifically, the EDs were equal to 81.0 ± 10.7 and $90.5 \pm 2.8\%$ for the powders prepared at 5.0

and 6.0% (w/v) feeds, respectively. The spherical powders, however, possessed d_A larger than the 5- μm threshold ($\approx 11\text{ }\mu\text{m}$) due to their large d_G . Not unexpectedly, their FPF were low at 8.0 ± 1.1 and $16.3 \pm 1.9\%$ (Fig. 4-4D), respectively, which suggested that a majority of the emitted powders were deposited in the IP and PS of the NGI. Nevertheless, for the spherical powders that successfully entered stage 1 of the NGI, they were successfully deposited in stage 2 or later as indicated by their MMAD, which both were below 3.5 μm (Fig. 4-4D). Interestingly, in comparison to the spherical powders, the flake-like powders prepared at 1.0% (w/v) feed exhibited better ED ($94.4 \pm 2.0\%$) and FPF ($22.8 \pm 5.0\%$) with comparable MMAD ($2.5 \pm 0.2\text{ }\mu\text{m}$) (Fig. 4-4D). Their random morphology, however, would make it difficult to obtain consistent dosing.

Next, the effect of adding L-leucine was investigated for the spherical powders prepared at 5.0% (w/v) feed with the objective of improving the FPF. Compared to the powders prepared without L-leucine, the addition of L-leucine at Leu/Man = 1:10 produced smaller spherical powders having d_G in the range of $25.9 \pm 10.9\text{ }\mu\text{m}$ and ρ_{tap} of $0.044 \pm 0.004\text{ g/cm}^3$, resulting in d_A of $\approx 6.2\text{ }\mu\text{m}$ (Fig. 4-5A). Unlike SD, however, the addition of L-leucine at Leu/Man = 1:10 did not visibly increase the surface rugosity of the SFD powders (image not shown). Nevertheless, the powders formulated with L-leucine exhibited not only higher ED at $94.3 \pm 1.7\%$, but also higher FPF at $14.1 \pm 1.9\%$ (Fig. 4-5B) with MMAD of $3.1 \pm 0.2\text{ }\mu\text{m}$ (Fig. 4-5A). The higher ED was attributed to the surface presence of hydrophobic

L-leucine that made the powders less hygroscopic [148], whereas the higher FPF was likely attributed to the smaller d_A .

Figure 4-4 SEM images of spray-freeze-dried CIP nanoplex powders prepared at feed concentrations of (A) 1.0% and (B) 5.0% (w/v), resulting in flake-like and spherical particles, respectively; (C) a close-up view of the powder surface at 5.0% (w/v) feed reaffirms the porous structures; (D) the effects of feed concentrations on ED, FPF, and MMAD. The data for ED, FPF, and MMAD were presented as the mean $\pm$ standard deviation ($n = 4$).

Figure 4-5 The effects of Leu/Man on (A) d_G , d_A , and MMAD; (B) ED and FPF of spray-freeze-dried CIP nanoplex powders. The data for ED, FPF, and MMAD were presented as the mean $\pm$ standard deviation ($n = 4$).

Increasing the Leu/Man further to 1:4 led to the formation of significantly larger powders having d_G of $81.5 \pm 37.0 \mu\text{m}$ and ρ_{tap} of $0.037 \pm 0.001 \text{ g/cm}^3$. This resulted in d_A of $\approx 17.7 \mu\text{m}$ that far exceeded the $5\text{-}\mu\text{m}$ threshold. While the high ED at $92.4 \pm 1.6\%$ was not unexpected due to the increased presence of L-leucine, the powders surprisingly exhibited higher FPF at $29.1 \pm 0.9\%$ with MMAD of $2.8 \pm 0.1 \mu\text{m}$, despite their large d_A . Thus, the presence of L-leucine on the powders' surface could overcome the adverse effect of having large d_A in aerosolization. To conclude, the optimal SFD formulation exhibited ED of $\approx 92\%$ and FPF of $\approx 29\%$ with MMAD of $\approx 2.8 \mu\text{m}$ obtained at 5.0% (w/v) feed and Leu/Man of 1:4.

4.3.2 Aqueous Reconstitution

The volume-averaged size of the raw CIP nanoplex suspension determined by PCS

was 290 ± 25 nm with a major frequency peak at ≈ 205 nm and a much smaller peak at ≈ 460 nm that was attributed to nanoplex agglomeration (Fig. 4-6A). For the SD powders exhibiting the optimal aerosolization efficiency, the CIP nanoplex powders were found to possess a high degree of aqueous reconstitution with $S_{\text{final}}/S_{\text{initial}}$ of 1.0 ± 0.2 , denoting the spontaneous release of the nanoplex from the excipient matrix upon dissolution of D-mannitol in water. The size distribution of the nanoplex after reconstitution was highly comparable to that of the raw nanoplex suspension with only a slight shift in the frequency peaks to ≈ 220 nm and 580 nm due to the drying process (Fig. 4-6A).

In contrast, the optimal SFD formulation exhibited poorer aqueous reconstitution, where only a small fraction of the powders reconstituted freely as characterized by the presence of a small peak in the size distribution at ≈ 210 nm with a much larger peak at ≈ 630 nm. This translated to $S_{\text{final}}/S_{\text{initial}}$ of 2.2 ± 0.4 for a majority of the reconstituted nanoplex (Fig. 4-6A). As both the optimal SD and SFD formulations were prepared at the same CIP nanoplex to D-mannitol ratio (i.e. 1:1), the poorer aqueous reconstitution of the SFD powders was postulated to be caused by either (1) the difference in the solid contents between the SD and SFD formulations (i.e. feed concentrations of 1.0% versus 5.0%, higher feed concentration got higher chance for agglomeration of nanoplex in feed which resulted in poor reconstitution after spray freeze drying), (2) the difference in their morphology (i.e. small-solid versus large-porous spheres), or (3) the difference in their L-leucine content (i.e.

Leu/ Man of 1:10 versus 1:4).

To investigate, aqueous reconstitutions of the SFD powders prepared at different feed concentrations (i.e. 1.0, 3.0, and 6.0% w/ v) and at different Leu/Man (i.e. 1:10, 1:4) were examined. These powders, whose morphologies varied from flake-like to spherical at different sizes, were found to exhibit similar aqueous reconstitutions as the optimal SFD formulation. Their $S_{\text{final}}/S_{\text{initial}}$ values were all larger than 2 due to the presence of a major frequency peak in the reconstituted size distributions at > 600 nm (data not shown). Therefore, a majority of the SFD powders were not readily reconstituted to the individual nanoplex in an aqueous environment, regardless of their solid content, morphology, or L-leucine content. Instead, the reconstituted nanoplex existed as agglomerates of several nanoplexes in the size range of $\approx 600\text{--}800$ nm, rendering them potentially less effective for mucus penetration. The stresses exerted by the ice crystal formation in the freezing step of SFD were believed to cause the irreversible agglomeration of the nanoplex.

4.3.3 Antibiotic Release Profile and Antimicrobial Activity

Despite their incomplete aqueous reconstitution, the in vitro antibiotic release profile of the SFD powders was highly comparable to that of the raw CIP nanoplex suspension and the SD powders (Fig. 4-6B), where $80 \pm 3\%$ of the antibiotic was released from the nanoplex after 1 h, before it plateaued at $\approx 85 \pm 3\%$ release after 3 h. In fact, the SFD powders exhibited a slightly faster antibiotic release rate than

their SD counterparts owed to their high porosity. Nevertheless, the antibiotic release rates of the SD and SFD powders were slightly slower than that of the raw nanoplex suspension that exhibited $95 \pm 1.5\%$ release after 3 h. This was not unexpected as the antibiotic release in the SD and SFD powders could only take place after the dissolution of D-mannitol.

Figure 4-6 (A) Aqueous reconstitution and (B) antibiotic release profiles of the optimal spray-dried (SD) and spray-freeze-dried (SFD) CIP nanoplex powders. The data for aqueous reconstitution ($n = 3$) and antibiotic release profile ($n = 2$) were presented as the mean $\pm$ standard deviation.

Lastly, the MIC values of both the SD and SFD powders in the absence of mucus against the clinically derived *P. aeruginosa* were determined to be equal to 0.18 mg/mL, which was identical to the MIC values of the native CIP and raw CIP nanoplex suspension. Thus, the dry-powder transformation by either SD or SFD did not have any adverse impact on the antimicrobial activity of CIP as it was freely released from the SD and SFD powders.

4.4 Conclusion

Carrier-free DPI formulations of antibiotic nanoplex intended for lung infection therapy were successfully prepared by SD and SFD in the form of spherical small-solid ($\approx 6 \mu\text{m}$, 0.4 g/cm^3) and large-porous ($\approx 80 \mu\text{m}$, 0.04 g/cm^3) nanostructured aerosols, respectively. The addition of L-leucine was required to obtain acceptable aerosolization efficiency, particularly for the FPF, for both the SD and SFD powders. For the SD preparation, the L-leucine content greatly influenced the resultant particle morphology, where crumpled particles were produced in place of spherical particles at higher L-leucine contents, hence limiting the amount of L-leucine that could be used to 1:10. The effect of the drying temperature on the aerosolization efficiency of the SD powders was found to be minimal. For the SFD preparation, the L-leucine content had a minimal influence on the particle morphology, where instead the feed concentration was the governing variable. Hence, a higher L-leucine content at 1:4 could be used for the SFD powders. Furthermore, the L-leucine content was found to have a more significant influence on the aerosolization efficiency of the antibiotic aerosols than their aerodynamic diameter.

For the optimal formulations, the SFD powders exhibited superior aerosolization efficiency to their SD counterparts in terms of the ED (i.e. 92% versus 66%), FPF (i.e. 29% versus 23%), and MMAD (i.e. $3 \mu\text{m}$ versus $6 \mu\text{m}$). The higher ED of the SFD powders was attributed to their large size and low density, which made them

easily entrained off the inhaler, whereas the better FPF and MMAD were owed to the higher L-leucine content used (i.e. 1:4 in SFD versus 1:10 in SD). However, unlike the SD powders, the SFD powders exhibited incomplete aqueous reconstitution, which might jeopardize their mucus penetrating ability in bronchiectasis lungs. Despite their poorer aqueous reconstitution, the antibiotic release profile and antimicrobial activity of the SFD powders were not adversely affected. To improve the aqueous reconstitution of the SFD powders, the effect of introducing a secondary cryoprotectant besides D-mannitol is currently under investigation in our laboratory. These results signified the better suitability of the SFD technique to prepare carrier-free DPI formulation of antibiotic nanoplex.

CHAPTER 5. EFFECT OF DRYING ON THE SUPERSATURATION GENERATION CAPABILITY OF CIP NANOPLEX

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5.1 Introduction

A large majority ($\approx 80\%$) of the recently marketed drugs and new drugs currently in the pharmaceutical pipeline possess poor aqueous solubility due to their increasingly intricate chemistry, resulting in their low systemic bioavailability upon administration [78]. As mentioned in Chapter 2, amorphous nanoparticle formulations of such pharmaceuticals have been widely demonstrated to be more effective in enhancing the bioavailability of poorly soluble drugs in vitro and in vivo than their crystalline nano-pharmaceutical counterparts [79]–[82].

The enhanced drug bioavailability, nevertheless, is only realized when the high supersaturation level generated by the amorphous NP is maintained over a time period sufficient for the drug absorption across the gastrointestinal lumen [150],

[151]. To this end, amorphous NP formulations typically incorporate water-soluble biocompatible polymers having crystallization inhibiting properties, such as polyvinylpyrrolidone (PVP) and hydroxypropylmethylcellulose (HPMC) [152]. In the presence of these polymer stabilizers, the supersaturation generation capability of amorphous NP in their aqueous suspension form has been well established [79], [82]. Typically, the drug release from the amorphous NP suspension follows a “spring and parachute” profile, where the dissolution initially produces a peak in the drug solubility denoting the maximum achievable supersaturation level of the amorphous form (“spring”). Subsequently, the supersaturation level decreases gradually due to the crystallization of the dissolved drug (“parachute”), before it eventually reaches equilibrium at higher apparent solubility than the solubility of the crystalline form [153].

The supersaturation generation capability of the amorphous NP, however, has been demonstrated in several studies to be greatly diminished upon drying [110], [112], [154], which is a mandatory step in the oral solid dosage form preparation of any pharmaceuticals. The amorphous NP powders, which were made up of microscale aggregated nanoparticles, were found to be unable to generate the “spring and parachute” supersaturation profile. This was believed due to poor disassociation of the nanoparticle aggregates upon dissolution [110], [112], [154], which resulted in reduced surface areas available for dissolution, hence a slow dissolution velocity. The slow dissolution velocity in turn increased the crystallization propensity of the

remaining solid phase undergoing dissolution, thus significantly lower supersaturation levels were generated after drying.

Among the drying techniques, spray drying has been widely established as the most suitable drying technique for nanoparticles as it enables direct transformation of the aqueous nanoparticle suspension to powders having controlled morphology and good flowability [94], [95], [155], [156]. For this reason, the present work aimed to develop spray drying adjuvant formulations to mitigate the adverse effect of spray drying on the supersaturation generation capability of amorphous NP and as it was discussed in Chapter 1, there was few study related to dried powder form of nanoplex was carried out before. In this chapter, two types of water-soluble adjuvants were investigated, i.e., (1) fast dissolving adjuvant (i.e., trehalose, mannitol), whose role was to act as “interstitial bridges” to prevent the formation of irreversible aggregates of the NP upon drying [95], and (2) slowly dissolving HPMC, whose functions were to suppress (i) crystallization of the amorphous solid phase undergoing dissolution, as well as (ii) crystallization of the dissolved drug from the supersaturated solution.

Using antibiotic nanoparticles (i.e., ciprofloxacin) as the model amorphous NP, we first investigated the effect of employing only a single-adjuvant (either the fast-dissolving adjuvants or HPMC) on the supersaturation generation. This was followed by studies employing the double-adjuvant formulations in which we also

examined the effect of the HPMC incorporation method (i.e., whether it was co-spray dried, or physically mixed with the spray-dried NP and fast-dissolving adjuvant). Lastly, we examined the effects of the adjuvant formulation on the physical characteristics (i.e., morphology, flowability, and amorphous form stability) of the spray-dried amorphous NP.

5.2 Materials and Methods

5.2.1 Materials

Ciprofloxacin (CIP), mannitol, trehalose, HPMC, glacial acetic acid, Pluronic F68, and phosphate-buffer saline (PBS, pH 7.4) were purchased from Sigma-Aldrich (USA). Dextran sulfate (MW 5000 Da) was purchased from Wako Pure Chemical (Japan).

5.2.2 Methods

5.2.2.1 Preparation of Amorphous NP Suspension

The amorphous CIP nanoparticles were prepared by self-assembly electrostatic complexation between ionized CIP molecules and oppositely charged polysaccharides as described in Cheow, Kiew, and Hadinoto [110]. The details for suspension preparation has been mentioned in Chapter 4 hence will not be repeated here.

5.2.2.2 Spray Drying of Amorphous NP

The aqueous suspension of the amorphous CIP nanoparticles was mixed with the aqueous solution of the spray-drying adjuvants at the mass ratios prescribed in Table 5-1. The total solid concentrations were fixed at 1.0% (w/v) for the different adjuvant formulations investigated. The resultant suspension was then spray dried in Büchi B-290 mini spray dryer (Büchi, Switzerland) at 120 °C, 6 mL/min feed rate, and 355 L/h air flow rate. The operating condition of the spray dryer was selected from a prior study aimed at maximizing the production yield. The spray-dried amorphous NP were stored in a humidity cabinet at 25 °C and 55% relative humidity for 48 h prior to characterizations.

5.2.2.3 Physical Characterizations of Spray-Dried Amorphous NP

The morphology of the spray-dried amorphous NP was examined by scanning electron microscope (SEM) model JSM-6700 F (JEOL, USA), where the mean size was determined from the SEM images using ImageJ software (NIH, USA) from a minimum of 200 particle counts. The powder flowability was assessed by the Carr's Index (Eq. (2-6)) from the bulk and tap densities, where Carr's Index ≥ 33 signified poor flowability and Carr's Index ≤ 21 signified good flowability [157]. In this regard, the bulk density (ρ_{bulk}) was determined by measuring the volume of a known mass of the powders without tapping, whereas the tap density (ρ_{tap}) was determined using tap densitometer (Quantachrome, USA) after 2000 taps.

The amorphous form stability of the spray-dried products was assessed by thermal analysis using differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA). In the former, 2 mg of the spray-dried powders were filled into a sealed aluminium crucible and heated from 25 °C to 330 °C at 10 °C/min in DSC 822e (Mettler Toledo, USA), whereas in the latter 5 mg of the powders were filled into an alumina pan and heated from 30 °C to 400 °C at 10 °C/min in SDT Q600 (TA Instruments, USA). To supplement the thermal analysis, powder X-ray diffraction (PXRD) of the spray-dried products was carried out 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.

5.2.2.4 Supersaturation generation of spray-dried amorphous NP

The supersaturation generation was evaluated by adding excess amount of the spray-dried amorphous NP to 8.5 mL PBS in a shaking incubator at 37 °C. The excess amount was defined as CIP concentration in the feed equal to $\approx 13\times$ above the CIP saturation solubility (C_{Sat}), which was equal to ≈ 0.14 mg/mL. Subsequently, 0.2 mL aliquot was withdrawn at specified time intervals over 4 h. The aliquot was filtered immediately in 0.22 μm membranes after which it was diluted tenfold with fresh PBS to prevent drug crystallization from the supersaturated solution. The CIP concentration in the aliquot (i.e., C) was measured in triplicates by UV–vis spectrophotometer (UV Mini-1240, Shimadzu, Singapore) at 324 nm. After taking into account the tenfold dilution, the

supersaturation level was determined from the ratio of C to C_{Sat} .

Prior to the above, the supersaturation level generated by the aqueous NP suspension was compared with that generated by the spray-dried amorphous NP without any drying adjuvant. This comparison was used to confirm the adverse effect of spray drying on the supersaturation generation of the amorphous NP. For this comparison, the supersaturation generation was evaluated in 8.5 mL PBS solution containing 1.8 mg/mL of HPMC and at feed concentrations equal to $\approx 20\times$ above the CIP saturation solubility.

In addition to the supersaturation level, the in vitro drug release profile of the spray-dried amorphous NP was characterized under a sink condition with the aim of evaluating the rate of the supersaturation generation from the different adjuvant formulations. Briefly, 1mg of the spray-dried amorphous NP was immersed in 20 mL PBS in a dialysis bag in a shaking incubator at 37 °C. At specified time intervals over 1.5 h, 2 ml aliquot was withdrawn and replaced with fresh PBS of the same volume. The CIP concentration in the aliquot was measured in triplicates using UV–vis spectrophotometer at 324 nm.

5.3 Results and Discussion

5.3.1 Physical Characteristics of Spray-Dried Amorphous NP

For the single-adjuvant formulations, the amorphous NP co-spray dried with the

fast-dissolving adjuvants (i.e., “SD w/Trehalose” and “SD w/Mannitol” in Table 5-1) were found to be roughly spherical in shape in the size range of $\approx 5\text{--}10\ \mu\text{m}$ as shown in the SEM image in Fig. 5-1A, and with tap densities in the range of $\approx 400\text{--}550\ \text{g/cm}^3$. Thus, they shared similar morphology with the amorphous NP spray dried without any adjuvant (i.e., “SD w/o adjuvant”). In contrast, the amorphous NP co-spray dried with HPMC (i.e., “SD w/HPMC”), despite the relatively similar size, possessed a considerably lower tap density at $\approx 50\ \text{g/cm}^3$, which was caused by their hollow structure as seen from the SEM image in Fig. 5-1B. In fact, lower tap densities were also observed for the double-adjuvant formulations containing HPMC (i.e., “SD w/Trehalose & HPMC” and “SD w/Mannitol & HPMC”) (Table 5-1). Thus, the inclusion of HPMC in the spray-drying solution was found to produce particles with a hollow morphology.

SD= spray drying; Phys. Mix. = physical mixture.

Powders	NP (wt%)	Mannitol or trehalose (wt%)	HPMC (wt%)	Mean size (μm)	$\rho_{\text{tap}}\ (\text{g/cm}^3)$	Carr's Index
SD w/o adjuvant	100	0	0	5.6 ± 2.7	426 ± 5	39.8 ± 2.7
SD w/ HPMC	50	-	50	12.2 ± 6.1	52 ± 4	30.7 ± 1.9
SD w/ trehalose	50	50	-	7.5 ± 2.5	407 ± 11	32.0 ± 1.0
SD w/ mannitol	50	50	-	5.7 ± 3.0	548 ± 5	40.0 ± 1.3
SD w/ trehalose & HPMC	33.33	33.33	33.33	7.5 ± 3.6	55 ± 2	32.5 ± 1.2
SD w/ mannitol & HPMC	33.33	33.33	33.33	9.8 ± 4.5	68 ± 2	30.9 ± 2.8
Phys. Mix. of HPMC + SD w/ trehalose	33.33	33.33	33.33	NA	404 ± 18	32.9 ± 1.1
Phys. Mix. of HPMC + SD w/ Mannitol	33.33	33.33	33.33	NA	656 ± 34	37.6 ± 1.6

Table 5-1 A summary of the adjuvant composition and physical characteristics of the spray-dried NP.

Figure 5-1 SEM images of the spray-dried amorphous NP for adjuvant formulations (A) without HPMC and (B) with HPMC. Spray-dried powders from “SD w/Mannitol” and “SD w/HPMC” formulations were used as the representative samples, respectively.

In spray drying of nanoparticle suspension, droplet evaporation caused the nanoparticles at the receding liquid–vapor interface to be exposed to the vapor phase. As the surface energy of the solid–vapor interface was greater than that of the liquid–vapor interface, the nanoparticles had the tendency to migrate toward the droplet center to minimize their surface energy. Hollow particles were produced when the rate of nanoparticle migration towards the center was slower than the droplet evaporation rate [94]. The inclusion of HPMC, which increased the viscosity of the spray-drying solution, was believed to slow down the nanoparticle migration rate due to the greater fluid resistance, resulting in the hollow morphology. Incorporating HPMC in the form of physical mixture with the spray-dried amorphous NP (i.e., “Phys. Mix. of HPMC + SD w/Trehalose” and “Phys. Mix. of HPMC + SD w/Mannitol”), however, did not lead to particles with

lower tap densities, which was not unexpected as HPMC was not present in the spray drying solution.

The Carr's Index values of the spray-dried amorphous NP were found to be between 31 and 33 for five out of the eight adjuvant formulations investigated, with three formulations (i.e., “SD w/o adjuvant”, “SD w/Mannitol” and “Phys. Mix. of HPMC + SD w/Mannitol”) exhibited Carr's Index values greater than 33 (Table 5-1). The high Carr's Index values signified the far-from-ideal flowability of the spray-dried amorphous NP, which was likely caused by their small size. To improve the flowability, the spray-dried amorphous NP ought to undergo a further granulation process typically performed in oral solid dosage form preparation [158].

The DSC thermographs of the spray-dried amorphous NP (with or without adjuvants) indicated the absence of a sharp endothermic peak at $\approx 255\text{ }^{\circ}\text{C}$ (Fig. 5-2A). This peak was attributed to the crystalline melting point of CIP as reflected by its presence in the native crystalline CIP's thermograph. Similarly, the sharp endothermic peaks at $\approx 165\text{ }^{\circ}\text{C}$ apparent in the DSC thermographs of “SD w/Mannitol” and “SD w/Mannitol & HPMC” were attributed to the melting point of mannitol. On this note, the slight downward peaks at $\approx 170\text{ }^{\circ}\text{C}$ observed in the DSC thermographs of the adjuvant formulations containing HPMC were attributed to the glass transition temperature of HPMC. The TGA thermographs, however,

showed that all of the spray-dried amorphous NP decomposed earlier (at $\approx 210 - 220$ °C) than the native crystalline CIP (≈ 280 °C) (Fig. 5-2B) due to the early decomposition of dextran sulfate. Hence, the peaks above 210 °C in the DSC thermographs of the spray-dried products were attributed to the decomposition events, such that they could not be used to evaluate the amorphous form stability.

The DSC/TGA results, nevertheless, did indicate that the spray-dried amorphous NP possessed low crystallization propensity prior to their decomposition. Importantly, the amorphous form stability of the spray-dried amorphous NP was demonstrated by the PXRD patterns of the three representative samples after three month storage (i.e., “SD w/o adjuvant,” “SD w/Trehalose & HPMC,” and “SD w/Mannitol & HPMC”) (Fig. 5-3). Amorphous halos with very small peaks (if any) were observed in the PXRD patterns of the spray-dried NP, which were in contrast to the sharp peaks exhibited by the native crystalline CIP.

Figure 5-2 Thermal analysis of the spray-dried amorphous NP revealed the amorphous form stability prior to their decomposition (A) DSC and (B) TGA.

Figure 5-3 PXRD patterns of the spray-dried amorphous NP (with or without adjuvants) after three month storage at 25 °C and 55% relative humidity showed the amorphous halos, in contrast to the sharp crystalline peaks of the native drug crystals. Note: “SD w/o adjuvant”, “SD w/Trehalose & HPMC,” and “SD w/Mannitol & HPMC” formulations were used as the representative samples.

5.3.2 Supersaturation Generation of the Single-Adjuvant Formulations

The adverse effect of spray drying on the supersaturation generation capability of the amorphous NP was demonstrated in the significantly lower supersaturation level (C/C_{Sat}) of the “SD w/o adjuvant” formulation compared to that of the aqueous NP suspension (Fig. 5-4A). In the presence of HPMC in the dissolution medium, the supersaturation level generated by the aqueous NP suspension rapidly reached its maximum at $\approx 17\times$, which was close to the theoretical maximum achievable supersaturation level of $\approx 20\times$ (based on the CIP concentration in the feed). Subsequently, the supersaturation level decreased to eventually reach $\approx 6\times$ at

equilibrium (after 4 h), thus exhibiting the aforementioned “spring and parachute” supersaturation profile. In contrast, the supersaturation level generated by the “SD w/o adjuvant” plateaued at a significantly lower value of $\approx 4\times$, without the appearance of a “spring” in the supersaturation profile.

A look at the in vitro drug release profiles showed that the aqueous NP suspension exhibited a faster drug release rate, where $\approx 70\%$ of the drug was released in 0.5 h compared to $\approx 50\%$ for the “SD w/o adjuvant” formulation (Fig. 5-4B). Furthermore, the “SD w/o adjuvant” formulation resulted in only $\approx 70\%$ of the drug released after 1.5 h compared to a nearly complete release for the aqueous NP suspension. The faster drug release rate of the aqueous NP suspension signified its faster rate of supersaturation generation, which reduced the crystallization propensity of the remaining solid phase, thus resulting in the higher supersaturation level. The slower drug release rate of the “SD w/o adjuvant” formulation was caused by poor disassociation of the nanoparticle aggregates in the absence of spray-drying adjuvants acting as the “interstitial bridges”.

Figure 5-4 Supersaturation and in vitro drug release profiles of (A, B) aqueous NP suspension versus spray-dried amorphous NP without adjuvant and (C, D) spray-dried amorphous NP of single-adjuvant formulations containing HPMC, trehalose, or mannitol.

Co-spray drying the amorphous NP with a single-adjuvant type, however, was not found to greatly improve the supersaturation generation. For CIP concentration in the feed equal to $\approx 13\times$ of the saturation solubility, the “SD w/HPMC” formulation resulted in a stagnant supersaturation level (i.e., without the appearance of a

“spring”) that plateaued at $\approx 3.5\times$. Thus, it did not represent a significant improvement over that generated by the “SD w/o adjuvant” formulation that plateaued at $\approx 2.5\times$ (Fig. 5-4C). Likewise, even though co-spray drying the amorphous NP with trehalose or mannitol resulted in the “spring and parachute” supersaturation profiles (where the peak supersaturation level of $\approx 3.7\times$ was achieved after 0.25 h), the supersaturation levels quickly decreased to $\approx 2.0\times$ after 1.0 h. The supersaturation level remained at that value after 4 h, which in fact was lower than the equilibrium supersaturation level of the “SD w/o adjuvant” formulation (Fig. 5-4C).

A look at the in vitro drug release profiles, nevertheless, revealed that the single-adjuvant formulations improved the rate of the supersaturation generation (as reflected by the faster drug release rates) when compared to the “SD w/o adjuvant” formulation (Fig. 5-4D). This was attributed to the improved nanoparticle aggregate disassociation in the presence of the water-soluble adjuvants acting as the “interstitial bridges”. A minimal difference was observed in the drug release rates from the different single-adjuvant formulations. Thus, HPMC was as effective as the fast-dissolving adjuvants in preventing the formation of irreversible nanoparticle aggregates, despite the slow-dissolving nature of HPMC compared to trehalose and mannitol.

The lack of improvement in the supersaturation generated by the single-adjuvant

formulations was reaffirmed by the calculated area under the curve (AUC) of the supersaturated concentration versus time plot, which is typically used as an in vitro indicator for drug bioavailability [12]. The calculated AUCs of the “SD w/Trehalose” and “SD w/Mannitol” formulations were found to be similar in magnitude to that of the “SD w/o adjuvant” formulation at 9 ± 1 ($\times C_{\text{Sat}}$ mg mL⁻¹ h) (Fig. 5-5), despite the improved nanoparticle aggregate disassociation in the former. Their similar AUCs, however, were not unexpected because the supersaturation levels of both the “SD w/Trehalose” and “SD w/Mannitol” formulations decreased rapidly upon reaching the maximum values. The rapid decrease was caused by crystallization of the dissolved drug from the supersaturated solution and crystallization of the remaining solid phase, due to the absence of crystallization inhibitors (e.g. HPMC) in the two formulations.

The calculated AUC of the “SD w/HPMC” formulation, on the other hand, was found to be higher at 11 ± 0.8 ($\times C_{\text{Sat}}$ mg mL⁻¹ h) (Fig. 5-5) as the presence of HPMC contributed to both aggregate disassociation and crystallization inhibition. A more significant improvement in the AUC remained desirable as the calculated AUC of the “SD w/HPMC” formulation represented less than 20% improvement when compared to the “SD w/o adjuvant” formulation. For this reason, the supersaturation generation of the double-adjuvant formulations was investigated next.

Figure 5-5 The calculated areas under the curve (AUCs) of the supersaturation versus time plot of spray-dried amorphous NP from the different adjuvant formulations.

5.3.3 Supersaturation Generation of the Double-Adjuvant Formulations and Physical Mixtures

In comparison to the “SD w/Trehalose” formulation, the double adjuvant formulation containing both trehalose and HPMC at 1:1 ratio (i.e., “SD w/ Trehalose & HPMC”) resulted in not only a higher peak in the supersaturation level (at $\approx 4.3\times$) but also a prolonged supersaturation level over 4 h at the peak value (Fig. 5-6A). The same trend was observed for the double-adjuvant formulation containing mannitol and HPMC (i.e., “SD w/Mannitol & HPMC”) (Fig. 5-6B). As a result, despite not exhibiting the spring and parachute”

supersaturation profile, the calculated AUCs of the double-adjuvant formulations were found to be significantly higher at 15 ± 0.6 ($\times C_{\text{Sat}}$ mg mL⁻¹ h) (Fig. 5-5). This represented $\approx 70\%$ improvement over the AUC of the “SD w/o adjuvant” formulation.

Figure 5-6 Supersaturation profiles of the spray-dried amorphous NP from the double adjuvant formulations and physical mixtures containing (A) trehalose and (B) mannitol.

As seen from the initial slopes in the supersaturation versus time plot (Fig. 5-6), the larger AUCs of the double-adjuvant formulations were obtained despite their slower supersaturation generation rates compared to the single-adjuvant formulation. The slower supersaturation generation rates of the double-adjuvant formulations, which indicated their less effective nanoparticle aggregate disassociation, were reaffirmed by the in vitro drug release profiles, where only $\approx 75\%$ of the drug was released after 1.5 h compared to $\approx 85 - 90\%$ release for the

single-adjuvant formulations (Fig. 5-7). In this regard, as the use of HPMC as a single adjuvant in “SD w/HPMC” formulation was not found earlier to result in a slower drug release rate, it was believed that the fast-dissolving adjuvants might interact with HPMC upon spray drying, resulting in slower dissolution rates for both.

Figure 5-7 In vitro drug release profiles of the spray-dried amorphous NP from the double adjuvant formulations and physical mixtures containing (A) trehalose and (B) mannitol.

The lower degree of supersaturation generated in the beginning by the double-adjuvant formulations had the positive impact of reducing the crystallization driving force of the supersaturated solution, resulting in the abovementioned higher and prolonged supersaturation levels. This signified that effective disassociation of the nanoparticle aggregates (which was manifested in the fast supersaturation generation rate) should not be the only objective in

spray-drying formulation of amorphous NP. Instead, the formulation had to ascertain that the fast supersaturation generation was accompanied by sufficient crystallization inhibition for improvement in the AUC to be achieved.

When HPMC was physically mixed with the single-adjuvant formulation of trehalose or mannitol (i.e., “Phys. Mix. of HPMC & SD w/Trehalose” and “Phys. Mix. of HPMC and SD w/Mannitol”), similar supersaturation profiles as those of the double-adjuvant formulations (albeit at lower overall supersaturation levels) were obtained (Fig. 5-6). Importantly, the improved supersaturation generation exhibited by the physical mixtures was also obtained at slower supersaturation generation rates (Fig. 5-7). Thus, it was concluded that the co-presence of HPMC and the fast-dissolving adjuvants led to lower supersaturation generation rates.

Not unexpectedly, the calculated AUCs of the physical mixtures at 14 ± 0.6 ($\times C_{\text{Sat}}$ mg mL⁻¹ h) were found to be slightly lower than the AUCs of the double-adjuvant formulation, which represented $\approx 60\%$ improvement over the “SD w/o adjuvant” formulation. For the physical mixtures, the absence of HPMC in the spray-dried products led to lower crystallization inhibition of the remaining solid phase, resulting in the lower supersaturation level compared to the double-adjuvant formulations. Thus, co-spray-drying amorphous NP with the two adjuvant types was determined to be the best method in mitigating the adverse effect of spray drying on the supersaturation generation capability of amorphous NP.

5.4 Conclusion

In the present work, the inevitable decrease in the supersaturation generation capability of amorphous NP after spray drying was mitigated by co-spray drying the amorphous NP with two types of water-soluble adjuvants (i.e., the crystallization inhibiting HPMC and fast-dissolving trehalose/mannitol). First, the different adjuvant formulations had relatively insignificant impacts on the physical characteristics of the spray-dried amorphous NP and the amorphous form of the spray dried products was shown to remain stable after three-month storage. Second, the calculated AUC of the supersaturation versus time plot for the double-adjuvant formulations represented approximately 70% improvement when compared to spray drying without adjuvant. This was attributed to the prolonged high supersaturation level after the supersaturation peak was reached. Third, compared to the double adjuvant formulations, co-spray drying the amorphous NP with only one adjuvant type resulted in inferior supersaturation generation, which was characterized by either stagnant supersaturation profile (for HPMC), or high yet short-lived (i.e., burst) supersaturation levels (for trehalose and mannitol). This was caused by the higher crystallization propensity of the single-adjuvant formulations due to their faster supersaturation generation rate. Fourth, while they exhibited similar supersaturation profiles as the double-adjuvant formulations, physical mixtures of HPMC and the single-adjuvant formulations containing either trehalose or mannitol exhibited slightly smaller AUC in their supersaturation versus time plot. This was attributed to the less crystallization inhibition of the

remaining solid phase due to the absence of HPMC in the spray-dried products. In short, our study clearly demonstrated that formulation efforts aimed at enhancing the supersaturation generation rate alone were not sufficient to mitigate the adverse effect of spray drying on the supersaturation generation capability of amorphous NP if crystallization inhibition of the dissolved drug and the remaining solid phase was not addressed.

CHAPTER 6. DUAL DRUG NANOPLEX DELIVERY SYSTEM

6.1 Introduction

As discussed in Chapter 2, multi-drug administration is required for lung infection therapy sometimes due to growth of multi-type of microorganisms and emergence of drug resistant strains from abuse of antibiotics. The effectiveness of combined therapy with two types of antibiotics has been found by Doring et al. [99]. In fact, the application of dual drug delivery system have many promising advantages like achievement of synergistic effects, repression of drug resistance and manipulation of dosage ratio of different drugs in a single nanoparticle [103]. Most of studies about dual drug delivery system are focused on anticancer studies. Studies related to lung infections are mainly about conventional nanoparticles such as liposomes. These studies about co-delivery of multi-drugs used either one nanoparticle with native drug in crystalline form [52] or dual drug encapsulated liposomes for inhalation therapy of lung infection [100]. However, crystalline form of drug is not suitable for mucus penetration and the payload for drugs are quite low which will cause increase of dose administration leading to increased burdens towards patients' airways. Furthermore, the preparation methods are not cost-effective or solvent-free during which large amount of organic solvent will be used for sample preparation and in washing steps.

In order to solve above mentioned problems, we used a well-established method by

Cheow and Hadinoto [14], [17], [18] in this chapter to prepare dual drug nanoplex which is more cost-effective and solvent-free. Most importantly, the pay load of both drugs can be improved greatly. Here we used two antimicrobial drugs, ciprofloxacin and itraconazole, as model drugs. Ciprofloxacin (CIP) is a kind of widely used broad-spectrum antibiotic and especially effective towards many pathogens in pulmonary systems. Itraconazole (ITZ) is a kind of anti-fungal drug and is also studied here considering the fact that it has been reported that some fungi such as *Sporothrix schenckii* also be found in airways [2]. ITZ has been studied a lot in improving its solubility because the saturated solubility of its native crystalline form is quite low ($\approx 1.8 \mu\text{g/mL}$). Here we tried to produce amorphous form for ITZ in dual drug nanoplex so that the solubility will be greatly enhanced. In this study, feasibility about formation of dual drug nanoplex by using CIP and ITZ was investigated and a self-assemble method via electrostatic complexation was applied. This method is much more cost-effective compared to other methods such as antisolvent precipitation or melt extrusion. It is promising for studies about possible application of inhaled dual drug delivery system in therapy of lung infections with different types of pathogens such as bacteria and fungi. This dual drug delivery system is expected to enhance therapeutic efficacy and efficiency.

In this chapter, we firstly investigated the effects of pH on the formation of dual drug nanoplex in terms of size, surface charge, complexation efficiency, payload and yield. Secondly, some studies were carried out to figure out a way for

manipulating the dosage ratio of CIP and ITZ in one nanoplex and lastly the supersaturation of dual drug nanoplex was tested as well as dissolution rate in *in vitro* environment.

6.2 Materials and Methods

6.2.1 Materials

Ciprofloxacin (CIP), glacial acetic acid, HPMC, phosphate buffer saline (PBS, pH 7.4), potassium dihydrogen phosphate(KH_2PO_4) were purchased from Sigma-Aldrich (USA), itraconazole (ITZ) was purchased from Alfa Aesar (USA), low molecular weight dextran sulfate (LMW DXT) (5 kDa of molecular weight) was purchased from Wako Pure Chemical (Japan), acetonitrile, methanol were purchased from Merck Millipore (USA).

6.2.2 Methods

6.2.2.1 Preparation of CIP-ITZ Dual Nanoplex

The method for preparing CIP-ITZ dual nanoplex is based on Cheow et al.'s work about ITZ nanoplex [112]. To prepare dual nanoplex, LMW DXT was dissolved in 1 mL of deionized water (DI water) with 7.3 mg/mL of concentration. CIP and ITZ were dissolved in 1 mL of 50, 60, 70 or 80% (v/v) of acetic acid solution simultaneously with 5 mg/mL of concentration for both drugs. Hence drug molecules will be positive charged. Then 1 mL of drug solution was added into equal volume of LMW DXT solution, followed by 10 s vortexing and the mixture

was left still for 30 min for complexation. Afterwards the suspension was washed 3 times with centrifugation ($13,000 \times g$, 30 min) and redispersion in 1 mL of DI water.

In the study of tuning dosage ratio of both drugs in one nanoplex, the acetic acid concentration was fixed as optimal value based on results from above experiments. LMW DXT and CIP concentrations are set as invariable with 7.3 mg/mL and 5 mg/mL respectively. Mass ratio of ITZ over CIP ($M_{ITZ/CIP}$) was varied from 0.2 to 1.6 with 0.2 of step size. The following procedures are the same as above experiment and the final suspensions were forwarded for further physical characterizations.

6.2.2.2 Physical Characterizations for CIP-ITZ Dual Nanoplex

In the optimizations of CIP-ITZ dual nanoplex, complexation efficiency (CE), yield and payload were compared among nanoplex prepared with different conditions. CE for both drugs and yield were calculated from Eq. (2-1) and Eq. (2-2), respectively. The details about the methods have been described in Chapter 3 and will not be repeated here.

Payload of nanoplex is tested by measuring both CIP and ITZ concentrations after completely dissolving a known mass of CIP-ITZ dual nanoplex in a certain volume of acetic acid solution. All these three parameters were tested at least two replicates.

For measuring CIP and ITZ concentration, HPLC method was used in Agilent 1100 (Agilent Technologies, Singapore) installed with ZORBAX Eclipse Plus C18 column (250×4.6 mm and 5- μ m particle size) at 262 nm of wavelength. The mobile phase for testing both drugs simultaneously is a mixture of acetonitrile and 0.002 M KH_2PO_4 (pH 3.2) (80 : 20, v/v) with a flow rate of 1 mL/min which results in retention times for CIP and ITZ are 2.4 min and 5.4 min respectively.

CIP-ITZ dual nanoplex size and zeta potential were measured by dynamic light scattering (DLS) with Brookhaven 90 Plus Nanoparticle Size Analyzer (Brookhaven Instruments Corporation, USA). Fourier Transform Infrared Spectroscopy (FTIR) was used to verify existence of CIP and ITZ in nanoplex. Spectrum One (Perkin-Elmer, USA) was applied for FTIR determination and the wavenumber range was set between 400 and 4500 cm^{-1} with 1 cm^{-1} of spectral resolution. The amorphous pattern of CIP-ITZ dual nanoplex was performed with freeze dried powders and Powder X-ray diffraction (PXRD) was carried out for determination. The details have been mentioned in Chapter 3 and will not be repeated here.

Differential Thermal Analysis (DTA) was performed for dual drug nanoplex by assessing their differential scanning calorimetry (DSC) patterns. Details for DSC measurement have been described in Chapter 5 and will not be mentioned here.

6.2.2.3 Supersaturation Determination of CIP-ITZ Dual Nanoplex

As mentioned in Chapter 4, the supersaturation was performed by adding excess amount of drugs in 8.5 mL of PBS solution (pH 7.4) which was incubated at 37°C in shaking incubator (150 rpm). Considering the lower solubility of ITZ in PBS (1.8 µg/mL), here we chose saturated solubility of CIP in PBS (0.186 mg/mL) as standard and the initial CIP concentration was set as 10 times of its saturated solubility. The following procedures were as it was described in Chapter 4 except that the filtered solution was diluted tenfold with 50% (v/v) of acetic acid to prevent crystallizations of both drugs. The concentration measurement for CIP and ITZ were performed with HPLC as described above. In addition to supersaturation determination, dissolution tests were also carried out in sink condition to evaluate supersaturation generation rate and the details were also mentioned in Chapter 4 except for the PBS volume was replaced with 100 mL.

6.3 Results and Discussion

6.3.1 Complexation of Dual Drugs (CIP and ITZ) with LWM DXT

6.3.1.1 Pre-studies about Dual Drug Nanoplex ($M_{ITZ/CIP} = 1$)

Considering the fact that ITZ has a quite low solubility in aqueous phase when in lower acid concentration environment ($\ll 10$ µg/mL) [159], we firstly investigated effects of acetic acid concentration on dissolving both CIP and ITZ for reaction of nanoplex formation. In order to dissolve ITZ with higher concentration which will be used for complexation with LMW DXT later, higher concentration of acetic

acid was needed. The concentration range of acetic acid tested was between 50% (v/v) and 80% (v/v) when both drug concentrations were 5 mg/mL. Drugs were not be able to be completely dissolved when acetic acid concentration was below 50% (v/v).

The pre-study data exhibited that there was no obvious difference in terms of particle size and zeta potential for formed products indicating that AA concentration had no obvious effects on product characteristics in aspects of size and zeta potential (Fig. 6-1A). In Fig. 6-1A, it showed that particle size of products ranged from 200 nm to 300 nm and all products exhibited negative zeta potential ($-30 \text{ mV} \sim -36 \text{ mV}$) due to presence of LMW DXT chains at outside part of particles. Furthermore, AA concentration also affected payload of both drugs minimally. Payloads of CIP (32%) and ITZ (42%) did not change a lot when AA concentration ranged from 50% (v/v) to 80% (v/v) which were not shown in this chapter.

Even though AA concentration had minimal effects on particle properties in aspects of size, zeta potential as well as payloads of both drugs, it greatly affected utilization rates of CIP and ITZ during reactions. As it was showed in Fig. 6-1B, CE of both drugs decreased (from $54 \pm 1 \%$ to $22 \pm 2 \%$ for CIP and from $90 \pm 2 \%$ to $23 \pm 2 \%$ for ITZ) when AA concentration was higher despite of the fact that higher solubility of drugs at acid environment. This phenomenon was possibly

resulted from less charge for LMW DXT molecules when pH was too low ($\ll$ pKa of LMW DXT). Less charge for LMW DXT chains diminished electrostatic driving force in formation of nanoplex with drug molecules. Yields also decreased due to lower CE values for both drugs which dropped from $51 \pm 1\%$ to $13 \pm 2\%$ as it was shown in Fig. 6-1B.

Figure 6-1 Effects of acetic acid concentration on (A) particle size, zeta potential, (B) yield and CE of both drugs for CIP-ITZ dual drug nanoplex.

Considering the fact that AA concentration has minimal effects on physical properties of CIP-ITZ dual drug nanoplex in aspects of size, zeta potential and the fact that drug utilization significantly decreased at higher AA concentration. We used 50% (v/v) of AA concentration for further studies. Also controlled experiments were performed with absence of LMW DXT and exhibited no appearance of precipitation after mixing drug solutions with LMW DXT solutions. This proved formation of CIP-ITZ dual drug nanoplex via complexation process.

6.3.1.2 Studies about Effects of $M_{ITZ/CIP}$

In studies about effects of $M_{ITZ/CIP}$ on characteristics of CIP-ITZ dual drug nanoplex, $M_{ITZ/CIP}$ were set to varied from 0.2 to 1.6 resulting in 0.51 to 1.07 of range for $R_{drug/LMW\ DXT}$ (charge ratio). Except for $M_{ITZ/CIP}$ equals to 1.6, other runs have charge ratios less than 1 which means LMW DXT molecules were sufficiently charged for complexation with drug molecules so that CE of drugs were able to be maximized. Since CIP and LMW DXT concentrations were set as constant parameters (5 mg/mL and 7.3 mg/mL respectively), higher $M_{ITZ/CIP}$ values meant more drugs (ITZ) in drug solutions for nanoplex formation with the same amount of LMW DXT.

Figure 6-2 Effects of $M_{ITZ/CIP}$ on (A) particle size, zeta potential, (B) payload of CIP-ITZ dual drug nanoplex.

In Fig. 6-2A, it exhibited that CIP-ITZ dual drug nanoplex had larger particle size when $M_{ITZ/CIP} > 1$ (360 nm – 410 nm) compared to it of nanoplex with $M_{ITZ/CIP} \leq 1$ (160 nm – 280 nm). FESEM images also verified particle size of CIP-ITZ dual

drug nanoplex (Fig. 6-3). It showed amorphous morphology of nanoplex when $M_{ITZ/CIP}$ was 1. Zeta potential of nanoplex in Fig. 6-2A showed that it had a narrow range of value when $M_{ITZ/CIP}$ was higher than 1. Basically it ranged from -36 mV to -49 mV among all $M_{ITZ/CIP}$ values and these high zeta potentials indicated better colloidal stability for dual drug nanoplex.

Figure 6-3 FESEM images of CIP-ITZ dual drug nanoplex ($M_{ITZ/CIP} = 1$).

From Fig. 6-2B, it was interesting that payloads of CIP and ITZ in nanoplex were able to be tuned by simply changing mass ratio of ITZ over CIP in drug solution. Higher mass ratio increased ITZ payload while CIP payload decreased. When $M_{ITZ/CIP}$ was 0.2, payloads of CIP and ITZ were $62 \pm 1 \%$ and $11 \pm 1 \%$, respectively. Payloads of CIP and ITZ changed to $31 \pm 2 \%$ and $45 \pm 2 \%$

respectively when $M_{ITZ/CIP}$ increased to 1.6. Though payloads of CIP and ITZ changed when varied $M_{ITZ/CIP}$ values, total payload of drugs remained almost constant (about 75%) as showed in Fig. 6-2B.

Fig. 6-4 showed that CE of CIP and ITZ increased when increasing $M_{ITZ/CIP}$ values. There were 53 ± 1 % of CIP and 69 ± 3 % of ITZ encapsulated into nanoplex when $M_{ITZ/CIP}$ was 0.2 and CE values increased to 69 ± 1 % and 96 ± 2 % for CIP and ITZ respectively at $M_{ITZ/CIP} = 1.6$. Increase of CE for both drugs at higher $M_{ITZ/CIP}$ values meant more drug molecules participated in the formation of nanoplex. Since total drug payload remained constant no matter how $M_{ITZ/CIP}$ varied, it also indicated that more LMW DXT formed nanoplex so that mass ratio of drugs over LMW DXT remained the same. More drugs and LMW DXT participated in formation of dual drug nanoplex also proved higher production yields (Fig. 6-4). Also more drugs and LMW DXT molecules participated in nanoplex complexation explained aforementioned larger particle size when $M_{ITZ/CIP}$ values were higher than 1.

Figure 6-4 Effects of $M_{ITZ/CIP}$ on yield and CE for CIP-ITZ dual drug nanoplex.

Furthermore, Fig. 6-4 also showed that CE of ITZ was higher than CE of CIP among all $M_{ITZ/CIP}$ values. It was predictable that CE of ITZ was higher when $M_{ITZ/CIP}$ value was higher than 1 because more ITZ was dissolved in drug solution. However, higher CE of ITZ also be observed when $M_{ITZ/CIP}$ was less than 1. This indicated that ITZ was more competitive in nanoplex formation compared to CIP. Lei et al [160] have raised a cooperative mechanisms for this complexation process. In the complexation process, free drug molecules hydrophobically interacted with electrostatically polyelectrolyte-bounded drug molecules to form nanoplex. Hence ITZ which has higher hydrophobicity than it of CIP was easier to bind to LMW DXT chains resulting in higher CE.

6.3.1.3 FTIR and PXRD Data Analysis

In order to verify existence of CIP and ITZ in nanoplex, FTIR analysis was applied

and CIP-ITZ dual drug nanoplex with $M_{ITZ/CIP}$ equals to 1 was used as model sample. In Fig. 6-5A, C=O bond in pyridone functional group showed at 1628 cm^{-1} proved presence of CIP because this was characteristic peak for ciprofloxacin [161]. This peak also presented in FTIR spectrum of CIP nanoplex. However, compared to spectrum of native CIP, peak of this functional group in nanoplex slightly moved due to ionization of CIP molecules (Fig. 6-5A). Cheow and Hadinoto [17] have also reported disappearance of peak at 1595 cm^{-1} as evidence of CIP-LMW DXT interaction in CIP nanoplex and this also happened for CIP-ITZ dual drug nanoplex.

Peaks at 1698 cm^{-1} indicated existence of ITZ in CIP-ITZ dual drug nanoplex because it was characteristic peak of itraconazole resulting from presence of C=O bond in triazole functional group[162]. Also C=C bond at 1510 cm^{-1} due to aromatic groups in itraconazole molecules proved existence of ITZ in dual drug nanoplex. FTIR results of native ITZ and ITZ nanoplex also attested above conclusion due to presence of these two peaks. Interactions between ITZ and LMW DXT molecules could be verified by disappearance of peak at 1610 cm^{-1} and this was found in both ITZ nanoplex and dual drug nanoplex.

Figure 6-5 (A) FTIR and (B) PXRD results of native CIP, native ITZ, native LMW DXT, CIP nanoplex, ITZ nanoplex and CIP-ITZ dual drug nanoplex ($M_{ITZ/CIP} = 0.4$, 1, 1.6)

Fig. 6-5B showed PXRD data for CIP-ITZ dual drug nanoplex to verify amorphous state of nanoplex. Samples with mass ratio of ITZ over CIP equaled to 0.4, 1, 1.6 were tested here. Unlike PXRD patterns of native drugs, results of dual drug nanoplex obviously lacked of sharp peaks which indicated crystalline pattern. When $M_{ITZ/CIP} = 1$ or 1.6 (higher ITZ payload), PXRD patterns showed a broad curve which was similar to it of ITZ nanoplex. When mass ratio was 0.4, PXRD pattern showed similar curves as it was in CIP nanoplex. It seems that amorphous state of CIP-ITZ nanoplex changed based on payloads of CIP and ITZ in nanoplex. Higher ITZ payload in dual drug nanoplex led to ITZ nanoplex-similar PXRD pattern and higher CIP payload led to CIP nanoplex-similar PXRD pattern.

6.3.2 DTA Analysis and Dissolution Determination of Dual Drug Nanoplex

6.3.2.1 DSC/TGA Patterns of Dual Drug Nanoplex

In order to further verify presence of CIP and ITZ in dual drug nanoplex, differential thermal analysis were carried out by using DSC and TGA analysis. Here nanoplex with mass ratio of ITZ over CIP equaled to 0.4, 0.8 and 1.6 were examined considering differences of payloads for both drugs among these three ratios. When $M_{ITZ/CIP}$ was 0.4, CIP payload was 50% and ITZ payload was 25%. When $M_{ITZ/CIP}$ was 0.8, CIP payload was 40% and ITZ payload was 35%, when $M_{ITZ/CIP}$ equaled to 1.6, CIP payload dropped to 30% while ITZ payload increased to 45%. CIP nanoplex, ITZ nanoplex and corresponding native drugs were compared as controlled experiments here.

Fig. 6-6 confirmed that CIP-ITZ dual drug nanoplex possessed amorphous state. Fig. 6-6 showed that peaks at 265°C and 170°C which were observed in DSC data of native CIP and native ITZ respectively were absent in DSC data of dual drug nanoplex. Furthermore, an obvious glass transition property for amorphous state was found at around 210°C - 220°C for CIP-ITZ dual drug nanoplex and this glass transition property was also found in data of both CIP nanoplex and ITZ nanoplex (Fig. 6-6).

Figure 6-6 DSC data of native CIP, native ITZ, native LMW DXT, CIP nanoplex, ITZ nanoplex and CIP-ITZ dual drug nanoplex ($M_{ITZ/CIP} = 0.4, 0.8, 1.6$).

6.3.2.2 Dissolution Tests for Dual Drug Nanoplex

Dissolution characteristics in sink condition of dual drug nanoplex were discussed in this section by comparing dissolution behaviors of CIP nanoplex and CIP-ITZ dual drug nanoplex with $M_{ITZ/CIP} = 0.4, 0.8, 1.6$. Considering lower solubility of ITZ at pH 7.4 (PBS solution) which is $1.8 \mu\text{g/mL}$, dissolution tests were carried out by only measuring CIP concentration. From Fig. 6-7, it clearly showed that dissolution rate of CIP in dual drug nanoplex diminished compared to it of native CIP and CIP nanoplex. Only 50% of CIP was released from dual drug nanoplex after 4h. for native CIP and CIP nanoplex, they reached almost 70% and 100% release of CIP respectively after 4h.

Figure 6-7 Release of CIP from native CIP crystals, CIP nanoplex and CIP-ITZ dual drug nanoplex ($M_{ITZ/CIP} = 0.4, 0.8, 1.6$)

6.3.3 Supersaturation Generation of CIP-ITZ Dual Drug Nanoplex

Supersaturation determination for dual drug nanoplex were carried in PBS solution (pH 7.4). In Fig. 6-8A, it showed that supersaturation generation ability for CIP in dual drug nanoplex was obviously lower than it of CIP nanoplex. Similar to data in Chapter 3, CIP nanoplex went a “spring and parachute” profile in which supersaturation level went as high as $8.3 \times C_{Sat}$ in 10 min and afterwards dropped to around $2 \times C_{Sat}$ in 4h resulting from recrystallization of CIP (Fig. 6-8A).

Figure 6-8 Supersaturation determinations for (A) CIP and (B) ITZ in CIP-ITZ dual drug nanoplex ($M_{\text{ITZ/CIP}} = 0.4, 0.8, 1.6$) in PBS solution.

However, CIP-ITZ dual drug nanoplex exhibited poor supersaturation generation ability. When $M_{\text{ITZ/CIP}} = 0.4$, the highest supersaturation level for CIP was only around $3 \times C_{\text{Sat}}$ in 3 min and dropped to $1 \times C_{\text{Sat}}$ soon (in twenty minutes) (Fig. 6-8A). When mass ratio of ITZ over CIP was higher (0.8, 1.6), the highest supersaturation level was further lower which was no more than $1.1 \times C_{\text{Sat}}$. This indicated that higher ITZ payload in dual drug nanoplex would result in worse supersaturation generation ability for CIP in dual drug nanoplex. When supersaturation determination was performed in PBS with presence of 2 mg/mL HPMC as crystallization inhibitor (Fig. 6-9), supersaturation generation ability for CIP in dual drug nanoplex was not improved indicating that this behavior was not resulted from fast recrystallization of CIP in solution.

Figure 6-9 Supersaturation determinations for CIP in CIP-ITZ dual drug nanoplex ($M_{ITZ/CIP} = 0.4, 0.8, 1.6$) in PBS solution with 2 mg/mL of HPMC.

Despite of lower supersaturation generation capability for CIP in dual drug nanoplex, ITZ in dual drug nanoplex exhibited typical supersaturation profiles. As it was shown in Fig. 6-8B, the highest supersaturation level could reach $184 \times C_{Sat}$ when $M_{ITZ/CIP} = 0.4$ and when mass ratio increased to 0.8 and 1.6, highest supersaturation levels dropped to $76 \times C_{Sat}$ and $35 \times C_{Sat}$ respectively. ITZ level could still be able to maintain higher level ($4 - 5 \times C_{Sat}$) for ITZ in dual drug nanoplex even after 24h (data were not shown here). Lower supersaturation generation ability for ITZ in dual drug nanoplex at higher $M_{ITZ/CIP}$ values (higher ITZ payload) was probably due to increased tendency of recrystallization for ITZ supersaturated solution. Dual drug nanoplex with higher $M_{ITZ/CIP}$ values which means higher ITZ payload would generate an ITZ solution with higher degree of

supersaturation and this would increase the driving force of recrystallization.

6.4 Conclusion

In this chapter, a dual drug nanoplex system using CIP and ITZ as model drugs was successfully developed by using a proof-of-concept method. Drugs underwent electrostatic complexation to bind to opposite charged LMW DXT chains simultaneously. Firstly, we confirmed the existence of CIP and ITZ in nanoplex by verifying unique peaks for CIP or ITZ using FTIR analysis. Afterwards, preparation conditions for making dual drug nanoplex such as AA concentration and $M_{ITZ/CIP}$ were studied. Dual drug nanoplex possessed a particle size in the range from 160 nm to 410 nm and negative zeta potential (-35 mV - -50 mV). The payload of each drug could be manipulated by simply change the mass ratio of CIP over ITZ but the total payload would always maintain at around 75%. Higher $M_{ITZ/CIP}$ value led to higher ITZ payload in dual drug nanoplex. Also PXRD data and DSC/TGA analysis confirmed amorphous state of dual drug nanoplex.

Furthermore, $M_{ITZ/CIP}$ also had effects on particle size of dual drug nanoplex and CE of both drugs. Increased $M_{ITZ/CIP}$ value would result in higher CE of both drugs, higher yield as well as larger particle size. In the formation of dual drug nanoplex, ITZ was more competitive in complexation with LMW DXT compared to CIP due to higher hydrophobicity. Lastly, CIP-ITZ dual drug nanoplex exhibited much lower supersaturation generation ability for CIP in dual drug nanoplex when

compared to it of CIP nanoplex. This behavior was much more obvious when nanoplex was prepared with higher $M_{ITZ/CIP}$ values. Studies about dual drug nanoplex system using different drugs are ongoing in lab to confirm and improve this.

CHAPTER 7. DRY POWDER INHALER FORMULATION OF CURCUMIN-CHITOSAN NANOPLEX BY SPRAY FREEZE DRYING

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7.1 Introduction

Curcumin (CUR) – a natural flavonoid isolated from turmeric plants – has been well documented to possess excellent anti-inflammatory [40] and wide-ranging antimicrobial and anticancer activities [164], [165]. As persistent inflammation of the airways represents the common symptom in many major lung diseases, such as asthma, cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD), and bronchitis, the anti-inflammatory effects of CUR in various lung diseases have been extensively investigated [42], from which the ability of CUR to alleviate airway inflammations was successfully established [43]–[45]. In addition to its anti-inflammatory effect, CUR was also shown to be able to partially correct phenotypic lung defects in CF [166], inhibit the growth of bacteria isolated from

CF patients [167], and suppress the invasion and metastasis of lung cancer cells [168], [169].

These many therapeutic benefits of CUR, however, can only be realized if effective localized delivery of CUR to the lung is achieved. Besides lung disease therapies, pulmonary delivery of CUR is also useful in enhancing the systemic bioavailability of CUR for the treatment of non-lung diseases [170], [171] as oral delivery of CUR is ineffective due to the rapid intestinal metabolism of CUR [47]. The pulmonary delivery of CUR in its native form, however, faces a major obstacle in the inherently low aqueous solubility of CUR (∞ 1 $\mu\text{g/mL}$). The low solubility limits the deliverable dosage when CUR is delivered as nebulized solution, and when CUR is delivered as dry powder aerosols, the low solubility leads to its slow dissolution upon reaching the airways lumen. As a result, pulmonary delivery of CUR in its native form would inevitably result in low bioavailability. Thus, there is a need to develop pulmonary delivery formulation of CUR that can address the issue of its low solubility.

CUR nanoparticles, where CUR is encapsulated or physically dispersed in nanoparticle carriers, have been demonstrated in numerous studies to enhance the dissolution rate of CUR attributed to their large specific surface areas [172]. CUR nanoparticles intended for pulmonary delivery in the form of CUR nanocrystals themselves [171], or CUR encapsulated in polymeric micelles [173], dequalinium

vesicles [174], and lipid vesicles [175] have been developed. However, these inhaled CUR nanoparticle formulations had a major drawback in their intricate preparation, where it required multiple steps or heavy use of organic solvents in order to obtain high CUR payloads, or in the case of CUR nanocrystals, its preparation was time and energy-intensive.

Previously, we developed a simple and highly efficient solvent-free preparation method of high-payload CUR nanoparticles in the form of amorphous CUR-chitosan nanoparticle complex (or nanoplex in short) [46]. The CUR nanoplex was prepared by electrostatically driven self-assembly complexation between CUR and the oppositely charged chitosan (CHI). The nanoplex was prepared at high CUR utilization rates (>90%) by simply mixing of the aqueous solutions of CUR and CHI under ambient condition. Importantly, the CUR nanoplex was safe as only Generally Recognized as Safe (GRAS) materials were used in its preparation [46].

In this method, ionized CUR molecules were mixed with oppositely charged CHI molecules to form soluble CUR-CHI complex (Fig. 7-1). Owing to hydrophobic interactions among the bound CUR molecules, aggregates of the CUR-CHI complex were formed after which they precipitated upon reaching a critical concentration to form the CUR nanoplex. The strong electrostatic interactions between CUR and CHI prevented the former from assembling into ordered

crystalline structures during its precipitation, resulting in the formation of amorphous CUR nanoplex. Compared to crystalline nanoparticles, the supersaturation generation afforded by the amorphous state of the nanoplex led to high apparent solubility, hence higher bioavailability [153].

Figure 7-1 Schematics of curcumin (CUR) nanoplex preparation via self-assembly complexation with chitosan, and the subsequent spray freeze drying to transform CUR nanoplex to dry powder aerosols for pulmonary delivery.

Herein we developed dry powder inhaler (DPI) formulation of the CUR nanoplex by spray freeze drying (SFD), which represents one of the most widely used DPI

formulation methods for therapeutic nanoparticles [135]. In SFD, the CUR nanoplex suspension and cryoprotective adjuvants were sprayed into a cryogen (*i.e.* liquid nitrogen), resulting in instantaneous freezing of the sprayed droplets, hence preserving the original size and spherical shape of the droplets (Fig. 7-1). The frozen droplets subsequently underwent freeze drying, where sublimation of the frozen ice crystals in the interstitial space between the nanoplex took place. As a result, spherical particles with large and low-density morphology, which are ideal for aerosolization by DPI [176], were produced by SFD.

The first objective of the present work was to investigate the effect of adjuvant formulation in SFD, specifically the ratio of L-leucine to D-mannitol, on the aerosolization efficiency of the CUR nanoplex aerosols produced. L-leucine and D-mannitol were used as the adjuvants because L-leucine has been established as an effective aerosol dispersion enhancer attributed to the surface rugosity generated in its presence [146], while D-mannitol as a sugar alcohol is well-known for its cryoprotective properties [177]. Furthermore, D-mannitol was chosen among other cryoprotectants because of its mucolytic property [178] as many lung diseases are characterized by the presence of thick stationary mucus in the airways.

The second objective of the present work was to evaluate the effect of the dry powder transformation by SFD on the supersaturation generation capability of the CUR nanoplex, and how it subsequently affected the (1) antimicrobial efficacy of

the dry powder aerosols of the CUR nanoplex against clinically derived respiratory bacterial pathogens (*i.e. Pseudomonas aeruginosa*, *Burkholderia cepacia*, *Klebsiella pneumoniae*, and *Stenotrophomonas maltophilia*), and (2) their *in vitro* cytotoxicity towards lung epithelium cells. In this regard, CUR has been shown to exhibit synergistic antibacterial effects with several antibiotics [179], thus denoting its potential application in respiratory infection therapies.

7.2 Materials and Methods

7.2.1 Materials

7.2.1.1 Materials for CUR Nanoplex Preparation and Characterization

CUR (98% purity) was purchased from Alfa Aesar (UK). Low molecular weight CHI (50–190 kDa), potassium hydroxide (KOH), glacial acetic acid, d-mannitol (Man), l-leucine (Leu), phosphate buffered saline (PBS, pH 7.4), glacial acetic acid, and ethanol were purchased from Sigma-Aldrich (Singapore).

7.2.1.2 Materials for Antimicrobial Efficacy Test

P. aeruginosa, *B. cepacia*, *K. pneumonia*, and *S. maltophilia* were provided by clinical microbiology laboratory at the National University Hospital (Singapore). Mueller Hinton broth (MHB) was purchased from BD Diagnostics (USA).

7.2.1.3 Materials for Cytotoxicity Test

A549 human alveolar basal epithelial cells were purchased from ATCC (USA).

0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) solution, penicillin-streptomycin, dimethyl sulfoxide (DMSO), and (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT, 98% purity) were purchased from Gibco (Canada), PAA Laboratories (Austria), Sigma-Aldrich (USA), and Alfa Aesar (UK), respectively. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were purchased from HyClone (Thermo Scientific, USA).

7.2.2 Methods

7.2.2.1 Preparation and Characterization of CUR Nanoplex

The CUR nanoplex was prepared at the optimal condition determined previously in Nguyen et al. [46]. Briefly, CUR was dissolved in 0.1 M KOH at 5 mg/mL and CHI was dissolved in 1.2% (v/v) aqueous acetic acid solution at 5.8 mg/mL. The CUR solution was then immediately mixed with an equal volume of the CHI solution to minimize alkaline degradation of CUR. The resultant nanoplex suspension was vortexed for 10 s followed by ultrasonication for 25 s using a probe sonicator (CV24, Sonics, USA). Afterwards, the nanoplex underwent two cycles of centrifugation ($14,000 \times g$ for 25 min) and rinsing. The CUR nanoplex was then re-suspended in deionized water for characterizations, followed by its transformation to dry powder aerosols by SFD.

The size and zeta potential of the CUR nanoplex were characterized by photon

correlation spectroscopy (PCS) after 100-fold dilution using Brookhaven 90 Plus Nanoparticle Size Analyzer (Brookhaven Instruments Corporation, USA). The morphology of the nanoplex was examined by Field Emission Scanning Electron Microscope (FESEM) (JSM-6700F, JEOL, USA) using the freeze-dried powders as samples after sputter coating them with platinum. The payload, which was defined as the ratio of the CUR mass in the nanoplex to the total mass of nanoplex, was characterized by dissolving a known mass of freeze-dried nanoplex powders in 80% (v/v) aqueous ethanol solution. The mass of CUR recovered in the ethanol solution was then quantified by UV–vis spectrophotometry (UV Mini-1240, Shimadzu, Japan) at the optimal absorbance wavelength of 423 nm.

7.2.2.2 Spray Freeze Drying of CUR Nanoplex

SFD of the CUR nanoplex suspension was carried out in a modified Büchi B-290 mini spray dryer (Büchi, Switzerland) by removing its drying chamber as described in Cheow et al. [29]. A polypropylene vessel containing 400 mL of liquid nitrogen under constant stirring at 500 RPM was placed beneath the spray dryer's two-fluid atomizer (ID = 1.5 mm). The distance between the atomizer and the polypropylene vessel was fixed at 10 cm to minimize the loss of the sprayed droplets due to impaction at the vessel wall. The atomization was performed at feed rate of 0.25 L/h, atomizing air flowrate of 300 L/h, and feed solid concentration of 5% (w/v). After the atomization, the frozen droplets were freeze dried at $-52\text{ }^{\circ}\text{C}$ and 0.05 mbar in Alpha 1–2 LD Plus freeze dryer (Martin Christ,

Germany) for 24 h. The mass ratio of the nanoplex to D-mannitol was fixed at unity at 2.5% (w/v) each, while the ratio of L-leucine to D-mannitol (*i.e.* Leu/Man) was varied between 0.10 and 0.50.

7.2.2.3 Aerosolization Efficiency

Aerosolization efficiency of the CUR nanoplex aerosols was characterized in a seven-stage Next Generation Impactor (NGI) equipped with an induction port (IP) and a pre-separator (PS) (Copley Scientific, UK). The details are the same as it mentioned in Chapter 4 which will not be described again here. The determination of aerodynamic diameter (d_A) is as Eq. (2-5).

7.2.2.4 Supersaturation Generation and Dissolution Rate

The supersaturation generation capabilities of the CUR nanoplex in its aqueous suspension and dry powder aerosol forms were evaluated by adding excess nanoplex at $5 \times$ of the saturation solubility of CUR (*i.e.* $C_{Sat} = 4.15 \mu\text{g/mL}$ in PBS at 37°C) to 8.5 mL PBS in a shaking incubator maintained at 37°C . Next, 0.2 mL aliquot was withdrawn at fixed time intervals over a 12 h period, and was immediately filtered and diluted tenfold with fresh PBS to prevent precipitation of CUR from the supersaturated solution. The CUR concentration in the aliquot (*i.e.* C) was quantified by High Performance Liquid Chromatography (HPLC) (Agilent 1100, Agilent Technologies, USA) using ZORBAX Eclipse Plus C18 column ($250 \times 4.6 \text{ mm}$, $5 \mu\text{m}$ particle size) at detection wavelength of 423 nm. Aqueous 90%

(v/v) ethanol solution was used as the mobile phase at 1 mL/min resulting in CUR retention time of approximately 2.5 min. The supersaturation level was reported as the average ratio of C to C_{Sat} from three replicates.

The rate of the supersaturation generation in the absence of crystallization was characterized from the dissolution rate of the nanoplex under sink condition. Briefly, 2 mg of the CUR nanoplex in its aqueous suspension or dry powder aerosol forms were placed inside a dialysis bag filled with 2 mL PBS. The dialysis bag was then immersed in 18 mL PBS maintained at 37 °C in a shaking incubator. The following steps are same as dissolution tests in former chapters (Chapter 3, 4, 5). The CUR concentration in the aliquot was then determined by HPLC from which the dissolution time-profile of the CUR nanoplex was constructed based on three replicates.

7.2.2.5 Aqueous Reconstitution

Aqueous reconstitution of the dry powder aerosols of the CUR nanoplex was investigated to examine its influence on the nanoplex's dissolution rate. Aqueous reconstitution characterized the ability of the SFD powders to reconstitute back to individual nanoparticles upon the dissolution of the water-soluble matrix (*i.e.* D-mannitol) in an aqueous environment. The method is same as it in Chapter 4 except that 80 µg of the dry powder aerosols were dispersed in triplicates in 20 mL deionized water for the size determination.

7.2.2.6 Antimicrobial Efficacy

Antimicrobial efficacy of the CUR nanoplex in its aqueous suspension and dry powder aerosol forms was evaluated from the minimum inhibitory concentration (MIC) against clinically derived isolates of *P. aeruginosa*, *B. cepacia*, *K. pneumonia*, and *S. maltophilia*. MIC of the native CUR was also determined to assess if the complexation with CHI had any adverse effect on the antimicrobial efficacy of CUR. The procedures of antimicrobial efficacy test are similar to those in Chapter 4. 200 μ L of the bacterial cell suspension was transferred to 96-well microplate wells. Next, 200 μ L of the CUR nanoplex was added to the cells at CUR concentrations in the range of 50–300 μ g/mL obtained by diluting the nanoplex in MHB. For the native CUR, DMSO was used to dissolve the native CUR powders prior to their addition to the bacterial cells.

7.2.2.7 Cytotoxicity

The A549 lung epithelial cells were cultivated in a 24-well plate containing DMEM supplemented with 10% (w/v) fetal bovine serum and 1% (w/v) penicillin-streptomycin solution. The cells were incubated for 24 h at 37 °C in 5% CO₂ incubator to produce cell densities of 1.0×10^5 cells/mL per well. Afterwards, the CUR nanoplex in its aqueous suspension and dry powder aerosol forms was added to 1 mL DMEM and the resultant suspension was subsequently added to the cells at two CUR concentrations (*i.e.* 0.02 and 0.04 mg/mL). Wells containing only the cells and supplemented DMEM were used as the control. The cells exposed to

the CUR nanoplex were then incubated for 24 h at 37 °C and 5% CO₂. Afterwards, the medium was replaced with serum-free DMEM containing 100 µL of 0.5% (w/v) MTT dye solution in PBS and the cells were then incubated for another 4 h.

Subsequently, 2 mL of DMSO was added to dissolve the formazan crystals produced by the enzymatic reduction of MTT by the viable cells. The microplate was then gently shaken for 15 min to fully dissolve the formazan crystals after which the amount of formazan was determined by optical density measurement at 550 nm (OD₅₅₀) using a microplate reader. The cytotoxicity was reported as a percentage of survived cells in experimental group based on two biological replicates among the control's cells. The cytotoxicity test was also performed for the native CUR, native CHI, raw D-mannitol, and raw L-leucine. The amount of CHI used in the cytotoxicity test was determined from the CHI content in the CUR nanoplex, whereas the amounts of D-mannitol and L-leucine used were determined from their respective amounts used in the SFD.

7.2.2.8 Statistical Analysis

The results of the physical characteristics (d_G , ρ_{tap}), aerosolization efficiency (ED, FPF, and MMAD), supersaturation generation, dissolution rate, aqueous reconstitution, and cytotoxicity of the dry powder aerosols of the CUR nanoplex were presented as the mean $\pm$ standard deviation of the experimental replicates. The statistical significance between the treatment groups and control was analyzed

by unpaired two-tailed Student's *t*-test at significance level of 0.05.

7.3 Results and Discussion

7.3.1 Physical Characteristics of Spray-Freeze-Dried CUR Nanoplex

Prior to SFD, the CUR nanoplex prepared by the self-assembly complexation between CUR and CHI was roughly spherical in shape as shown in the FESEM image in Fig. 7-2. The size of the CUR nanoplex from the PCS measurement was 290 ± 50 nm with zeta potential of 25 ± 4 mV and payload of $85 \pm 4\%$ (w/w). The positive zeta potential of the CUR nanoplex was attributed to the presence of excess cationic CHI on its surface [46], where its reasonably high value signified the colloidal stability of the CUR nanoplex despite the absence of conventional surfactant stabilizers in its formulation. The excess CHI on the nanoplex's surface acted as both charge and steric stabilizers that ensured its colloidal stability.

After SFD, the CUR nanoplex existed as solids that were physically dispersed in a highly spherical matrix made up of the adjuvants (*i.e.* D-mannitol and L-leucine) (Fig. 7-3A). A close-up view of the adjuvant matrix revealed its high porosity, hence low density, denoting the prominent characteristics of SFD powders (Fig. 7-3B). While the low density of the SFD powders of the CUR nanoplex made them readily aerosolized, the low density must be accompanied by a sufficiently large d_G as indicated by Eq. (2-5) to prevent the dry powder aerosols from exhibiting too small d_A ($MMAD \ll 1 \mu m$), which could cause the aerosols to pass through the

airways and subsequently exhaled without being deposited [143]. For this purpose, the atomization parameters in SFD had been determined in our preliminary study to produce powders having sufficiently large d_G in the range of 30–120 μm .

Figure 7-2 FESEM image of the CUR nanoplex prior to spray freeze drying and its physical characteristics.

In the absence of L-leucine, the SFD powders exhibited d_G in the range of $83 \pm 39 \mu\text{m}$ and ρ_{tap} of $24.6 \pm 1.3 \text{ kg/m}^3$ denoting their high porosity (Fig. 7-4). As L-leucine had been found in our previous study to influence d_G and ρ_{tap} of SFD powders prepared from nanoparticulate suspension [67], the effects of *Leu/Man* ratio on d_G and ρ_{tap} of the CUR nanoplex aerosols were investigated in the present work.

Figure 7-3 (A) FESEM images of the CUR nanoplex aerosols prepared by spray freeze drying and (B) a closed-up look on the surface reveals their high porosity.

Figure 7-4 Geometric size and density of the CUR nanoplex aerosols as a function of the ratio of L-leucine to D-mannitol (i.e. Leu/Man) used in spray freeze drying.

While d_G varied upon the addition of L-leucine, the variations did not exhibit a definite trend as a function of the Leu/Man ratio. Specifically, d_G was found to initially decrease to $56 \pm 29 \mu\text{m}$ upon the introduction of a small amount

of L-leucine at Leu/Man ratio = 0.10. Increasing the Leu/Man ratio to 0.25, however, resulted in an increase in d_G to $72 \pm 35 \mu\text{m}$, which then again decreased to $59 \pm 29 \mu\text{m}$ at Leu/Man ratio = 0.50 (Fig. 7-4). Thus, in overall, smaller mean d_G values were obtained upon the addition of L-leucine, where the decrease in d_G fluctuated within 30% of the original value, while the widths of the size distribution remained relatively unchanged. Nevertheless, the magnitudes of the d_G variations were found to be statistically insignificant ($p\text{-value} > 0.05$).

In contrast, the variations in ρ_{tap} as a function of the Leu/Man ratio showed a more definite and statistically significant trend (Fig. 7-4). Overall, ρ_{tap} was found to increase with increasing Leu/Man ratio, albeit slightly, to reach $30.6 \pm 1.3 \text{ kg/m}^3$ at the highest Leu/Man ratio investigated. Hence, the porosity of the SFD powders was gradually reduced at higher Leu/Man ratio, which was not unexpected considering the higher solid content used. To conclude, the effects of Leu/Man ratio on d_G and ρ_{tap} of the CUR nanoplex aerosols were found to be fairly insignificant.

7.3.2 Aerosolization Efficiency

The CUR nanoplex aerosols exhibited high ED ($\geq 90\%$) denoting their successful entrainment off the inhaler (Fig. 7-5). The high ED was attributed to their large d_G and low ρ_{tap} , where the large d_G reduced the interparticle surface force interactions making the dry powder aerosols less cohesive, while the low ρ_{tap} made

the aerosols remain light despite their large size. The effect of L-leucine inclusion on ED was not evident in the range of Leu/Man ratios investigated, where the variations in ED were found to remain within the experimental uncertainties (Fig. 7-5).

Figure 7-5 Aerosolization efficiency characteristics (i.e. ED, FPF, and MMAD) of the CUR nanoplex aerosols as a function of the ratio of L-leucine to D-mannitol used in spray freeze drying.

On the contrary, the L-leucine inclusion increased the FPF significantly from $27 \pm 6\%$ without L-leucine to $49 \pm 1\%$ at Leu/Man ratio = 0.25 (Fig. 7-5). The higher FPF indicated that a higher fraction of the emitted dose reached Stage 1 or higher of the NGI. In other words, fewer aerosols were deposited in the IP and PS sections of the NGI. Hence, the addition of L-leucine improved the mid-air dispersion of the CUR nanoplex aerosols after their collective entrainment off the

inhaler, resulting in fewer agglomerates of the aerosols entering the NGI. The present results thus reaffirmed the role of L-leucine as an aerosol dispersion enhancer in DPI delivery as established in numerous studies previously.

The improved aerosol dispersion upon the addition of L-leucine also led to improvement in the MMAD, where it decreased from $1.37 \pm 0.18 \mu\text{m}$ without L-leucine to $1.07 \pm 0.12 \mu\text{m}$ at Leu/Man ratio = 0.25 (Fig. 7-5). Increasing the Leu/Man ratio to 0.50, however, did not increase the FPF or decrease the MMAD further. Instead, the FPF decreased to $40 \pm 2\%$, while the increase in the MMAD to $1.16 \pm 0.06 \mu\text{m}$ was found to be statistically insignificant (Fig. 7-5). It was postulated that the continuous increase in the density of the CUR nanoplex aerosols with increasing Leu/Man ratios eventually became too large to overcome, resulting in increased deposition of the aerosols in the earlier stages of the NGI.

To conclude, the nearly 100% ED, relatively high FPF ($\approx 50\%$), and MMAD $\ll 5 \mu\text{m}$ exhibited by the CUR nanoplex aerosols at Leu/Man ratio = 0.25 signified their successful aerosolization and subsequent deposition in the targeted lung region *in vitro*, which bodes well for their successful application *in vivo*.

7.3.3 Supersaturation Generation

Prior to SFD, the CUR nanoplex suspension produced the characteristic “spring and parachute” supersaturation profile of an amorphous form upon dissolution (Fig.

7-6A), where the supersaturation level exhibited a peak at $\approx 4.6 \times$ of C_{Sat} in less than 5 min (“spring”), followed by its gradual decrease to $\approx 1.8 \times$ of C_{Sat} after 3 h (“parachute”). The supersaturation level eventually dropped to the thermodynamic saturation solubility (*i.e.* $1.0 \times C_{\text{Sat}}$) after 12 h (data not shown). In contrast, the CUR nanoplex aerosols did not produce the “spring and parachute” supersaturation profile upon dissolution (Fig. 7-6A). Instead, the supersaturation level reached a plateau at $\approx 1.5 \times$ of C_{Sat} after 5 min that eventually decreased to $1.0 \times C_{\text{Sat}}$ after 12 h (data not shown). In other words, the CUR nanoplex aerosols could only generate apparent solubility that was approximately 50% higher than the thermodynamic solubility.

Furthermore, the effect of Leu/Man ratio on the supersaturation generation of the CUR nanoplex aerosols was found to be insignificant. Therefore, only the supersaturation profile at Leu/Man ratio = 0.25 was plotted in Fig. 7-6A for clarity. Significantly, the results indicated that the supersaturation generation capability of the CUR nanoplex was compromised by the transformation to dry powder aerosols. In this regard, the diminished supersaturation generation of the CUR nanoplex aerosols was found to be caused by their significantly slower dissolution rate compared to that of the nanoplex suspension (Fig. 7-6B).

Figure 7-6 (A) Supersaturation generation and (B) dissolution rate of the CUR nanoplex aerosols in comparison to that obtained from the CUR nanoplex suspension.

Specifically, the CUR nanoplex suspension was shown to dissolve very rapidly under sink condition, resulting in more than 80% of CUR released in 5 min (Fig. 7-6B). The presence of salt in the dissolution medium provided a charge screening effect that weakened the electrostatic interactions between CUR and CHI in the nanoplex, resulting in their decomplexation and subsequently the fast release of

CUR. On the contrary, the CUR nanoplex aerosols dissolved at a considerably slower rate in which less than 20% of CUR was released within the same period (Fig. 7-6B). In fact, merely 35% of the CUR was released from the nanoplex aerosols after 3 h and 80% CUR release was only achieved after 12 h (data not shown). Not unlike the trend in the supersaturation profile, the effect of Leu/Man ratio on the dissolution rate was found to be minimal, where there were less than 5% variations in the dissolution rates obtained from different Leu/Man ratios (Fig. 7-6B).

The slow dissolution rates of the CUR nanoplex aerosols stemmed from their poor aqueous reconstitution as indicated by the high $S_{\text{Final}}/S_{\text{Initial}}$ values, which were equal to 5.2 ± 1.0 , 3.8 ± 0.2 , 10.1 ± 0.9 , and 18.5 ± 0.9 at Leu/Man ratios equal to 0 (no L-leucine), 0.10, 0.25, and 0.50, respectively. The poor aqueous reconstitution indicated that the CUR nanoplex formed irreversible aggregates during the freezing step of SFD despite the use of cryoprotectant. As a result of the aggregate formation, smaller surface areas of the nanoplex were exposed to the dissolution medium, resulting in the slow dissolution rate.

The increase in $S_{\text{Final}}/S_{\text{Initial}}$ with increasing Leu/Man ratio was caused by the increased presence of hydrophobic L-leucine, resulting in less effective dissolution of the water-soluble matrix of D-mannitol. In this regard, aqueous reconstitution of nanoparticles after freeze drying was known as a complex interplay between the

freezing rate, cryoprotectant's type, and its concentration [177]. Thus, a separate study must be conducted to determine the optimal freeze drying condition to ensure aqueous reconstitution of the CUR nanoplex aerosols.

7.3.4 Antimicrobial Activity

The MICs derived from the OD₆₀₀ measurements showed that the antimicrobial activities of CUR against the four respiratory pathogens were not compromised by either the complexation with CHI, or by the dry powder transformation by SFD (Table 7-1). The CUR nanoplex was most effective against clinical isolates of *S. maltophilia* and *B. cepacia* with MIC equal to 100 µg/mL and was less effective against *P. aeruginosa* and *K. pneumoniae* with MICs equal to 150 and 200 µg/mL, respectively (Table 7-1).

Bacteria	MIC (µg/mL)		
	Native CUR	CUR nanoplex suspension	CUR nanoplex aerosols
	(OD ₆₀₀)	(OD ₆₀₀)	(OD ₆₀₀)
<i>P. aeruginosa</i>	150 (0.056 ± 0.008)	150 (0.046 ± 0.007)	150 (0.066 ± 0.008)
<i>K. pneumoniae</i>	200 (0.020 ± 0.001)	200 (0.011 ± 0.012)	200 (0.040 ± 0.002)
<i>S. maltophilia</i>	100 (0.063 ± 0.004)	100 (0.016 ± 0.004)	100 (0.086 ± 0.006)
<i>B. cepacia</i>	100 (0.028 ± 0.017)	100 (0.070 ± 0.008)	100 (0.018 ± 0.006)

Table 7-1 MICs of the CUR nanoplex and native CUR against common respiratory pathogens.

The comparable MICs between the native CUR and the CUR nanoplex suspension indicated that CUR and CHI readily decomplexed upon addition of the nanoplex to

the bacterial suspension owed to the presence of salt from the bacterial growth medium, resulting in the release of CUR from the nanoplex. Despite their slower dissolution rates, the CUR nanoplex aerosols exhibited the same antimicrobial activities as the CUR nanoplex suspension as the 24 h incubation provided more than enough time for the CUR nanoplex aerosols to fully dissolve in the bacterial suspension medium.

7.3.5 Cytotoxicity

The CUR nanoplex suspension was found to exhibit similar cytotoxicity towards the A549 cells as the native CUR, where the cell survival rates upon exposures to 0.02 and 0.04 mg/mL of CUR were found to equal to approximately 75% and 65%, respectively (Fig. 7-7). The cytotoxicity of the CUR nanoplex suspension upon incorporation of D-mannitol and L-leucine was also similar, hence reaffirming the non-cytotoxicity of the adjuvants used. On the other hand, the presence of the CUR nanoplex aerosols resulted in slightly higher cell survival rates of $85.0 \pm 3.7\%$ and $79.2 \pm 1.1\%$ upon CUR exposures at 0.02 and 0.04 mg/mL CUR, respectively. The higher cell survival rates in the presence of the CUR nanoplex aerosols were likely attributed to their slower dissolution rates that resulted in the lower overall CUR exposures. The results thus indicated that the cytotoxicity of CUR was not adversely affected by the complexation with CHI and by the dry powder transformation.

Figure 7-7 *In vitro* cytotoxicity of the CUR nanoplex aerosols towards lung epithelium cells in comparison to that obtained from the CUR nanoplex suspension and the native CUR.

7.4 Conclusion

The present work successfully employed SFD to develop dry powder aerosol formulation of the CUR nanoplex exhibiting excellent aerosolization efficiency as characterized by $ED \geq 90\%$, $FPF \approx 50\%$, and $MMAD \approx 1.1 \mu\text{m}$. The excellent aerosolization efficiency of the CUR nanoplex aerosols was attributed to (1) their large and low-density morphology and (2) the inclusion of L-leucine as an aerosol dispersion enhancer at an optimal ratio. The CUR nanoplex aerosols, however, exhibited a diminished supersaturation generation capability in comparison to the CUR nanoplex suspension. The diminished supersaturation generation was caused by the slower dissolution of CUR from the aerosols due to the formation of irreversible aggregates of the nanoplex during the freeze-drying step of SFD,

which in turn reduced the surface areas of nanoplex available for dissolution.

Nevertheless, compared to the native CUR, the CUR nanoplex aerosols still produced apparent solubility that was about 50% higher than the thermodynamic solubility, hence signifying their dissolution enhancement capability. Despite their slower dissolution rate, the CUR nanoplex aerosols possessed the same antimicrobial activity against common respiratory pathogens as that of the CUR nanoplex suspension and the native CUR. Lastly, the CUR nanoplex aerosols did not exhibit any enhanced cytotoxicity towards the lung epithelium cells compared to the native CUR.

CHAPTER 8. IMPROVING CHARACTERISTICS OF CURCUMIN NANOPLEX VIA COMPLEXATION WITH BOVINE SERUM ALBUMIN

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8.1 Introduction

In Chapter 7, we developed amorphous CUR-chitosan nanoparticle complex (or nanoplex in short) exhibiting a high payload (> 80%) prepared by a simple, highly efficient, cost-effective method based on drug-polysaccharide complexation [46]. Most importantly, the nanoplex was capable of producing high apparent solubility of CUR upon dissolution as a result of the supersaturation generation of its amorphous state, thus making it an ideal bioavailability enhancement strategy [46]. The CUR-chitosan nanoplex, however, exhibited a tendency to agglomerate in its aqueous suspension form during short-term storage (i.e. after 6 h), hence necessitating its immediate transformation to dry powders [46].

Herein we hypothesized that the use of proteins, in place of polysaccharides, as the complexation partner for CUR could improve the colloidal stability of the resultant CUR nanoplex. Bovine serum albumin (BSA) was used as the model protein because of its low cost, biodegradability, non-toxicity, and wide acceptance for pharmaceutical uses [181]–[183]. Most importantly, several studies have reported the ability of BSA to provide excellent steric and electrostatic stabilization of nanoparticles by virtue of the presence of adsorbed protein corona on the nanoparticle's surface [48]–[50]. Furthermore, BSA has been known to possess high binding capacity for various drugs, including CUR and other flavonoids [184], [185], where CUR binding with serum albumins (including BSA) has been found to improve the chemical stability of CUR in physiological condition [186]–[188].

The schematic of the CUR-BSA nanoplex formation is presented in Fig. 8-1. The high affinity of CUR to BSA attributed to the hydrophobic binding of CUR to the hydrophobic cavities of BSA [189] led to the formation of soluble CUR-BSA complex. Depending on the preparation pH, the electrostatic binding between BSA and the oppositely charged CUR could also play a role in the CUR-BSA complex formation [187]. Similar to the particle formation mechanism in drug-polysaccharide or protein-polyelectrolyte complexation [17], [190], aggregates of the soluble CUR-BSA complex subsequently formed due to the hydrophobic interactions among the bound CUR and BSA molecules (Fig. 8-1). The aggregates of CUR-BSA complex then precipitated upon reaching a critical

concentration to produce the CUR-BSA nanoplex.

Figure 8-1 Schematic of CUR complexation with BSA driven by their hydrophobic and electrostatic interactions resulting in the formation of CUR-BSA nanoplex.

In the present work, the first objective was to investigate the effects of the two key variables in drug-protein complexation (i.e. pH and mixing ratio of BSA to CUR) on the (1) physical characteristics (i.e. size, zeta potential, payload, and amorphous state) and (2) preparation efficiency (i.e. curcumin utilization rate and production

yield) of the CUR-BSA nanoplex produced, from which the optimal preparation condition was determined. Subsequently, the second objective was to analyze and compare the (1) physical characteristics, (2) preparation efficiency, (3) colloidal stability, (4) dissolution rate, and (5) supersaturation generation capability of the CUR-BSA nanoplex prepared at the optimal condition, with those exhibited by the conventional CUR-chitosan nanoplex. In addition, we assessed whether the bioactivities of CUR were adversely affected by its complexation with BSA by using antimicrobial assay as the representative bioactivity test among the many bioactivities of CUR.

Lastly, the third objective of the present work was to equip the CUR-BSA nanoplex with modulated release functionality achieved by post-preparation thermal treatment of the nanoplex. As proteins, BSA unfolds upon heating causing the disruption of its compact helical structures [191], [192]. We hypothesized that the conformational change of BSA in the nanoplex after the thermal treatment would enable us to modulate the release of CUR from the nanoplex. For this purpose, the effect of heating on the nanoplex's structural integrity was examined first in order to determine the onset of thermal denaturation of BSA that led to the structural collapse of the nanoplex.

8.2 Materials and Methods

8.2.1 Materials

CUR ($\geq 98\%$ purity) and Mueller Hinton broth (MHB) were purchased from Alfa Aesar (USA) and BD Diagnostics (USA), respectively. BSA (MW = 69 kDa, $\geq 96\%$ purity), chitosan (CHI) (MW = 50–190 kDa, 75–85% deacetylation), potassium hydroxide (KOH), glacial acetic acid (AA), ethanol, and phosphate buffered saline (PBS) (pH 7.4) were purchased from Sigma-Aldrich (Singapore). Clinically derived strain of *P. aeruginosa* was provided by the clinical microbiology laboratory of National University Hospital (Singapore).

8.2.2 Methods

8.2.2.1 Preparation of CUR-BSA Nanoplex

Acidic CUR having pK_a of 8.4, 9.9, and 10.5 [193] was dissolved in 0.1 M KOH (pH 13) at 5 mg/mL to produce negatively charged CUR molecules. Amphoteric BSA with pI in the range of 4.8 to 5.6 [194] was dissolved in aqueous AA solution at AA concentration between 0.4% (v/v) (pH 2.95) and 1.2% (v/v) (pH 2.71) to produce BSA molecules with net positive charges. Equal volumes of the CUR and BSA solutions were then mixed immediately after their preparation to minimize alkaline degradation of CUR [193], as well as acidic degradation of BSA in strong acids ($pH \leq 2.0$) [195]. The resultant CUR-BSA nanoplex suspension was then washed by two cycles of centrifugation at $14,000 \times g$ for 25 min, followed by its re-suspension in deionized water for characterizations. Four independent replicates

from different days were used for the characterizations.

To study the pH effect, the mass ratio of BSA to CUR ($M_{\text{BSA/CUR}}$) was fixed at 1.2 (w/w), where a value > 1.0 for $M_{\text{BSA/CUR}}$ was arbitrarily chosen to ensure sufficient BSA available for complexation with CUR. Subsequently, $M_{\text{BSA/CUR}}$ was varied between 0.2 and 2.5 (w/w) at a fixed amount of CUR (i.e. 5 mg/mL) to study the effect of $M_{\text{BSA/CUR}}$ at the optimal pH. For the CUR-CHI nanoplex, the detailed preparation method had been presented in Nguyen, Yu, Kiew and Hadinoto [46] as well as in Chapter 7, hence the method was not repeated here for brevity.

The preparation efficiency was characterized by the complexation efficiency (CE) and the yield defined in Eqs. (2-1); (2-2), respectively. The details have been described in former chapters so will not be mentioned here. The mass of CUR recovered in the supernatant was quantified by UV–vis spectrophotometry (UV Mini-1240, Shimadzu, Japan) at 423 nm.

8.2.2.2 Physical Characterizations of CUR-BSA Nanoplex

The size and zeta potential of the nanoplex were characterized by photon correlation spectroscopy (PCS) using Brookhaven 90 Plus Nanoparticle Size Analyzer (Brookhaven Instruments Corporation, USA). The particle morphology was examined by Field Emission Scanning Electron Microscope (FESEM) (JSM-6700F, JEOL, USA) using the freeze-dried sample of the nanoplex sputter

coated with platinum. The payload was characterized by dispersing a known mass of the freeze-dried nanoplex in 80% (v/v) aqueous ethanol solution after which the amount of the dissolved CUR was determined by UV–vis spectrophotometry at 423 nm.

The existence of CUR in the nanoplex was verified by Fourier Transform Infrared Spectroscopy (FTIR) using Spectrum One (Perkin-Elmer, USA). The FTIR was performed between 400 and 4500 cm^{-1} at 1 cm^{-1} spectral resolution for the native CUR, native BSA, CUR-BSA nanoplex, and physical mixture of the two natives. The amorphous state of the nanoplex was assessed from its Powder X-ray Diffraction (PXRD) patterns from 5° to 70° (2 θ) with a step size of 0.02°/s using D8 Advance X-ray diffractometer equipped with Cu K α radiation (Bruker, Germany). The PXRD analysis was performed for the native CUR, native BSA, and for the CUR-BSA nanoplex 48-h after six-month storage at 25 °C and 55% relative humidity.

8.2.2.3 Dissolution Rate and Supersaturation Generation

The dissolution time-profile of the nanoplex under sink condition was reported in terms of % Dissolution defined in Eq. (8-1), where the amount of CUR recovered in the dissolution medium represented the net difference between the amount of CUR dissolved from the nanoplex and the amount of the dissolved CUR that had undergone hydrolytic degradation in PBS [196].

$$\% \text{Dissolution} = \frac{\text{Mass of CUR recovered in the dissolution medium}}{\text{Mass of CUR in the nanoplex prior to dissolution}} \times 100 \quad (8-1)$$

Briefly, 0.1 mL of the nanoplex suspension was placed inside a microtube filled with 1.9 mL PBS, where one tube was used for each time interval investigated. The nanoplex suspension in PBS was then placed in a shaking incubator at 37 °C. At fixed time interval over a 30-min-period, 0.5 mL of the nanoplex suspension was withdrawn and filtered after which the amount of the dissolved CUR was determined by High Performance Liquid Chromatography (HPLC). The HPLC analysis was performed using ZORBAX Eclipse Plus C18 column (250 × 4.6 mm, 5 μm particle size) at detection wavelength of 423 nm. Aqueous ethanol solution 90% (v/v) was used as the mobile phase at 1 mL/min to produce CUR's retention time of approximately 2.5 min.

The supersaturation generation capability of the nanoplex was characterized in triplicates by the ratio of the supersaturated concentration generated upon dissolution (C) to the thermodynamic saturation solubility of CUR (C_{Sat}) determined previously to be equal to 4.15 μg/mL [46]. The supersaturation was generated by adding the nanoplex in excess (i.e. $15 \times C_{\text{Sat}}$) to 8.5 mL PBS in a shaking incubator at 37 °C. At a fixed time interval over a 24-h-period, 0.2 mL aliquot was withdrawn, filtered, and immediately diluted tenfold with aqueous 80% (v/v) ethanol solution to prevent CUR precipitation from the supersaturated

solution. The amount of CUR in the aliquot was then determined by HPLC following the same protocols described earlier. The effect of the inclusion of hydroxypropyl methylcellulose (HPMC) as crystallization inhibitor in the dissolution medium on the supersaturation level (C/C_{Sat}) was investigated at HPMC = 2 mg/mL.

8.2.2.4 Antimicrobial Assay and Colloidal Stability

Antimicrobial activity of the CUR-BSA nanoplex was evaluated from the minimum inhibitory concentration (MIC) against clinically derived isolate of *P. aeruginosa* which have been described in former chapters. For the nanoplex prepared at the optimal pH and $M_{\text{BSA/CUR}}$, its colloidal stability during ambient storage was examined in triplicates by monitoring the changes in the size and zeta potential of the nanoplex at fixed time intervals over a 24-h-period.

8.2.2.5 Post-preparation Thermal Treatment of CUR-BSA Nanoplex

The thermal treatment was performed on the CUR-BSA nanoplex prepared at $M_{\text{BSA/CUR}}$ of 0.6, 1.0, and 2.5. Briefly, 1 mL of the nanoplex suspension was added to 9 mL of deionized water that had been preheated at an elevated temperature in the range of 65 °C to 100 °C. The resultant suspension was then incubated in a water bath at the elevated temperature for 10 min after which aliquot of the heat-treated nanoplex suspension was withdrawn for measurements of its size and zeta potential. The rest of the nanoplex suspension was then centrifuged, followed

by freeze drying of the sediments. The payload of the heat-treated nanoplex was determined using the same method described in Section 8.2.2.2. The dissolution rate of the nanoplex after the thermal treatment was characterized over a 30-min-period by the same method described in Section 8.2.2.3.

8.3 Results

8.3.1 Effects of pH at fixed $M_{BSA/CUR}$

At fixed $M_{BSA/CUR}$ of 1.2, the CUR-BSA nanoplex was successfully prepared for all the pH values investigated (i.e. pH 4.4 to 11.6) as indicated by the results of the size measurement in Fig. 8-2A. On this note, the pH displayed in Fig. 8-2A represented the pH of the mixed CUR and BSA solutions. The nanoplex exhibited the smallest size (i.e. 67 ± 2 nm) at pH 11.6 and the largest size (i.e. 103 ± 4 nm) at pH 4.9. The nanoplexes prepared at the other pH showed little size variation as a function of pH with sizes lying in the narrow range of 80 to 90 nm (Fig. 8-2A). The zeta potential of the nanoplex was also relatively constant as a function of the preparation pH in the range of -19 to -25 mV denoting its good colloidal stability (Fig. 8-2A). Significantly, the negative zeta potentials of the nanoplex after washing in a neutral pH condition indicated that BSA, which carried net negative charges above its pI [190], had a predominant presence on the nanoplex's surface as expected.

Not unlike the trends in the size and zeta potential, the payload was not greatly

affected either by the preparation pH (Fig. 8-2B). The payloads at pH 11.6, 5.1, and 4.9 were similar in magnitude at $53 \pm 7\%$, $58 \pm 4\%$, and $59 \pm 4\%$, respectively. The payloads were found to be slightly higher at $70 \pm 8\%$ and $71 \pm 9\%$, respectively, upon decreasing the pH to 4.7 and 4.4. The variation in payload, however, was found to be statistically insignificant (two-tailed Student's *t*-test at significance level of 0.05).

Figure 8-2 Effects of preparation pH on the (A) size, zeta potential; (B) payload, CE, and yield of the CUR-BSA nanoplex produced.

In terms of the preparation efficiency, the CUR-BSA nanoplex prepared at pH 11.6 exhibited low CE of $39 \pm 4\%$ and low yield of $7 \pm 1\%$ denoting a very small amount of CUR was transformed to the nanoplex (Fig. 8-2B). In contrast, at pH 5.1 to 4.4, the CE was significantly higher ranging from $92 \pm 1\%$ at pH 5.1 to $83 \pm 5\%$ at pH 4.4 (Fig. 8-2B), denoting high CUR utilization rates at acidic preparation pH. Likewise, the yield was also improved greatly to reach $62 \pm 2\%$ and $47 \pm 2\%$ at pH 5.1 and 4.4, respectively (Fig. 8-2B).

The optimal pH for the CUR-BSA nanoplex preparation was thus determined to be at pH 5.1 (i.e. AA = 0.6% v/v) based on the high CE of > 90%, high yield of > 60%, and small nanoplex size (i.e. 84 ± 6 nm) obtained under this condition. While comparable CE and yield were produced at pH 4.9, the nanoplex size was larger (> 100 nm) with a lower zeta potential rendering it inferior to the nanoplex produced at pH 5.1. To confirm that pH 5.1 represented the optimal condition, we carried out an additional run at AA = 0.5% (v/v) corresponding to the preparation pH of 5.6 (data not plotted for clarity). While the CUR-BSA nanoplex produced exhibited similar physical characteristics as the nanoplex prepared at pH 5.1 (i.e. 98 ± 1 nm, -20 ± 7 mV), the preparation efficiency was highly inferior with CE of and yield of $67 \pm 10\%$ and $35 \pm 6\%$, respectively.

The successful preparation of the CUR-BSA nanoplex at pH 5.1 was verified by the FESEM image in Fig. 8-3A, which showed the presence of nanoparticles with slightly elongated shapes having sizes < 100 nm, hence they were in agreement with the size measurements by PCS. The PXRD patterns of the CUR-BSA nanoplex after six-month storage revealed the amorphous state of the nanoplex as reflected by the appearance of broad amorphous halos, which were in contrast to the sharp crystalline peaks observed in the PXRD pattern of the native CUR (Fig. 8-3C). In this regard, the strong binding between CUR and BSA inhibited the former from assembling into ordered crystalline structures upon precipitation of the CUR-BSA complex, resulting in the amorphous state formation.

The presence of CUR in the CUR-BSA nanoplex was verified by the appearance of the characteristics bands of CUR at 1626, 1508, and 1272 cm^{-1} in the FTIR spectrum of the nanoplex (Fig. 8-3B). These bands, which also appeared in the FTIR spectra of the native CUR and the physical mixture of CUR and BSA, corresponded to the stretching vibrations of the $\text{C}=\text{C}-\text{C}_{\text{ring}}$, $\text{C}=\text{O}$, and enol $\text{C}-\text{O}$ bonds of CUR, respectively [197]. On this note, the characteristic bands of BSA were typically presented at 1656 and 1545 cm^{-1} corresponding to the $\text{C}=\text{O}$ stretching (amide I) and $\text{C}-\text{N}$ stretching coupled with $\text{N}-\text{H}$ bending (amide II), respectively [184]. The amide bands, however, were not distinctly visible in the FTIR spectra of the nanoplex and the physical mixture because they overlapped with the characteristic bands of CUR (Fig. 8-3B).

8.3.2 Effects of $M_{\text{BSA/CUR}}$ at the Optimal pH

At pH 5.1, the nanoplex size was found to be minimally affected by $M_{\text{BSA/CUR}}$ at $0.4 \leq M_{\text{BSA/CUR}} \leq 2.5$, where the sizes fell within the narrow range of 75 to 90 nm (Fig. 8-4A). The nanoplex prepared at $M_{\text{BSA/CUR}} \leq 0.4$, on the other hand, were considerably larger at 126 ± 13 nm and 116 ± 18 nm for $M_{\text{BSA/CUR}} = 0.2$ and 0.3, respectively (Fig. 8-4A). The zeta potential was also found to be relatively constant as a function of $M_{\text{BSA/CUR}}$ with a majority of the runs produced nanoplexes having zeta potentials that fell within the narrow range of -21 to -27 mV (Fig. 8-4A). The exception was for the nanoplex produced at $M_{\text{BSA/CUR}} = 0.7$ in which a higher zeta potential of -31 ± 0.4 mV was observed. In contrast, the payload exhibited a

distinct trend as a function of $M_{BSA/CUR}$, where it gradually decreased with increasing $M_{BSA/CUR}$ from $80 \pm 3\%$ at $M_{BSA/CUR} = 0.2$ to $62 \pm 5\%$ at $M_{BSA/CUR} = 1.0$, before it further decreased to $46 \pm 4\%$ at $M_{BSA/CUR} = 2.5$ (Fig. 8-4B).

Figure 8-3 (A) FESEM image, (B) FTIR spectra and (C) PXRD patterns of the CUR-BSA nanoplex prepared at the optimal pH.

The effects of $M_{BSA/CUR}$ on the preparation efficiency were only evident for $1.0 \leq M_{BSA/CUR} \leq 2.5$, where a decrease in the CE and yield with increasing $M_{BSA/CUR}$ was observed, hence suggesting that preparation at $M_{BSA/CUR} > 1.0$ was inefficient. Specifically, the CE and yield steadily decreased from $92 \pm 2\%$ and

$60 \pm 3\%$ at $M_{\text{BSA}/\text{CUR}} = 1.0$, respectively, to $46 \pm 4\%$ and $24 \pm 2\%$ at $M_{\text{BSA}/\text{CUR}} = 2.5$ (Fig. 8-4B). At $M_{\text{BSA}/\text{CUR}} < 1.0$, CE was not affected by the $M_{\text{BSA}/\text{CUR}}$ variation, where it remained at nearly 100% denoting excellent CUR utilization rates at pH 5.1 (Fig. 8-4B). Similarly, after taking into account the experimental uncertainties, the yield was fairly constant at approximately 80% at $0.2 \leq M_{\text{BSA}/\text{CUR}} \leq 0.8$, before it began to decrease at $M_{\text{BSA}/\text{CUR}} = 0.9$ to $71 \pm 3\%$ (Fig. 8-4B).

Figure 8-4 Effects of $M_{\text{BSA}/\text{CUR}}$ on the (A) size, zeta potential; (B) payload, CE, and yield of the CUR-BSA nanoplex produced.

Considering that $M_{\text{BSA}/\text{CUR}} < 0.4$ resulted in larger nanoplex and $M_{\text{BSA}/\text{CUR}} > 0.9$ led to lower preparation efficiency, the optimal $M_{\text{BSA}/\text{CUR}}$ for the CUR-BSA nanoplex preparation was thus determined to lie between 0.4 and 0.9. While there was little variation in the size, CE, and yield at $0.4 \leq M_{\text{BSA}/\text{CUR}} \leq 0.9$, the payloads of the nanoplex prepared in this $M_{\text{BSA}/\text{CUR}}$ range were noticeably lower starting at $M_{\text{BSA}/\text{CUR}} = 0.7$ (Fig. 8-4B). This suggested that the optimal $M_{\text{BSA}/\text{CUR}}$ lay between

0.4 and 0.6. Subsequently, the optimal $M_{BSA/CUR}$ was determined to be at 0.6 attributed to the slightly higher zeta potential of the nanoplex produced at this condition (Fig. 8-4B).

To conclude, the optimal preparation condition was determined to be at pH 5.1 and $M_{BSA/CUR} = 0.6$, resulting in CUR-BSA nanoplex in the size range of 87 ± 9 nm and zeta potential of -27 ± 0.8 mV with payload of $69 \pm 2\%$, where the nanoplex was prepared at nearly 100% CE (i.e. $99.8 \pm 0.03\%$) and $80 \pm 2\%$ yield. On the significances of pH and $M_{BSA/CUR}$ variations, a slight change in the pH from 5.1 to 5.6 as AA was varied from 0.6% to 0.5% (v/v) caused a significant drop in both the CE and yield. Likewise, a slight pH change from 4.9 to 4.7 as AA was varied from 0.8 to 1.0% (v/v) caused a considerable drop in the yield. In contrast, the impacts of the $M_{BSA/CUR}$ were not as significant, where varying $M_{BSA/CUR}$ from 0.2 to 2.5 (> 12-fold increase) only had an impact on the CE and yield at $M_{BSA/CUR} > 1.0$. Hence, the preparation efficiency was more sensitive to pH than $M_{BSA/CUR}$.

8.3.3 Physical Characteristics of the Optimal CUR-BSA Nanoplex Formulation

8.3.3.1 Colloidal Stability after Preparation

Aqueous suspension of the CUR-BSA nanoplex prepared at the optimal condition was found to exhibit prolonged colloidal stability after its preparation as indicated by the minimal variations in its size and zeta potential during its 24-h storage in

ambient condition owed to the absence of irreversible agglomeration (Fig. 8-5A). The size, which was equal to 87 ± 9 nm after preparation, decreased slightly to 74 ± 6 nm after 1 h, before it went back up to 82 ± 2 nm after 6 h and remained at approximately that value after 24 h. Likewise, the zeta potential fluctuated within a narrow range of -27 to -31 mV with time, and it dropped to below -25 mV only after 24 h (Fig. 8-5A).

Figure 8-5 (A) Colloidal stability of the CUR-BSA nanoplex during ambient 24-h storage; (B) dissolution time profile of the CUR-BSA nanoplex in comparison to that of the CUR-CHI nanoplex.

8.3.3.2 Antimicrobial Activity

The CUR-BSA nanoplex possessed the same antimicrobial activity against the clinically derived *P. aeruginosa* as the native CUR, where both exhibited MICs equal to $150 \mu\text{g/mL}$. This finding served as evidence that the complexation with BSA did not have any adverse effect on the bioactivity of CUR.

8.3.3.3 Dissolution Rate

The CUR-BSA nanoplex exhibited a rapid dissolution rate under a sink condition with a burst release pattern, where approximately 80% of the CUR in the nanoplex was recovered in the dissolution medium in < 1 min (Fig. 8-5B). The burst release pattern was not unexpected as it was attributed to the nano-size and amorphous state of the nanoplex. The % Dissolution reached the maximum at $86 \pm 2\%$ after 5 min, followed by its gradual decrease to $68 \pm 1\%$ after 30 min due to hydrolytic degradation of the dissolved CUR in PBS. For comparison, the % Dissolution of the CUR-CHI nanoplex reached approximately 60% after 1 min and was at the maximum at $73 \pm 5\%$ after 5 min, hence denoting its slower dissolution rate compared to the CUR-BSA nanoplex (Fig. 8-5B).

8.3.3.4 Supersaturation Generation

The CUR-BSA nanoplex exhibited the typical “spring and parachute” supersaturation profile of amorphous drug formulations, which was characterized by rapid generation of a supersaturation peak (“spring”), followed by its gradual decrease to the thermodynamic saturation solubility (“parachute”) [128]. In the absence of HPMC in the dissolution medium, the maximum supersaturation level generated by the CUR-BSA nanoplex was equal to $(9.0 \pm 0.1) \times C_{\text{Sat}}$ achieved in < 5 min (0.083 h) after dissolution (Fig. 8-6A). The supersaturation level then gradually decreased to $(4.7 \pm 0.2) \times C_{\text{Sat}}$ after 1 h due to the precipitation of CUR from the supersaturated solution (Fig. 8-6A). The supersaturation level continued

to decrease to $(2.8 \pm 0.3) \times C_{\text{Sat}}$ after 8 h before it eventually bottomed out at nearly C_{Sat} after 24 h (Fig. 8-6B).

The inclusion of HPMC in the dissolution medium greatly reduced the rate of decrease of the supersaturation level, resulting in a more prolonged supersaturation level, while maintaining the same maximum supersaturation level (Fig. 8-6). Herein the presence of HPMC not only suppressed the precipitation of CUR from the supersaturated solution, but also the Ostwald-ripening crystallization of the remaining solid phase of the nanoplex undergoing dissolution. Specifically, the supersaturation level reached the maximum at $\approx 9 \times C_{\text{Sat}}$ after 5 min, followed by its slow decrease to $(7.8 \pm 0.9) \times C_{\text{Sat}}$ after 1 h (Fig. 8-6A). The supersaturation level then continued to decrease to $(6.6 \pm 0.1) \times C_{\text{Sat}}$ and $(2.3 \pm 0.1) \times C_{\text{Sat}}$ after 8 h and 24 h, respectively (Fig. 8-6B).

Figure 8-6 Supersaturation time-profiles of the CUR-BSA nanoplex in the first (A) 1-h and (B) 24-h of dissolution in comparison with the CUR-CHI nanoplex.

For comparison, the CUR-CHI nanoplex achieved a slightly lower maximum supersaturation level at $(7.5 \pm 0.2) \times C_{\text{Sat}}$ in the absence of HPMC (Fig. 8-6). The supersaturation level then rapidly decreased to $(1.9 \pm 0.1) \times C_{\text{Sat}}$ after 1 h and bottomed out at C_{Sat} after just 2 h. The inclusion of HPMC increased the maximum supersaturation level to the level achieved by the CUR-BSA nanoplex, while also significantly slowed down its rate of decrease as expected, where the supersaturation level only decreased slightly to $(7.3 \pm 0.4) \times C_{\text{Sat}}$ after 1 h and did not reach C_{Sat} after 24 h. These results showed that while the maximum supersaturation level achieved by the CUR-BSA nanoplex and CUR-CHI nanoplex was comparable, the rate of decrease of the supersaturation level was significantly faster in the latter, regardless of whether HPMC was present or not in the dissolution medium.

8.3.4 Modulated CUR Release

8.3.4.1 Effect of Thermal Treatment on the Nanoplex's Structural Integrity

The effect of heating temperature on the payload of the CUR-BSA nanoplex after the thermal treatment was presented in Fig. 8-7A. For the nanoplex prepared at $M_{\text{BSA/CUR}} = 0.6$, the payload decreased from $69 \pm 2\%$ before heating to $46 \pm 6\%$ after heating at $65\text{ }^{\circ}\text{C}$ as the heat-induced unfolding of the BSA chains in the nanoplex caused the release of CUR molecules that were originally bound to the hydrophobic cavities of these chains. The payload continued to decrease with increasing temperature to eventually result in payload $< 20\%$ at temperatures

above 75 °C, which denoted the onset of structural collapse of the nanoplex.

Likewise, for the nanoplex prepared at $M_{BSA/CUR} = 1.0$, the payload decreased from $62 \pm 5\%$ before heating to $45 \pm 3\%$ after heating at 65 °C. The after-treatment payload, nonetheless, remained at $\approx 45\%$ for heating up to 80 °C. For the nanoplex prepared at $M_{BSA/CUR} = 2.5$, the after-treatment payload only started to decrease for heating above 80 °C, where it decreased from $48 \pm 2\%$ before heating to $< 25\%$ after heating. The onset temperatures for the structural collapse of the nanoplexes prepared at $M_{BSA/CUR} = 0.6$, 1.0, and 2.5 were thus equal to 75, 85, and 80 °C, respectively. The higher onset temperatures for the nanoplexes prepared at $M_{BSA/CUR} > 0.6$ were attributed to their lower CUR payload, hence higher BSA content, such that a higher degree of heating was needed to cause complete unfolding of the BSA chains in the nanoplex. Thermal treatments at 65 °C, 80 °C, and 80 °C were thus performed on the nanoplexes prepared at $M_{BSA/CUR} = 0.6$, 1.0, and 2.5, respectively, to produce nanoplexes having similar payloads after the thermal treatment at around 45% (Fig. 8-7A).

Next, the size and zeta potential of the heat-treated nanoplexes were characterized to investigate whether any structural changes had taken place upon heating. The size was found to only change slightly after the thermal treatment, where the size of the nanoplex prepared at $M_{BSA/CUR} = 0.6$, 1.0, and 2.5 changed from 108 ± 6 nm, 101 ± 2 nm, and 90 ± 3 nm, respectively, before heating to 83 ± 2 nm, 121 ± 21 nm,

and 74 ± 3 nm after heating (Fig. 8-7B), hence the size variations were not statistically significant. The change in the zeta potential after the thermal treatment was also statistically insignificant, where the zeta potentials remained in the narrow range of -24 to -30 mV (Fig. 8-7B). The minimal changes in the size and zeta potential of the nanoplex signified the absence of aggregation of BSA in the nanoplex that typically occurred upon protein denaturation. Hence, the thermal treatments had not adversely affected the nanoplex's structural integrity.

Figure 8-7 Effects of thermal treatment on the (A) payload; (B) size and zeta potential of the CUR-BSA nanoplex prepared at MBSA/CUR = 0.6, 1.0, and 2.5.

8.3.4.2 Effect of Thermal Treatment on the CUR Release

The dissolution time-profiles in the first 15 min of the 30-min dissolution period were shown in Fig. 8-8 to better distinguish the dissolution profiles between the non-treated and heat-treated nanoplexes. The thermal treatment had a negligible effect on the % Dissolution for the nanoplex prepared at $M_{BSA/CUR} = 0.6$ (Fig. 8-8C), whereas the heat-treated nanoplexes prepared at $M_{BSA/CUR} = 1.0$ and 2.5

exhibited faster dissolution rates than their non-heat-treated counterparts (Figs. 8-8A, B). On this note, the gradual decrease in the % Dissolution observed after 5 min was due to the aforementioned hydrolytic degradation of CUR in PBS.

More specifically, for the heat-treated nanoplex prepared at $M_{BSA/CUR} = 2.5$, the % Dissolution reached the maximum at $93 \pm 4\%$ in < 1 min compared to $67 \pm 6\%$ achieved by the non-treated nanoplex in the same period. A similar trend was observed for the nanoplex prepared at $M_{BSA/CUR} = 1.0$, however, the difference in the maximum % Dissolution between the heat-treated and non-treated nanoplexes was smaller at $90 \pm 3\%$ and $83 \pm 2\%$, respectively. The faster dissolution rate after the thermal treatment was likely caused by the unfolding of BSA chains after heating that in turn increased the exposure of the bound CUR molecules to the dissolution medium. To conclude, the effects of the thermal treatment increased in significance with increasing $M_{BSA/CUR}$ as higher $M_{BSA/CUR}$ resulted in a lower payload before the thermal treatment, hence higher BSA contents.

Figure 8-8 Dissolution time-profiles of the heat-treated and non-treated CUR-BSA nanoplexes prepared at $M_{\text{BSA/CUR}}$ of (A) 1.0, (B) 2.5 and (C) 0.6.

8.4 Discussion

8.4.1 Effects of pH

Like most proteins, the charge heterogeneity of BSA, where patches of positive and negative charges co-existed [190], enabled BSA to electrostatically interact with both positively and negatively charged species for a wide range of pH. In this study, both CUR and BSA were fully ionized with opposite charges in their respective solutions (i.e. KOH and AA, respectively) as we aimed to promote their electrostatic binding upon mixing, which together with the hydrophobic binding

led to the formation of soluble CUR-BSA complex. As the mixed solution equilibrated to the final pH, however, the degree of ionization of both CUR and BSA in the complex varied and consequently influenced the nanoplex formation process. While CUR-BSA nanoplexes having fairly similar physical characteristics (i.e. size, zeta potential, and payload) were successfully prepared independent of pH at $M_{BSA/CUR} = 1.2$, the preparation efficiency, on the contrary, exhibited a strong pH dependence.

For final pH in the range of 5.6 to 4.4, which fell within or slightly below the reported pI range of BSA, BSA essentially carried a zero net charge, while CUR also lost some of its charges as the pH fell below its pKa. Had the soluble CUR-BSA complex been predominantly held together by the electrostatic binding, the loss of charges would have led to disengagement of the complex and consequently a lack of nanoplex formation. The fact that the nanoplex remained formed at high CE (> 90%) indicated that significant disengagement of the complex did not take place. This signified the importance of the CUR-BSA hydrophobic binding in the CUR-BSA complex formation.

For final pH of 11.6, which lay above the pI of BSA and pKa of CUR, both BSA and CUR were negatively charged. The soluble complex, however, remained formed as CUR theoretically could still electrostatically bind to the positively charged patches of BSA, and the hydrophobic binding still took place. The weak

nanoplex formation, which was reflected by the low yield ($< 10\%$), however, indicated that aggregation of the CUR-BSA complex did not take place as effectively, resulting in a lack of critical mass for precipitation.

In this regard, the presence of the net negative charge of BSA in the complex was postulated to hinder the cooperative binding of CUR from taking place. Herein the cooperative binding referred to the binding of free CUR molecules to the CUR molecules already in the complex via inter-CUR hydrophobic interactions. The net negative charge of BSA exerted repulsive forces to the similarly charged CUR molecules, hence preventing the free CUR molecules from interacting with the complex. The lack of cooperative binding caused the aggregation of the complex to diminish, resulting in the weak nanoplex formation.

8.4.2 Effects of $M_{\text{BSA/CUR}}$

In addition to its pH dependence, the preparation efficiency also exhibited a strong dependence on $M_{\text{BSA/CUR}}$ at $M_{\text{BSA/CUR}} > 1.0$, where the increasing presence of excess BSA at higher $M_{\text{BSA/CUR}}$ resulted in lower CE and consequently lower yield. The same trend was observed in the preparation of CUR-CHI nanoplex in which the CE was lower at higher mixing ratio of CHI to CUR ($M_{\text{CHI/CUR}}$) [46]. The presence of an excess amount of unbound BSA chains likely hindered the cooperative binding of the free CUR molecules to the complex, resulting in the lower CE. The relatively constant CE at $M_{\text{BSA/CUR}} < 1.0$ indicated a critical

$M_{BSA/CUR}$ existed above which the cooperative binding became hindered. Importantly, the nearly 100% CE obtained at $M_{BSA/CUR} = 0.2$ indicated that only a small amount of BSA was needed to successfully form the CUR-BSA nanoplex.

Likewise, the payload also exhibited a strong dependence on $M_{BSA/CUR}$, where the payload decreased with increasing $M_{BSA/CUR}$. While the decrease in the payload at $M_{BSA/CUR} \geq 1.0$ was due to the lower CE observed at higher $M_{BSA/CUR}$, the decrease in payload at $M_{BSA/CUR} < 1.0$ occurred despite the CE remained relatively constant. This suggested that an increasing amount of BSA participated in the complexation for the same amount of CUR transformed to the nanoplex when there was an increased abundance of BSA. Thus, while only a small amount of BSA was needed to achieve nearly 100% CE for CUR, the abundant presence of BSA at higher $M_{BSA/CUR}$ caused more BSA to be transformed to the nanoplex.

8.4.3 Comparisons of CUR-BSA Nanoplex versus CUR-CHI Nanoplex

In terms of both the physical characteristics and preparation efficiency, the optimal formulation of the CUR-BSA nanoplex was found, for the most part, to be superior to that of the CUR-CHI nanoplex presented earlier in Nguyen, Yu, Kiew and Hadinoto [46]. The only aspect in which the CUR-CHI nanoplex was superior was in the payload, where the CUR-CHI nanoplex exhibited payload of $84 \pm 4\%$ [46] versus $69 \pm 2\%$ for the CUR-BSA nanoplex.

First, the CUR-BSA nanoplex (87 ± 9 nm) was significantly smaller than the CUR-CHI nanoplex (260 ± 9 nm) [46]. As shown earlier, the smaller size of the CUR-BSA nanoplex led to its faster dissolution rate owed to the larger specific surface area. The faster dissolution made the CUR-BSA nanoplex less prone to the Ostwald-ripening recrystallization of the amorphous solid phase while undergoing dissolution, which was known to be prevalent in amorphous solid dispersion exhibiting a slow dissolution rate [79]. The reduced crystallization propensity of the CUR-BSA nanoplex upon dissolution resulted in the increased supersaturation generation presented earlier, which explained for the slower rate of decrease of its supersaturation level compared to the CUR-CHI nanoplex.

Second, the CUR-BSA nanoplex exhibited higher colloidal stability than the CUR-CHI nanoplex during ambient storage, despite them having comparable zeta potentials of -27 ± 0.8 mV and 23 ± 3 mV [46], respectively. Specifically, the CUR-BSA nanoplex remained individually dispersed after 24 h compared to 6 h for the CUR-CHI nanoplex [46] hinting at the superior steric stabilizing role of BSA compared to CHI. The increased coverage of the nanoplex surface by BSA (as proteins are well-known to produce corona surrounding nanoparticles [50]) was likely responsible for the improved colloidal stability.

Third, while the preparation of both nanoplexes exhibited high CUR utilization rates with $CE > 90\%$, the yield of the CUR-BSA nanoplex preparation was

significantly higher at $80 \pm 2\%$ compared to $47 \pm 2\%$ for the CUR-CHI nanoplex [46]. The higher yield was attributed to the lower mixing ratio required to achieve the high CE for the CUR-BSA nanoplex, where the optimal $M_{BSA/CUR}$ was equal to 0.6 compared to the optimal $M_{CHI/CUR}$ equal to 1.2 [46]. The lower mixing ratio meant that the amount of BSA needed to be supplied in the feed to transform nearly 100% of the CUR into the nanoplex was less compared to that of CHI, resulting in the higher yield.

8.5 Conclusion

A bioavailability enhancement strategy of CUR was successfully developed in the form of amorphous CUR-BSA nanoplex prepared via a simple method based on drug-protein complexation. We have successfully demonstrated for the first time the self-assembled formation of drug-protein nanoparticle complex having well-defined morphology and high drug payload. The effects of two key variables in drug-protein complexation (i.e. pH and $M_{BSA/CUR}$) on the physical characteristics and preparation efficiency of the nanoplex produced were thoroughly investigated. While the effects of the preparation pH and $M_{BSA/CUR}$ on the physical characteristics of the nanoplex were found in general to be insignificant (with the exception of the payload's dependence on $M_{BSA/CUR}$), the same could not be said about the preparation efficiency. The CUR-BSA nanoplex formation was found to favor the acidic preparation pH and $M_{BSA/CUR}$ below unity as evidenced by the consistently high CE and yield observed under this condition. At the optimal

preparation condition (i.e. $\text{pH} = 5.1$ and $M_{\text{BSA/CUR}} = 0.6$), CUR-BSA nanoplex with size of ≈ 90 nm, zeta potential of ≈ -27 mV, and payload of $\approx 70\%$ were produced at nearly 100% CE and $\approx 80\%$ yield.

The amorphous state stability after six-month storage, colloidal stability during 24-h ambient storage, and antimicrobial activity of the CUR-BSA nanoplex prepared at the optimal condition were successfully established. Upon dissolution, the CUR-BSA nanoplex produced a prolonged supersaturation level at $\approx 9 \times$ of the saturation solubility that was maintained for > 4 h in the presence of HPMC, hence denoting its excellent supersaturation generation capability. We also successfully demonstrated the modulated release functionality of the CUR-BSA nanoplex achieved by thermal treatment of the nanoplex. The impact of the thermal treatment on the modified CUR release was found to increase in significance for nanoplex exhibiting high BSA contents before the thermal treatment. Most importantly, the CUR-BSA nanoplex was found to be superior in terms of the size, supersaturation generation, colloidal stability, and yield when compared to the CUR-CHI nanoplex prepared by drug-polysaccharide complexation.

CHAPTER 9. CONCLUSIONS AND RECOMMENDATIONS

9.1 Conclusions

This dissertation discussed the development of inhaled antimicrobial nanoparticle complex in terms of dry powder forms of CIP nanoplex, CUR nanoplex and development of dual drug nanoplex. As discussed in introduction and literature review chapters, inhalation therapy in dry powder forms for lung infections has numerous advantages such as less dosage administration, less side effects, portability and convenience. Thus the feasibilities of DPI forms for CIP nanoplex as well as CUR nanoplex were studied here.

Chapter 3 – 5 discussed the CIP nanoplex and its studies in DPI form using different adjuvants or drying methods. For CIP nanoplex, the length of polyelectrolyte (PE) chains in formation of CIP nanoplex affects payload and yield of nanoplex. Longer chain length of dextran results in lower payload and higher yield due to increase of inter-PE chain interaction. However, the dissolution profile which is the most important characteristic for drug formulations showed similarity for all CIP nanoplexes regardless of the length of PE chains proving less significance of PE chains on improving properties of CIP nanoplex.

When it comes to DPI delivery of CIP nanoplex, effects of different drying method and excipients on CIP nanoplex inhaled delivery were studied. No matter whether

the CIP nanoplex dried powders were prepared using spray drying or spray freeze drying method, addition of L-leucine is able to improve the aerodynamic characteristics of powders. In the comparison of two different drying method for preparing inhaled CIP nanoplex powders, SFD powders exhibited superior aerosolization efficiency to their SD counterparts. However, SD powders performed better in aqueous reconstitution than SFD powders which resulted in better mucus penetration ability in lungs.

In Chapter 5, different adjuvants were studied for the investigation of effects of drying method on supersaturation generation ability of CIP nanoplex. Compared to CIP nanoplex SD powders without adjuvants, the decreased supersaturation generation capability of CIP nanoplex which resulted from influence of drying process can be greatly improved by addition of adjuvants. Furthermore, double-adjuvant form has a better supersaturation generation ability compared to it of single-adjuvant form and it can achieve up to 70% of improvement compared to powders prepared without adjuvant. This is attributed to prolonged higher supersaturation level for double-adjuvant formulation. The crystallization inhibition capability of HPMC in double-adjuvant formulation is crucial in mitigating adverse effect of drying process on supersaturation generation capability of CIP nanoplex.

Chapter 6 investigated dual drug nanoplex system using CIP, ITZ as antimicrobial

drugs and LMW DXT as polyelectrolyte. The formed dual drug nanoplex exhibited smaller particles size (160 - 410 nm) and stable zeta potential (-35 – -50 mV) which is suitable for mucus penetration after reaching the lung system. Interestingly, the proportion of payloads of CIP and ITZ can be tuned by simply changed the mass ratio of ITZ over CIP during the preparation and the total payload was around 75% no matter how payload of each drug changed. Furthermore, ITZ was competitive in formation of dual drug nanoplex compared to CIP due to its higher hydrophobicity leading to higher CE of ITZ. The supersaturation generations of CIP and ITZ were both affected by the content of ITZ in nanoplex. Less ITZ in nanoplex resulted higher ITZ supersaturation level as well as CIP supersaturation level while more ITZ content in nanoplex will suppress CIP supersaturation level and caused faster crystallization of ITZ resulting in lower ITZ supersaturation level too. Even CIP supersaturation level was able to reach higher level with less ITZ in nanoplex, the level was significantly diminished compared to it of CIP-DXT nanoplex.

Chapter 7 and 8 mainly discussed the study of CUR nanoplex in terms of DPI form and complexation with BSA. In the study of DPI form of CUR nanoplex using spray freeze drying method, inclusion of L-leucine in the formulation also enhanced aerodynamic characteristics of CUR nanoplex SFD powders. However, the supersaturation generation capability of CUR nanoplex in SFD powder form was diminished compared to CUR nanoplex suspension which was caused by

slower dissolution rate due to formation of aggregates of nanoplex during freeze drying process. Nevertheless, CUR nanoplex SFD powder still exhibited enhanced solubility compared to CUR in native form.

As a new strategy for enhancing bioavailability of CUR, complexation of CUR with BSA was studied in Chapter 8. Compared to CUR nanoplex self-assembled with chitosan, CUR-BSA nanoplex showed superior characteristics in terms of size, supersaturation generation, colloidal stability and yield. CUR-BSA nanoplex was able to maintain a higher supersaturation level which was about 9-fold of saturation solubility for more than 4h with presence of HPMC indicating better supersaturation generation capability. The higher the content of BSA is in nanoplex, the more effects of thermal treatment has on CUR release from nanoplex.

9.2 Recommendations

Based on existing results from this dissertation, there are still some future works which are necessary and promising to be done:

- To modify the CIP-ITZ-DXT dual drug nanoplex in terms of supersaturation generation capability. From Chapter 6, it showed that supersaturation of CIP was suppressed by presence of ITZ. The highest level for CIP could only reached 3 at maximum and there was no supersaturation generation in the presence of HPMC. Develop method or strategy to minimize suppression

effect of ITZ on supersaturation generation of CIP in dual drug nanoplex and further improve solubility of ITZ (insoluble in aqueous phase) to achieve enhancement of supersaturation generation capabilities of both drugs in PBS are challenges for this study.

Another challenge is to modify structure of CIP-ITZ-DXT dual drug nanoplex. As discussed in Chapter 6, prepared nanoplex was fully dissolved in higher concentration of AA solution in payload determination. This indicates the fact that structure of nanoplex is difficult to be disrupted in mild conditions such as in PBS solution which is also attributed to incomplete release and lower supersaturation level of CIP. Hence modification of dual drug nanoplex in structure level also needs to be studied.

- To develop DPI form of CIP-ITZ-DXT dual drug nanoplex. Optimize the ideal drying conditions for preparing CIP-ITZ-DXT dual drug nanoplex dried powders with optimal aerosolization efficiency. Study the effects of drying process on supersaturation generation capability and compare effects of different drying methods on characteristics of CIP-ITZ-DXT dual drug nanoplex dried powders.

As discussed in Chapter 5, drying processes have negative effects on the supersaturation generation capability of nanoplex. DPI form of dual drug

nanoplex will further affect supersaturation generation ability of CIP in nanoplex which is already suppressed by ITZ in nanoplex. It is important to find suitable excipients that can mitigate this negative effect brought by drying process. Moreover, reconstitution ability of nanoplex in DPI form should also be considered because nanoplex tends to form aggregates during drying process which has influence on mucus penetration of nanoplex.

- To develop DPI form of CUR-BSA nanoplex. Optimize the drying conditions for CUR-BSA nanoplex dried powders. Investigate the effects of different drying methods on modified release of CUR from dried powders by denaturing BSA in nanoplex during different drying process. As thermally-sensitive component, denaturation of BSA during drying process is inevitable. Maintenance of CUR-BSA nanoplex structure and prevent CUR leakage in cryo-environment during spray freeze drying or high-temperature environment during spray drying are crucial in studies. Furthermore, comparison of supersaturation generation capability of CUR-BSA nanoplex dried powders with optimal drying conditions and different BSA contents is important for further studies.

CHAPTER 10. LIST OF PUBLICATIONS

H. Yu and K. Hadinoto, “Impacts of dextran sulfate’s chain length on the characteristics of its self-assembled colloidal complex formed with amphiphilic small-molecule drug,” *Int. J. Biol. Macromol.*, vol. 103, pp. 493–500, 2017

H. Yu, M.-H. Nguyen, W. S. Cheow, and K. Hadinoto, “A new bioavailability enhancement strategy of curcumin via self-assembly nano-complexation of curcumin and bovine serum albumin,” *Mater. Sci. Eng. C*, vol. 75, pp. 25–33, 2017.

H. Yu, T. T. Tran, J. Teo, and K. Hadinoto, “Dry powder aerosols of curcumin-chitosan nanoparticle complex prepared by spray freeze drying and their antimicrobial efficacy against common respiratory bacterial pathogens,” *Colloids Surfaces A Physicochem. Eng. Asp.*, vol. 504, pp. 34–42, 2016.

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CHAPTER 11. REFERENCES

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