EFFECT OF STORAGE HUMIDITY ON PHYSICAL STABILITY AND AEROSOL PERFORMANCE OF SPRAY-DRIED DRY POWDER INHALER FORMULATIONS

by

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To my loving grandparents, Krishna Shetty for being there with me in spirit and Ratna Shetty for her blessings

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LIST OF ABBREVIATIONS

AFM	Atomic force microscopy
Cipro	Ciprofloxacin hydrochloride monohydrate
DPI	Dry powder inhaler
DSC	Differential scanning calorimetry
DVS	Dynamic vapor sorption
ED	Emitted dose
FPF	Fine particle fraction
FTIR	Fourier transform infrared spectroscopy
Lac	Lactose
Leu	L-leucine
Man	Mannitol
MSLI	Multi-stage liquid impinger
PXRD	Powder X-ray diffractometer
RH	Relative humidity
RMS	Root mean square
SEM	Scanning electron microscopy
Suc	Sucrose
Tre	Trehalose
XPS	X-ray photoelectron spectroscopy

ABSTRACT

Author: Shetty, Nivedita. Ph.D.
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Dry Powder inhalers (DPIs) have been one of the most promising developments in pulmonary drug delivery systems. In general, DPIs are more effective than systemic administrations and convenient to use. However, delivering high-dose antibiotics through a DPI is still a challenge because high powder load may need a very large inhaler or increase the incidence of local adverse effects. Spray drying has been increasingly applied to produce DPI formulations for high-dose antibiotics; nevertheless, many spray-dried particles are amorphous and physically unstable during storage, particularly under the humid environment.

My research focuses on addressing critical challenges in physical stability of DPIs for spray-dried high-dose antibiotics. The effects of moisture-induced crystallization on physical stability and aerosol performance of spray-dried amorphous Ciprofloxacin DPI formulations stored at different humidity conditions were studied. Our study not only provided a mechanistic understanding in the impact of crystallization on aerosol performance but also developed novel approaches for improving stability of spray-dried formulations used in DPI.

Our work has shown that recrystallization of amorphous spray-dried Ciprofloxacin led to significant changes in aerosol performance of DPIs upon storage, which cause critical quality and safety concerns. These challenges have been solved through co-spray-drying Ciprofloxacin

with either excipient such as leucine or synergistic antibiotic like Colistin. Co-spray-drying Ciprofloxacin with Colistin not only improved physical and aerosol stability but also enhanced antibacterial activity which is a great advantage for treating ‘difficult to cure’ respiratory infections caused by multidrug resistant bacteria.

My research work is a sincere effort to maximize the utility and efficacy of high-dose DPI, an effective delivery tool for treating severe resistant bacterial respiratory infections.

CHAPTER 1. INTRODUCTION

1.1 Background

Lower respiratory tract infections (LRIs) continue to be a major health problem and a leading cause of illness and death. In 2015, LRIs caused 2.74 million deaths worldwide [1-3]. Inability to deliver the adequate concentration of antibiotics to the lungs and increasing incidence of multidrug resistant pathogens involved in LRIs have made the treatment a grand healthcare challenge.

In addition, controlling airway infections caused by virulent organisms such *Pseudomonas* in conditions like cystic fibrosis (CF) is a herculean task. *Pseudomonas aeruginosa* was also found to be highly prevalent among patients with ventilator-associated pneumonia (VAP) and hospital-acquired pneumonia (HAP), making treatment highly challenging [4]. Over the last decade newer treatment strategies have been tried to counter the twin problems of treating lower respiratory tract infections without causing systemic toxicity, e.g. delivering higher concentrations to the site of infections and overcoming the problem of antimicrobial resistance.

Inhalation therapies have gained popularity in treating LRIs because drugs can be delivered directly to the infection sites in the lungs with minimum systemic exposure [5]. The ability to deliver the active drugs direct to the site of action, rapid onset of action, better therapeutic efficacy and reduced systemic adverse effects have made these pulmonary drug delivery systems popular [5, 6]. Inhaled formulations and pulmonary drug delivery systems are successful in delivering drugs for treating both local and systemic diseases [7]. Asthma, cystic fibrosis and chronic obstructive pulmonary disease (COPD) are primarily being treated using inhalation

therapy [8, 9]. Dry powder inhalers (DPI) have been one of the most preferred inhaler devices because they can deliver high-dose of antibiotics directly to the lungs as a convenient administration with minimized systemic adverse effects caused by high plasma concentration of drugs [7]. DPIs are portable and easy to use, which are patient friendly [5, 10]. Furthermore, many drugs are more chemically stable in the powder form than the liquid form.

However, dry powder inhalation has its own challenges for antibiotic delivery such as coughing or throat infection due to deposition of the high-dose powder in the oropharynx by low efficiency inhaler [5]. For antibiotic therapies, the doses are usually much higher than the DPIs for asthma, ranging from 20 – 150 mg. Particle engineering is necessary to develop DPIs for high-dose antibiotics with relatively high aerosol efficiency. Success of the DPIs depends on the stability and aerodynamic behavior of the antibiotic particle – which is affected by powder properties such size, shape, density, crystallinity/ polymorphism, surface roughness, inter-particulate forces, etc (Figure 1-1). Hence, there is a critical need to optimize various parameters of DPI formulations by particle engineering techniques.

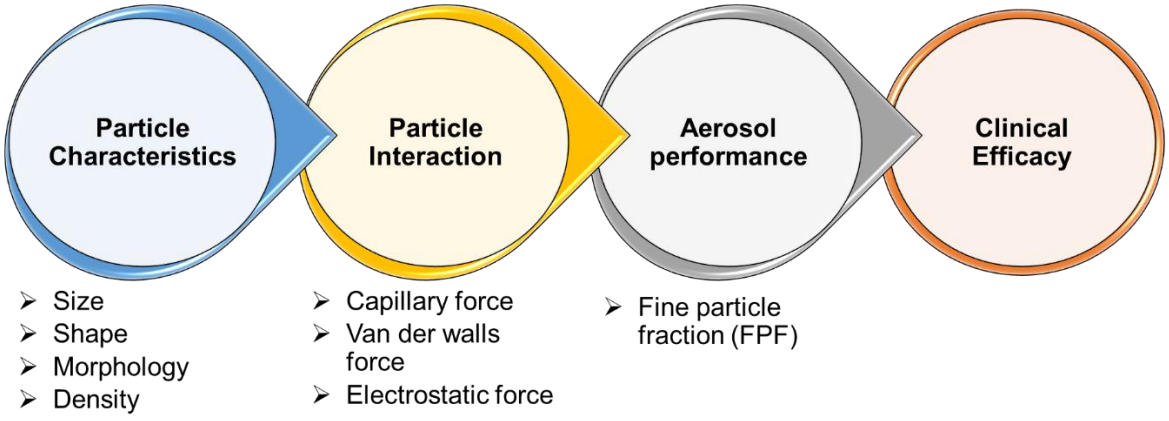


Figure 1-1: Factors influencing DPIs performance

Spray-drying is popular for producing DPI formulations as it enables engineering of drug particles; however, many spray-dried compounds are amorphous in nature and physically unstable. It has been observed that spray-dried amorphous powder of different material tends to crystallize at varying rates depending on the relative humidity (RH) and molecular weight [11]. Linna Wu et al. reported that the spray-dried amorphous lactose particles, a carrier for DPI formulations, tend to recrystallize when stored at the humidity > 32% [12]. Costantino et al. observed a drastic decrease in aerosol performance of a pharmaceutical protein rhuMabE25 (Recombinant humanized anti-IgE monoclonal antibody) due to crystallization of the excipient mannitol from the amorphous co-spray-died formulation upon storage [13]. However, there is a lack of studies that systematically examine the impact of crystallization of spray-dried drug particles on aerosol performance upon storage at various humidity conditions. Such significant changes in aerosol performance of DPIs upon storage may cause critical quality concerns in product stability. Therefore, there is a strong need to understand the mechanisms of such impact of crystallization on aerosol performance to ensure the quality and stability of the DPI products.

Ciprofloxacin, a broad spectrum antibacterial drug often used for the treatment of lower respiratory infections, was chosen as the model compound for our current study as it showed a tendency of crystallization upon storage post spray-drying in the literature [14, 15] and in our preliminary studies. Ciprofloxacin is a fluoroquinolone antibiotic with antimicrobial activities against *Pseudomonas aeruginosa* and *E.coli* [16]. It has been widely used for treatment of LRIs as oral dosage forms [17]. Commercial products of Ciprofloxacin DPIs are under the development with a promise to improve therapeutic efficacy and reduced systemic toxicity [18-

20]. **Our study has systematically examined the impact of crystallization of spray-dried drug particles on aerosol performance upon storage at different RHs.**

1.2 Hypothesis

The hypothesis of this thesis is

Recrystallization changes aerosol performance of amorphous spray-dried active pharmaceuticals for inhalation upon storage at humid environment.

1.3 Specific aims

Specific aims of this thesis are:

1. To determine change in solid state properties of spray-dried Ciprofloxacin dry powder inhaler formulation upon storage at humid conditions and its impact on aerosol performance.
2. To examine the effects of common excipients on physical stability and aerosolization of spray-dried DPI formulations of Ciprofloxacin.
3. To develop spray-dried combinational DPI formulations of Ciprofloxacin and Colistin with optimum aerosol performance and improved physical stability.

1.4 Innovation

This study proposes a novel solution to overcome physical instability associated with spray-dried amorphous Ciprofloxacin. Physical instability of spray-dried Ciprofloxacin hydrochloride is a critical quality concern for a safe and effective medication. Through this project, we will not only understand this problem, but also offer an innovative solution for preventing crystallization-induced instability of spray-dried powders of Ciprofloxacin, through co-spray-drying it with excipients or synergistic antibiotics of Colistin. The additional advantage of this novel approach through combinational drug formulations would be a better cure or control of respiratory tract infections associated with multidrug resistant bacteria, through synergistic activity of multiple drugs. Systemic administrations of combinational antibiotics may not result in synergy due to different pharmacokinetics (PK) and pharmacodynamics (PD) behavior of two or more drugs. We expect to incorporate synergistic antibiotics in a single particle which will ensure the simultaneous delivery of combinations to the same infection's sites, which will maximize the antimicrobial activities.

CHAPTER 2. LITERATURE REVIEW

*will be submitted for publishing to journal of Expert Opinion in Drug Delivery

2.1 Inhalation therapy

"*The only possible way of applying medicines directly to the lung is through the windpipe*"- This was the first acknowledgement of the feasibility of inhalation therapy for respiratory diseases by Philip Stern in 1764 [21]. At that time air was drawn from infusions of plants and other ingredients leading to development of ceramic inhalers. In the initial stages therapeutic aerosols were mainly prepared by the physicians and there was no commercial or large-scale manufacturing activity. But enhanced manufacturing capabilities and increased technical discoveries with the dawn of industrial revolution in 1760 totally changed this scenario. It paved the way for significant advances in the delivery of therapeutic aerosols [22]. Newer techniques were developed enhancing the safety and efficacy of these therapeutic entities. Dramatic improvements were made in the designs. Novel delivery systems were introduced such as nebulizers and 'early' dry powder inhalers. The real modern era of pharmaceutical aerosols began in 1950s [23]. Over a period of decades this inhalation route of administration has evolved and is useful to treat respiratory diseases by enabling local delivery of drugs and biopharmaceuticals. Continuous attempts have been made to improve the interaction between the formulation, the inhaler device, and the patient for a successful pulmonary administration [24]. It is a very ancient method of delivering drugs and become a mainstay for respiratory disease treatment in the 20^{th} century [6].

Pulmonary drug delivery has gained popularity for local delivery of drugs to the lungs over systemic administrations. Drugs delivered by the pulmonary route can bypass the first pass metabolism. Also the enzymatic activity in the lungs in lower as compared to gastrointestinal tract [25]. Since pulmonary drug delivery system offers the advantages of delivering drugs directly to the disease sites in the lungs, it has been explored extensively in the recent years for the treatment of various respiratory tract diseases such as chronic obstructive pulmonary disorder (COPD), asthma, pneumonia and chronic pulmonary infections. Inhalation also offers a non-invasive route for delivery of therapeutic drugs to treat systemic diseases [26]. Pulmonary drug delivery has become one of the most accepted systems for respiratory disorders [27].

Inhalation medicines are known as “complex products” as they consist of not only formulations but also a device. Advancement in the device and particle technology over the past decade has made the inhaler easier to use and the delivery of drugs to the lungs more efficient [5]. The most commonly used devices for pulmonary drug delivery are nebulizers, metered dose inhaler (MDIs) and dry powder inhaler (DPIs) [28-30].

2.2 Inhalation Devices

2.2.1 Nebulizer

The milestone in the advancement of therapeutic aerosols was the invention of devices that convert a medicated liquid to fine droplets for inhalation. Nebulizer devices contain a system which facilitates removal of coarse droplets from the air steam and enables aerosols to be deposited in the lungs. These devices used a variety of mechanisms for providing the pressurized airflow to atomize the liquid. Early nebulizer systems with baffles for removing the coarse droplets were described in 1862 by the German physician, L. Waldenburg, and Solis-Cohenin. It

is worth noting that the nebulizer technology had advanced from being a primitive simple set-up to commercial device for effective treatment of lung diseases during the period between 1850 and 1950 [23, 24].

In simple words, nebulizers generate an inhalable drug aerosol from a solution or suspension. They are used in the treatment of respiratory diseases as asthma, COPD and cystic fibrosis (CF) [5, 31]. Jet nebulizer which is the most common type of nebulizer used today makes use of compressed gas for generating aerosols from the liquid medicament (Figure 2-1). Though relatively inexpensive, there are certain disadvantages of jet nebulizers such as long treatment time, wastage of expensive medications due to high residual volume; the air compressors used in these nebulizers are bulky and noisy [5]. More recently introduced vibrating-mesh nebulizers takes care of most of the pitfalls of these older version of nebulizers as they are more portable and free from excessive noise [31]. They are found to be more efficient in delivering drugs to the lungs. But on the flip side, they are more expensive [5].

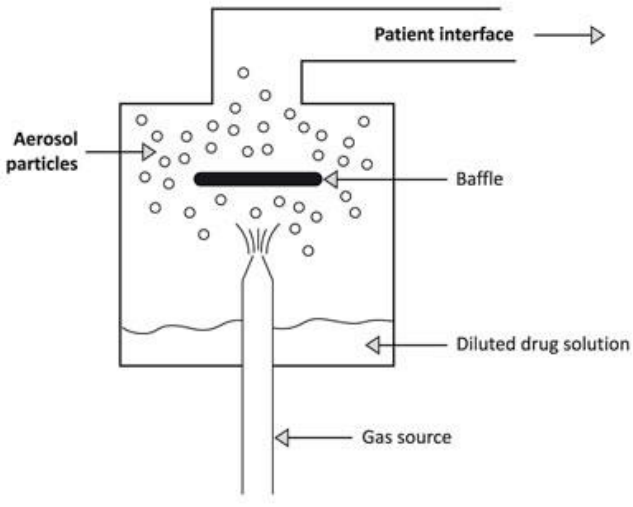


Figure 2-1**:** Schematic of a Jet Nebulizer [32].

2.2.2 Pressurized metered-dose inhaler (pMDI)

Pressurized metered-dose inhaler (pMDI) are one of the most popular inhaler devices used for the treatment of asthma and COPD [33]. Introduction of the first pressurized metered dose inhaler in 1956 by Riker Laboratories Inc., marked milestone as the beginning of a modern aerosol era. The

pMDI was the first truly portable and convenient inhaler which quickly gained widespread acceptance [24].

pMDI consist of a canister in which the drug is present in solution or suspension along with lubricants and propellant (Figure 2-2) [33]. The canister is lodged upside down in a plastic support. The propellant gas is kept in the liquid state with the help of canisters which endure high pressure. They are usually made up of inert materials like plastic, stainless steel, glass and aluminum etc. By pressing the bottom of the canister which is housed upside down in a plastic support, a pre-specified drug dose is released. To reduce particle aggregation and lubricate the delivery valve, surface-active agents such as sorbitan trioleate is added. pMDIs also consists of metering valve, actuator and a mouthpiece (Figure 2). When the device is actuated the metering valve helps in delivering precise aerosol amount. The propellant from the metering chamber is released into the actuator which consist of a spray nozzle (actuator orifice) and an expansion chamber where it partially volatizes due to the decrease in pressure. Metered drug-propellant mixture is released from the chamber as an aerosol when the canister is pressed by the patient for dose actuation. Newer actuators are also equipped with a dose counters [8, 34].

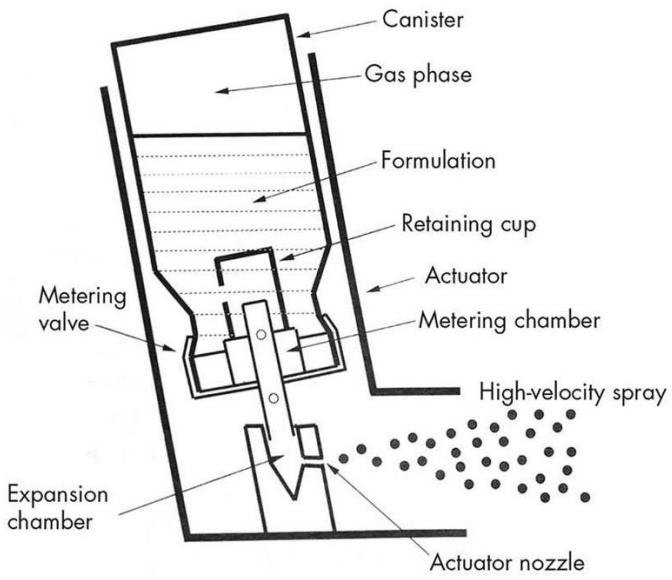


Figure 2-2: Pressurized metered dose inhaler with all its component parts [35].

2.2.2.1 Breath-actuated devices

Need for a breath-actuated pMDI became apparent it was realized that some patients had difficulty synchronizing the release of a dose from an pMDI with inhalation [36]. Autohaler™ was the first breath-actuated pMDI developed in early1970s [33, 37]. The Autohaler was a rugged pocket-sized device that patients found more convenient and simpler to use. This was followed by Duohaler (isoproterenol hydrochloride and phenylephrine bitartrate) and Iso-Autohaler (isoproterenol). Many other designs have been marketed since then. Though these actuated pMDIs helped the patients with poor co-ordination to an extent, there was definitely a need for simpler, better and cost-effective device. I-Breath coordination cap with a flexible coordination cap designed to fit onto a conventional pMDI is a good attempt in this direction [37]. On pressing the canister, the, multiple airtight sealing slits in the coordination cap are forced to open by compression, allowing airflow during patient inhalation. Clinical studies have proved that I-Cap provided ease and convenience to the patients as compared to earlier devices

[5, 24].

2.2.2.2 Add-on devices

Aerosols generated by pMDI have a high velocity, leading to loss of drugs in patients' oropharyngeal regions. In addition, elderly people and children may have difficulty in properly coordinating device actuation and inhalation. This problem can be addressed by adding a spacer [8, 38, 39]. Spacer device is available either as a tube or as a holding chamber with a port at one end and contains a mask at the other end. The drug is dispensed into the spacer by the patient and inhaled by breathing normally through the mask. Hence there is no need for patient coordination between actuation of the pMDI and inhalation of the aerosol [34]. Thus, the drugs can be inhaled comfortably by the patients when add-on devices for pMDIs such as spacers and valve holding chambers are used.

One of the drawbacks associated with the use spacers made up of plastic material is the build-up of static charges on the spacer wall, which decreases the drug delivery efficiency. Antistatic plastics (AeroChamber Plus®, Trudell) and metal valve holding chambers (Vortex®, Pari) have helped in overcoming this problem and improved their performances of pMDIs [5, 40]. Another challenge for industrial development and manufacturing of pMDIs is related to the chlorofluorocarbons (CFCs) propellants. Molina and Rowland in an article published in 1974 demonstrated that on exposure to sunlight CFC propellants break down and release chlorine radicals which has an adverse impact on ozone layer [41]. In 1987, the Montreal Protocol was signed and called for the elimination of CFC propellants and January, 1996 was the month for implementation of the Protocol as agreed upon by the members. This has led to replacement CFC with hydrofluoroalkanes (HFAs) propellants (or the even more environmentally-friendly

hydrofluoroolefins (HFOs) [5, 24].

2.2.3 Dry Powder Inhalers (DPIs)

Dry powder inhaler (DPI) heralded a new era in the innovation history of inhalers. They represented for a simple, handy and effective mode of delivering drugs in the form of powders through aerosols directly to the lungs (Figure 2-3). The immense popularity and acceptance of DPIs are because they are easy to use, portable, cost effective and the active ingredients in the solid formulation are chemically more stable than the liquid counterparts [5]. Alfred Newton, in 1864, patented an inhaling apparatus for delivery of dry powder medications. This device probably was the earliest recorded dry powder inhaler. He made twin observations which still applies to dry powder inhalers i.e. the powder needed to be fine, and kept dry [42].

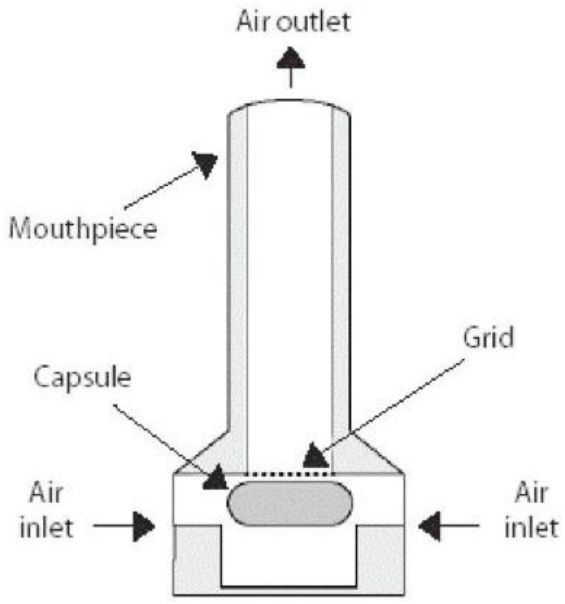


Figure 2-3: Diagram of a dry powder inhaler device (Aerolizer)

As shown in Figure 2-3, DPIs consist of a drug formulation and a device. Usually a dry powder inhaler (DPI) is a breath-activated device. The drug is micronized, present in the solid form and aerosolized into fine particles at a certain flow rate. The drug powders are usually loaded in either a capsule or blister in which holes are made prior to use. DPI powder formulation essentially consist of micronized drug either alone or with a carrier in the dry powder form. They are usually prepared using spray-drying or milling to ensure the fine aerodynamic particle sizes. In comparison to wet formulations used in case of pMDIs and nebulizers the dry powder inhalers are less likely to encounter microbiological contaminations. No propellants are needed for DPIs [43] but passive DPIs requires an adequate inspiratory flow for drug delivery [44, 45].

Dry powder inhalers (DPIs) has attracted increasing attention among all inhaler devices as they are easy to carry and use, and most drugs are more stable in the solid form [46, 47]. DPI make use of the patients own inspiration to generate powder aerosols; however, this could result in inconsistent flow rate and variable dose delivery [48]. The deposition of these fine powders in the deeper lungs depends upon the interactions between particles, lung deposition mechanisms and dispersion forces [5]. There is a need to formulate powders which are chemically stable and develop a metering system which can deliver a fixed dose of the powder to ensure efficient inhalation therapy using DPIs [48]. Thus, the powder formulation and the type of inhaler device has a great influence on the aerosol performance of the DPIs [5].

2.2.3.1 Devices

Passive devices based on traditional dispersion mechanisms have been the mainstream for aerosolizing powder formulations. The flow and dispersion of powders from passive devices

depend on the energy generated by patient inspiratory flow rates. The dose delivery does not require coordination with patients' inhalation, which is an advantage for passive devices. For passive device the dispersion mechanisms include air-turbulence, collision and acceleration [49, 50]. By increasing air-turbulence and particulate collisions, powder deagglomeration or drug-carrier detachment can be enhanced [5]. A marketed example of passive DPI device is Conix™ which enables powder deagglomeration by generating a high energy vortex using reverse flow cyclone technology [51]. However, the aerosolization performance of many passive devices suffers the dependence on the airflow [5], such as for elderly and children. Passive DPIs are not prescribed to patients aged below 6 years; leaving nebulization the only choice for the younger children. This problem could to some extent mitigated by using "Active' devices which utilize the energy provided externally such as compressed air (e.g. Exubera® and Aspirair®), electrical vibration (e.g. MicroDose), and heat (e.g. Staccato®) [5]. Yet another area where the active devices could be preferred is in the treatment of critically ill patients as these patients are unable to generate the required airflow to disperse the powder from passive inhalers. So far, active devices are not as popular as passive ones due to higher cost and reduced portability.

Based on dose numbers stored in the device, dry powder inhalers have been classified into 2 types: unit dose and multi-dose (Figure 2-4). Anti-asthma drugs are delivered using multi-dose inhaler as usually they should be taken for some days and the dose is low for such drugs. On the other-hand inhaled vaccinations which are administered less frequently through inhalation are administered using unit-dose DPI [5]. The single-unit dose inhaler has been used in the early DPIs such as the Spinhaler and Rotahaler. These inhalers essentially consist of capsules filled with micronized drug powder and the capsules are perforated in order for the powders to be

inhaled [48]. Over the years there has been significant improvements in DPIs with respect to design, functionality and ease of use.

Multiunit dose device contains pre-metered doses which are stored in individual protective packages such as blisters, disks, cartridges, or dimpled tapes (Advair/Seretide Diskus, GlaxoSmithKline, Research Triangle Park, NC). When the patient opened the mouthpiece cover of the device, a mechanism pierced the blister containing individual dose, thus making the powder available for inhalation. The Diskhaler used disks with 4-8 individual doses each dose separated in a foil blister [52]. “Multi reservoir system”, as the name indicates, contain the bulk powder formulation in a multidose reservoir, from which individual dose is metered, and dispensed by a built-in mechanism. In case of DPI devices like the Turbohaler™, the pure drug is contained in a reservoir sufficient to deliver up to 200 doses and had a mechanism which could deliver pre-determined single dose with every operation [53].

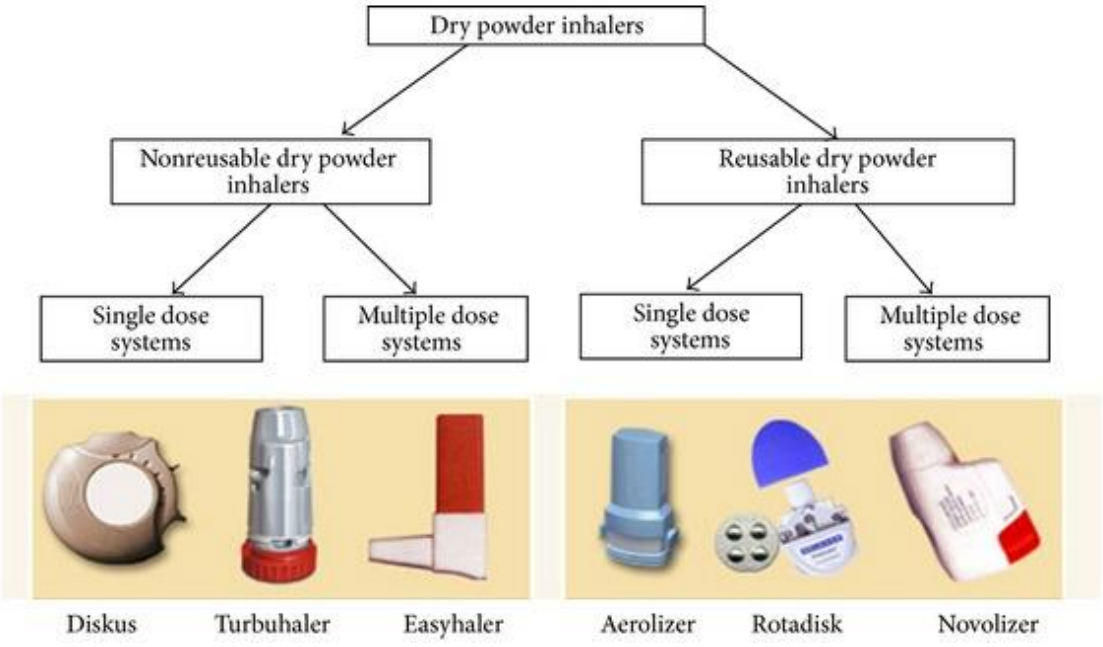


Figure 2-4: Types of dry powder inhalers [54].

There is a need to have ‘Disposable’ DPI devices for treating patients suffering from infectious diseases. This could minimize the transmission of viral, bacterial and protozoal infections from being transmitted from one person to another through common use of the devices such as DPI. The delivery of drug such as laninamivir, a novel neuramidase inhibitor used in the treatment of influenza A and B virus infections was enabled using disposable single-use DPI device such as TwinCaps® inhaler (Hovione, Loures, Portugal) [55]. Such disposable DPIs could replace reusable DPIs in patients suffering from other infectious airway diseases like cystic fibrosis-related *Pseudomonas* infections or pulmonary aspergillosis[56]. Additionally, vaccines could be delivered using disposable DPIs because only one or two doses are needed [57]. The disposable DPIs differ from the multi-dose counter parts as the operation procedure is simpler and cost can be lower. For example, the TwinCaps® has a simple design, with a main inhaler body and a pre-filled powder reservoir chamber. The Twincer™ is another example of a disposable DPI device consisting of 3 plastic plates easy to assemble and used to deliver Colistin for treating chronic infections in CF patients. Aespira's resQhaler™ is also a recently marketed disposable DPI device for single use based on the ActiveMesh™ technology [5].

In the last two decades, particle engineering techniques such as spray-drying, lyophilization, super critical fluid technology etc. have been employed to develop DPI formulations [27]. The effects of physico-chemical properties of particulate systems such as particle size, density, porosity, surface morphology, inter-particle force, surface energy etc. on the aerosol performance of DPIs have been extensively studied [58-61]. However, the solid-state characteristics and physical stability of the powder formulation in the DPIs seem overlooked; though they are also

critical to the quality and efficacy of the inhalation products. Here we provide a review on the physical stability of DPI formulations and its importance to the quality of DPIs.

2.3 Formulations

Therapeutic efficacy of dry powder inhalation technique depends on both the device and the powder formulation. Thus, careful considerations should be given to both the device and formulation properties. Dry powder inhaler formulation consists of either the drug alone or addition of a carrier. For the purpose of treating lower respiratory infections the aerodynamic particle size should preferably be between 1-5 μm. Strong cohesive and adhesive forces might be more prevalent at these small sizes than the dispersive forces, resulting in undispersed agglomerates. Thus, it is necessary that the particles are well dispersed and fluidized prior to entering the respiratory airways. In addition, satisfactory flowability is critical to ensure smooth manufacturing processes such as conveying and filling. To reduce the effects of such interparticulate forces and particularly to improve powder flow, carrier particles such as lactose are generally mixed with micronized drug in DPI formulations [62]. Better dose uniformity can be achieved by addition of excipients as the powder mass can be accurately metered.

In the early 1990s scientists discovered that carrier properties could have influences on the aerosol performance [63]. Improvements in powder dispersion was obtained by increasing carrier rugosity or adding fine particle lactose [64].However, addition of large amount of carrier may not be suitable for high-dose antibiotics (e.g. drug dose > 100 mg). In such cases, specialized particle engineering techniques have been sought to produce DPI formulations with improved flow and fluidization properties, without use of carriers [5]. Porous particles were formed using the PulmoSphere technology (by emulsion spray-drying) where the particles had a very low density

and relatively high aerosol performance [65, 66]. A commercial example is TOBI Podhaler that the aerosol performance of the drug, tobramycin, was less variable at varying temperature, relative humidity and air-flow rates [5]. Dispersion of dry powders was also enhanced by co-spray-drying two high dose drugs such as Colistin and rifampicin which produced wrinkled particles and got protected against moisture [67]. Another effective formulation approach to reduce cohesiveness between particles and improve aerosol performance was to dry coat the drugs with magnesium stearate [68]. Thus, optimizing formulation rather than device could also be beneficial for DPI performance.

Mathematically the inhalation efficiency can be calculated using the fraction of emitted dose (ED) and the dose delivered to the lung (i.e the fine particle fraction (FPF)). [69]. ED is the total amount of drug recovered from the impactor except for device and capsule [7]. FPF is the total amount of drug particles with an aerodynamic diameter of less than 5 µm.

2.4 Effects of physico-chemical properties on aerosolization

Aerosol performance of the DPIs depends on the powder properties i.e ability of the powder to flow and disperse out of the device and subsequent deposit in the lungs. Powder properties such size, shape, density, crystallinity and polymorphism, surface roughness and inter-particulate forces have a significant impact on the therapeutic efficacy of DPIs (Figure 1-1). Particle size expressed either as geometric or aerodynamic is one of the most important factor influencing aerosol performance [70]. In order to reach the lower regions of the respiratory system, the particles should have an aerodynamic diameter of 1-5 µm. The aerodynamic diameter (d_A) is an important parameter which helps determine how deep the particle can deposit in the airways. It depends on the air flow and particulate properties such as geometric size, shape and density [69].

It is defined as the diameter of a sphere of unit density with same terminal settling velocity as the particle in study when passing through air and can be calculated using the following equation:

$$d_A = d_g \sqrt{\rho/\chi\rho o} \qquad Eq.1$$

Where d_g is the geometric diameter, ρ is the particle density, ρ_o is the unit density and χ is the shape factor [69]. Based on equation 1, aerodynamic diameter and geometric diameter are directly related. Therefore, small geometric diameter with the same density would result in small aerodynamic diameter enabling the particles to deposit in the lower airways. However, this could increase the inter-particulate forces such as Van der Waals force between drug-drug and drug-carrier particles. Thus, particles with large geometric diameters and low density would be ideal with good dispersibility and better lung deposition [7].

Development of such porous drug particles was demonstrated to deposit efficiently in the lower airways and also escape the macrophage clearance mechanism of the lungs [65]. Dynamic shape factor χ is another important factor which reduced device-dependent aerosolization performance [71] and is inversely related to aerodynamic diameter. On comparing particles with different shapes, elongated particles were found to have large dynamic shape factor and thus lower aerodynamic diameter making it suitable for lung deposition [72, 73]. Enhancement in aerosol performance was observed by using elongated rifampicin and rifapentine particles [71, 74]. Additionally, solid state properties such as crystalline or amorphous nature of the powder formulation could affect product stability and aerosol performance by altering the surface energy or surface roughness [75, 76].

2.5 Particle engineering for DPI formulations

High aerosolization efficacy and accurate dosing can be achieved using specialized particle

engineering techniques. The physicochemical properties of powders can be altered by making use of particle-engineering technologies. Enhancing powder flow and thereby lung deposition is possible by reduction of particle size or density, modification of particle shape and surface characteristics, and alterations in crystalline form. Particle engineering has also enabled the development of carrier-free powder formulations, the delivery of nanoparticles encapsulated into biodegradable carriers, and fixed-dose combinations [53].

2.5.1 Jet milling

Raw drug materials usually have large particle sizes, which are jet-milled in order to achieve the inhalable particle size [77, 78]. There are several types of jet mills: fluid impact mills, opposed jet mills, spiral jet mills, oval chamber jet mills and fluidized bed opposed jet mills [79]. Micronized particles produced by the milling process are extremely cohesive and prone to aggregation and agglomeration as they carry high amounts of charge [69] and have high surface energy [80, 81]. These undesirable characteristics potentially reduce the aerosol performance [82]. Hence, to overcome these drawbacks, alternative and more innovative methods like spray-drying [83], spray freeze drying and supercritical fluid (SCF) precipitation [84] have been developed.

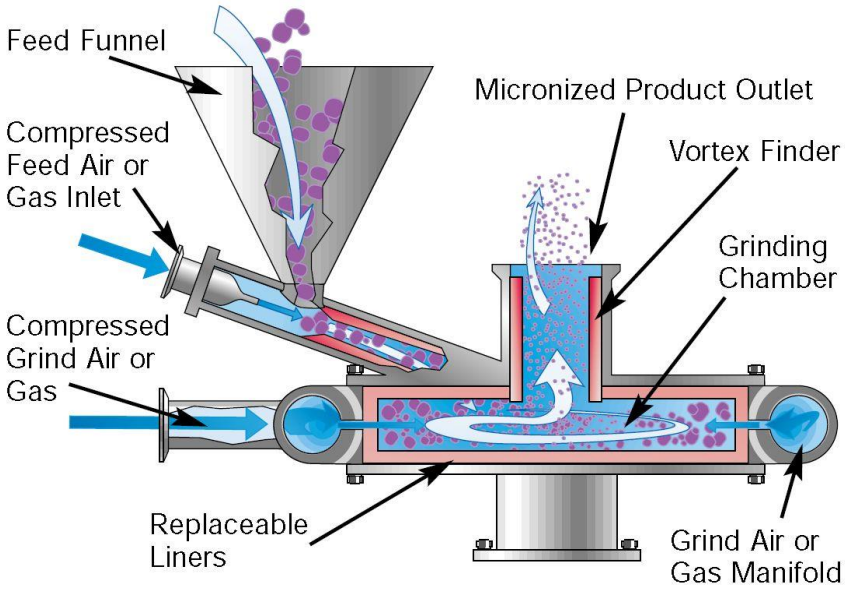


Figure 2-5: Schematic of jet mill.[85]

2.5.2 Spray-drying

Spray-drying involves atomizing the feedstock liquid into fine droplets that are rapidly evaporated in warm air to form dry particles (Figure 2-6). It is a one-step process that is scalable [83]. For decades, spray-drying has been an attractive approach to formulate DPI formulation because the formulator can control particle size, morphology and surface composition to enhance aerosol performance [77, 86]. Through spray-drying, particle shape, size, surface composition can be modified for the optimal delivery of DPIs. One of the challenges with the spray-drying is the amorphous nature of the spray-dried powder, which may cause a concern in physical stability because amorphous solids are usually unstable and have a tendency to crystallize [75, 87].

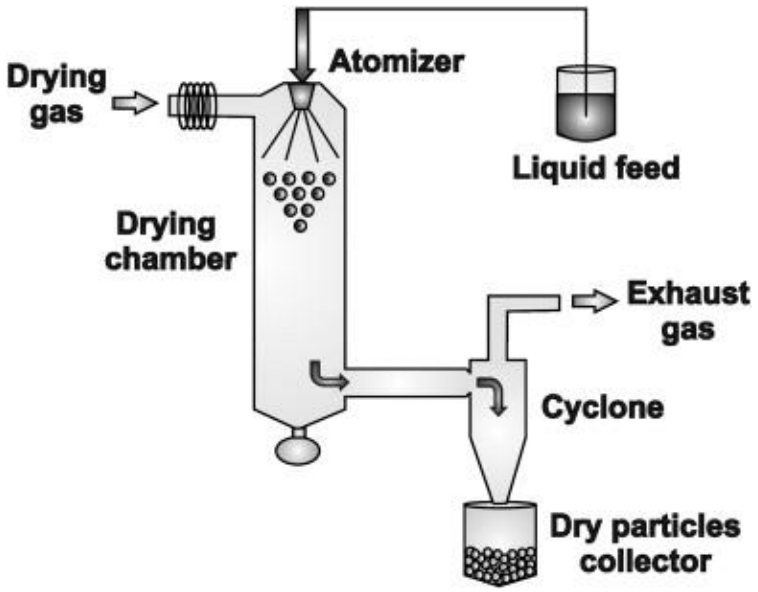


Figure 2-6: Schematic of a spray dryer [88].

Solid state properties influence both the aerodynamic behavior and stability. The particles may exist in two different states, namely, crystalline or amorphous [89]. The crystalline state is defined by the degree of ordered structure in a solid particle –comprising of repeating units of three-dimensional brick-like structures mutually connected by non-covalent forces [89-91]. On the other hand, the amorphous state lacks long-range crystal order. Amorphous solids may present another instability issue for DPIs, particularly for spray-dried particles of many small molecules. Amorphous solids are usually formed when a liquid containing the API is rapidly dried such that the molecules lose their mobility prior to the formation of a crystalline structure [89, 92]. The thermodynamically unstable powders are prone to chemical degradation and crystallization into the more stable crystal form [93]. Such undesirable crystallization may occur at ambient environment or at elevated temperature or humidity [92]. For instance, amorphous mannitol was

found to recrystallize at RH above 50% in a spray-dried mixture, showing temperature and humidity also have a significant impact on the physical stability of DPIs on storage [94].

2.5.3 Spray Freeze Drying

Spray freeze drying is an alternative drying method to spray-drying for the heat labile therapeutics. Spray freeze drying is a two-step technique that involves atomizing the feedstock into a freezing medium which turns the fine spray into frozen droplets [95, 96]. This step is followed by the second step, lyophilization, to remove the ice via sublimation, thereby leaving behind a powder (Figure 2-7). The twin advantages of Spray freeze drying are: low heat process for preserving the integrity of biotherapeutics [97, 98] and high production yield [99]. Porous particles obtained in the spray freeze drying process appear to have enhanced aerosol performance due to reduced particle density [100]. Study has demonstrated that the pulmonary administration of the influenza vaccine prepared by spray freeze drying process induced a potent and enhanced immunological response in mice as compared to that of conventional intramuscular delivery [101, 102].

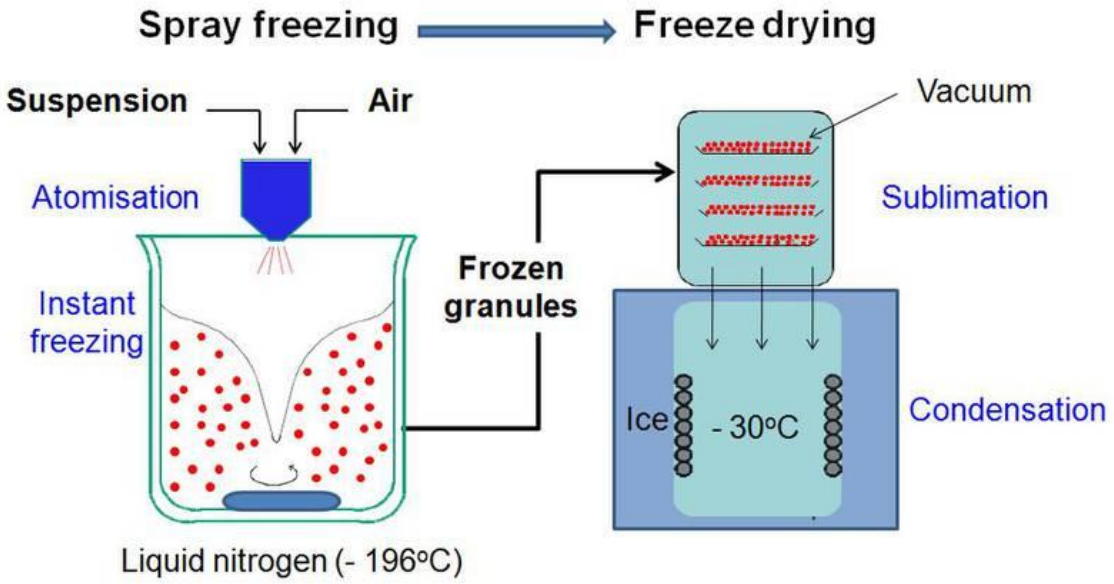


Figure 2-7**:** Schematic of spray freeze drying process. [103]

2.5.4 Super Critical Fluid Technology

Super Critical Fluid technology is one of the latest technologies used for powder production. One of the simple and common forms of Super Critical Fluid technology called Rapid Expansion of Supercritical Solution (RESS) [104] involves an extractor to solubilize drug particles in a SCF. The drug containing SCF is then passed through a nozzle allowing precipitation of the drug through rapid expansion of SCF. RESS possesses several advantages over other production methods [105] which include:

(1) No organic residue in the final product as no organic solvent is used in the process.

(2) Capability to produce fine particles suitable for inhalation.

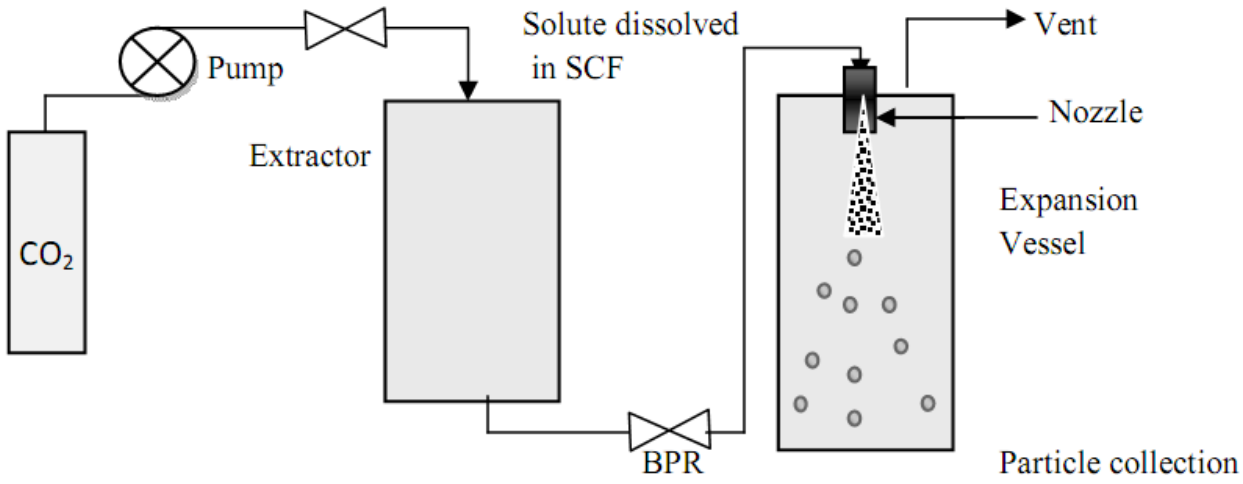


Figure 2-8: Schematic of supercritical freeze drying process

The major limitation of RESS relates to the poor solubility of most APIs in supercritical CO_2 [106].

SCF is getting more accepted for production of powders of DPI as it is capable of generating powders with more uniform shape, size distribution, morphology and crystallinity. SCF particles have showd enhanced flowability and dispersibility [107] as compared to those produced by

conventional micronization [108]. SCF technology has been successfully used in producing inhaled APIs like ipratropium [109], steroids such as budesonide [110], salbutamol, salmeterol [107], terbutaline [108] and steroids such as budesonide [110].

2.6 Effect of powder production methods on stability of dry powder inhalers

2.6.1 Milling

Micronization is a process that reduces the particle size down to micrometers by fracturing particles with mechanical energy or shear [111]. Jet milling the coarse particles into the inhalable size range is a common practice in the pharmaceutical industry for producing inhalable particles. Jet mills have different type of action or design including fluid impact mills, opposed jet mills, spiral jet mills, oval chamber jet mills and fluidized bed opposed jet mills [112].

Jet milling uses pressurized gas to create high particle velocity and high-energy impaction between particles and/or between particles and chamber walls to achieve micronization of particles. A jet mill essentially consists of a product inlet and a grinding chamber. The particles undergo multiple particle–particle and particle–wall impactions, which induce particle fracture and subsequent size reduction. The advantages of jet milling include micronization to a size suitable for inhalation, no contamination in the final micronized material, inexpensive and convenient to use [113].

Though jet milling is commonly used for DPIs, there are certain unfavorable characteristics of the inhalable particles produced by jet milling. The jet-milled powder is usually extremely cohesive and prone to strong agglomeration due to the increase in surface energy and electrostatic charge [80]. The excess energy supply during the milling process could lead to mechanical activation and development of local amorphous site at the surface of the particle,

which may subsequently lead to re-crystallization during storage at the high humidity environments [112, 114-116]. Young et al. studied the effect of freshly milled and milled-recrystallized lactose monohydrate on aerosol performance of 5% ((w/w)) nedocromil sodium trihydrate prepared by ball milling. The amorphous regions in freshly milled lactose monohydrate was intentionally re-crystallized upon storage at the relative humidity of 85% after milling for up to 60 mins. It was shown that ball milling of carrier such as α- Lactose monohydrate reduced the particle size while increased the level of fines and amorphous content [117]. For freshly milled lactose the amorphous content increased with increase in milling time. However, the milled-recrystallized lactose remained completely crystalline during milling with 5% ((w/w)) nedocromil sodium trihydrate. The freshly milled lactose sample was physically unstable and presence of increasing amorphous content resulted in decrease in fine particle fraction (FPF) compared to recrystallized lactose monohydrate (particles with aerodynamic diameter ≤ 5μm) [117].

This phenomenon has also been reported for jet-milled drugs such as revetropate hydrobromide, which agglomerated when subjected to grinding pressure of 0.31MPa and 0.52MPa [118]. The results indicate that intensity of micronization had an impact on the amount of amorphous content which is associated with higher degree of agglomeration [118]. It has been reported that low-level of amorphous materials present in the micronized samples can be identified using isothermal microcalorimetry and dynamic water vapor sorption (DVS). Using isothermal microcalorimetry it was observed that the micronized revatropate hydrobromide recrystallized at RH >30% [118]. Boshhiha et al. made a similar observation on salbutamol [119]. They found that the amorphous regions can be generated in crystalline materials during jet milling process,

which had significant impacts on physical stability [119]. Thus, re-crystallization of the amorphous regions can lead to changes in the physico-chemical properties of the micronized particles and formation of strong agglomerates, which has significant impacts on aerosol performance of the formulation [120-122].

Attempts were made to overcome such problems by “conditioning” or “relaxation process” following jet milling [112]. In the conditioning process, the storage environment is controlled with respect to relative humidity and temperature to enable intentional amorphous to crystalline conversion. The temperature of the surrounding environment is kept above the glass transition temperature (Tg) and relative humidity is such that solid powder adsorbs water reducing the Tg of the sample thereby increasing the molecular mobility and process of re-crystallization [123]. Accelerated temperature and relative humidity improve physical stability of the dry powder post-micronization by enhancing structural relaxation rate and reducing amorphous content and associated powder aggregation [124].

Another effective way to solve the problem is to coat the surface of jet-milled particles with anti-sticking materials [125]. Surface coating of jet-milled particles with appropriate excipients can be achieved using high-shear dry coating approach such as mechanofusion with no or little reduction in particle size [126, 127]. Significant improvements in powder flowability and aerosol performance of the mechanofused formulations have been demonstrated for both carrier-based and carrier-free DPI formulations attributed to the reduction in inter-particulate forces [128-131]. Such high-shear dry coating technique can de-agglomerate the strong aggregates of jet-milled

particles and coat the individual particles with a force control agent such as magnesium stearate [113, 132].

Co-milling of active pharmaceutical ingredient (API) such as Beclomethasone Dipropionate (BDP) with additives such as magnesium stearate has also been reported to improve aerosolization performance and aerosol stability [113]. It involves co-processing two or more components through a milling apparatus to achieve a homogenous powder blend, associated with particle size reduction. Lau et al., reported that addition of magnesium stearate 5% ((w/w)) had significant impact on physical stability and aerosol performance of co-milled BDP with lactose since it improved de-agglomeration by reducing adhesive force between BDP and lactose [133]. The formulation of 1% (w/w) BDP co-jet-milled with 99% (w/w) lactose but without magnesium stearate agglomerated at the elevated humidity of 75% RH at 25°C due to re-crystallization of the amorphous regions of micronized lactose, which led to decreased aerosol performance (p <0.05) as compared to the 1% BDP/94% lactose formulation with 5% magnesium stearate [133]. Co-milling method may also prevent changes in the solid-state property of the API such as salbutamol sulphate due to crystallization at the elevated humidity when the crystalline additives such as a-lactose monohydrate (LAC), adipic acid (AA) and magnesium stearate (MgSt) were added as crystallization inhibitors [134-136].

Wet milling was also studied by El-Gendy et al. to improve aerosol performance of DPIs [137]. They used wet milling to produce budesonide NanoClusters (NCs) an excipient-free dry powder formulation. The NanoClusters are irregular shaped agglomerates of drug nanoparticles with superior aerosol performance due to reduced contact area and minimized particulate interactions.

As the particles are milled in the aqueous media, the amorphous region would undergo crystallization immediately. Thus, the wet milling process minimizes crystallization of particle surface during the storage [138]. On comparison with the commercial budesonide DPI formulation, the NanoClusters showed superior aerosol performance due to lower density and lower adhesive and/or cohesive forces [137]. Such crystalline particles without amorphous regions may improve the physical stability of inhalable formulation, which deserve further investigations.

2.6.2 Spray-drying

Spray-drying is one of the commonly used particle engineering methods to produce fine particles suitable for inhalation [83, 139, 140]. In this method a feed solution or suspension is sprayed into a hot drying medium (air/nitrogen gas) so as to generate dry particles [141]. The unique advantage of this technology is its potential to control and tune the particle characteristics such as size, density, morphology and surface composition via the adjustment of formulation or the process parameters [140]. In addition, it is possible to incorporate multiple components into a single particle by this one-step continuous manufacturing process. This method is being increasingly used by the pharmaceutical industry to produce dry powders for inhalation within the aerodynamic size range of 1-5 µm, as exemplified by Aridol® (mannitol inhalation powder) and TOBI® Podhaler (tobramycin DPI).

2.6.2.1 Recrystallization of spray-dried amorphous particles

A grand challenge with the use of spray-drying technique for inhalable dry powder production is long-term physical stability, and its impact on aerosol performance. Many amorphous powders (particularly small molecules) obtained through the spray-drying technique are physically unstable and tend to crystallize (especially when they are exposed to moisture and high

temperature) [142]. In a study by Shetty et al., it was revealed that amorphous spray-dried drug particles such as Ciprofloxacin hydrochloride, tend to re-crystallize on storage at 55% RH and above thereby altering its aerosol performance [75]. Jeffry et al., in their review article described different approaches to maintain crystallinity of drugs during the process of spray-drying [143]. One of the suggested methods is to spray dry suspensions of micronized drug(s) from a liquid feed. But in this method, there is a need to minimize dissolution of drug particles in the liquid feed [143]. The review further discusses different formulations and engineering techniques useful to maintain physical and chemical stability of the crystalline drug in the formulation [143].

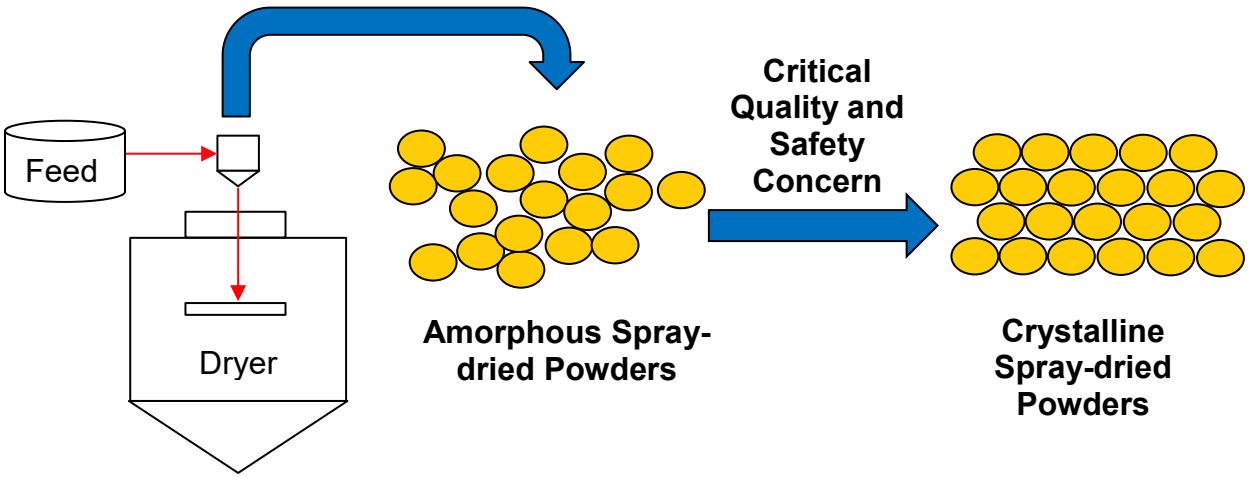


Figure 2-9: Physical instability with spray-dried powders upon storage

Challenges with physical instability due to spray-dried amorphous materials may be overcome by controlling formulation and process parameters. Adding suitable excipients to the spray-dried formulation could solve the crystallization problem and retain the physical stability and optimal aerosol performance in DPIs. Bosquillon et al. studied dependence of in-vitro aerosol

performance on excipients (i.e. lactose, albumin, dipalmitoylphosphatidylcholine (DPPC), trehalose and mannitol). The aerosolization property of human serum albumin powder was found to be improved by addition the albumin/lactose/DPPC (30/10/60 (w/w)/w) but reduced in presence of mannitol and trehalose. The physical characteristics of the particle was heavily influenced by the composition and type of excipient added: DPCC reduced surface energy and thereby powder cohesiveness whereas lactose and albumin reduced density and inter-particle adhesion forces enabling improved aerosol performance [144]. Recently, impacts of excipients such as lactose, sucrose, trehalose, mannitol and leucine on physical stability and aerosolization of the spray-dried Ciprofloxacin formulations were examined upon storage at 55% RH [145]. Leucine was shown as the best excipient with improved aerosol performance and enhanced physical stability for the spray-dried Ciprofloxacin formulations due to its surface-enrichment properties, even at a low concentration of leucine (10% (w/w)) [145]. Similarly, Li et al. studied the moisture protection effect of varying Leucine concentration (5-80% (w/w)) on aerosol performance of spray-dried salbutamol sulfate. In their study, 40% ((w/w)) leucine in the formulation did not absorb much water due to increased crystalline leucine content; and thus deterioration in aerosol performance of spray-dried amorphous salbutamol sulfate stored at 60% RH and 75% RH was significantly reduced compared to the formulations containing 5-20% ((w/w)) leucine [146]. Also, storage induced crystallization associated with high-dose spray-dried amorphous powders (such as antibiotics) has been avoided through co-spray-drying combination of antibiotics. Shetty et al., observed that co-spray-drying Ciprofloxacin with polypeptide antibiotic Colistin enhances the physical and aerosol stability of amorphous spray-dried Ciprofloxacin powder formulation stored at 55% RH. Colistin formed a polymer like matrix and enriched on the composite particle surface during co-spray-drying thereby reducing

molecular mobility of Ciprofloxacin and completely prevents its crystallization at 55% RH in the mass ratio (1-1). The composite formulation has also shown improved aerosol stability through intermolecular hydrogen bonding [147].

2.6.2.2 Hygroscopicity of spray-dried amorphous particles

Some spray-dried drugs can remain amorphous at the elevated humid conditions without crystallization; but usually these amorphous powders are hygroscopic and have a tendency to absorb moisture resulting in particle agglomeration and subsequent decrease in aerosol performance. For instance, Colistin is in the amorphous form for up to three months upon storage at the relative humidity (RH) above 60%. However, upon exposure to high humidity (>70%), the amorphous powders tend to absorb excess moisture and form stronger agglomerates due to increased capillary forces [148-151]. Zhou et al., was able to achieve enhanced aerosol performance at relative humidity (>70%), by co-spray-drying hygroscopic Colistin with other hydrophobic antibiotics like rifampicin and azithromycin [67, 152]. They used specialized surface characterization technique such as X-ray photoelectron spectroscopy (XPS) and time of flight secondary ion mass spectrometry (ToF-SIMS) and observed that with 50% ((w/w)) azithromycin in the formulation, the surface contained 96.5% azithromycin in the composite co-spray-dried formulation. Thus, surface enrichment with hydrophobic drugs such as azithromycin prevented degradation in aerosol performance of co-spray-dried Colistin at 75% RH.

Also, disodium cromoglycate (DSCG) powders were amorphous after spray-drying and underwent a significant mass increase of 50% due to water absorption at 90% RH [153]. After storage at 75% RH for one week, the fine particle fraction (FPF) of the spray-dried DSCG particles fell drastically to the extent that was not inhalable Also, Yu et al., found that sodium

stearate protected DSCG from moisture resulting in better aerosolization performance. This was attributed to enrichment of sodium stearate on the particle surface. There was improved dispersions as the particle-particle and particle–moisture interactions were reduced [153]. Li et al., observed that L-leucine added as an excipient not only improved aerosol performance but also prevent moisture-induced deterioration in aerosol performance of DSCG powders by surface enrichment [154]. Similarly, Cui et al., was able to improve aerosol performance of hygroscopic drug Netilmicin by co-spray-drying with different L-leucine ratios [155].Yu et al., also studied different hydrophobic amino acids like valine, isoleucine and methionine as an excipient to prevent deterioration in aerosol performance of highly hygroscopic spray-dried powders upon storage at high humidity and they also acted by the same surface-enrichment mechanism [150].

2.6.2.3 Spray-dried crystalline particles

Additionally, altering process parameters such as using high outlet temperatures with an insulated drying chamber and using ‘Spray blending’ methods have also shown to improve stability in some cases [112]. The study by Abiad et al., clearly showed that varying the spray-drying and storage conditions produce different structures in the material. According to the study initial relaxation occurring during spray-drying, affected the crystallization tendency of the produced dried powders. They were of the view that there is a need to monitor variations in spray-drying which may impact nucleation and subsequent crystal growth [156].

Spray-drying has also been shown to be a suitable method for preparing co-crystals i.e. crystalline solid forms composed of a drug and a conformer [157]. Co-crystals alter the solid-state properties of the drug and thereby improves solubility, dissolution, stability and bioavailability of drug molecules; but they are usually less hygroscopic than the amorphous

materials. Studies by Alhalaweh et al., on highly crystalline co-crystals of theophylline by spray-drying have demonstrated the greater potential of co-crystallization technology to control particle physico-chemical properties [158]. In comparison to milled theophylline, spray-dried co-crystals have considerably smaller surface energy and different micromeritic properties [158]. Studies have also demonstrated that micronized co-crystals were found to enhance the pulmonary absorption of poorly absorbed drugs like itraconazole. Karashima et al., prepared poorly soluble Itraconazole co-crystals with succinic acid (SA) or l-tartaric acid (TA) and successfully micronized using the jet milling system [159]. The particle shapes of micronized Itraconzole co-crystal powders were found to be different from those of the crystalline or spray-dried amorphous itraconazole, which showed better intrinsic dissolution rates (IDR) and pulmonary absorption profiles. It was demonstrated that DPI formulations of co-crystals were better than amorphous DPI formulation for pulmonary administration with respect to physical stability and dissolution [159].

2.6.2.4 Spray-dried biologics

More recently, spray-drying method is being explored as effective means of producing peptide or protein dry powders suitable for pulmonary delivery [5, 160]. However, there are concerns that spray-drying could cause denaturation of proteins due to the high drying temperature and air–water interfaces generated during spray-drying [161]. During the process of spray-drying the protein solution is forcefully pushed through the tubing, subjected to hot air, broken up into droplets and stripped from its aqueous environment. However, it has to be noted that the extent of protein degradation depends on the type of protein and the specific process conditions and configuration of the spray dryer [162]. Using a lower inlet air temperature has been suggested to reduce the potential thermal stress. Additionally, even at spray-drying temperatures above 100ºC

chances for protein denaturation is very unlikely since temperature of droplet barely exceeds the wet bulb temperature of water (~40ºC) [140, 141]. Also, incorporating a stabilizer (e.g. sugars, amino acids) into the protein formulation is reported to be advantageous since they serve as good water replacing agent [163, 164]. However, during the spray-drying process phase separation or inhomogeneous distribution of different components within a particle may occur due to difference in the solubility and diffusion rate and thereby could impact physical stability resulting in denaturation of proteins [164].

Solid-state stability is a critical quality concern for successful development of dry powder protein/peptide formulations. Spray-dried inhaled biomolecules must have a stable amorphous glass structure that would prevent molecular mobility of the biomolecule and keep them stable in the dry solid-state [165]. Mannitol in the amorphous state has shown to stabilize proteins however due to its low Tg (11ºC) it tends to re-crystallize [164, 166]. Such re-crystallization process may cause denaturation of proteins during the drying process. Use of high Tg excipient like trehalose (117ºC) has shown to form amorphous glasses after spray-drying and can be used to stabilize proteins on storage [167]. Hulse et al., showed in their study that the spray-dried lysozyme containing 50% trehalose as an excipient was not stable after storage at elevated temperature and humidity due to increased water uptake by trehalose compared to sample containing combination of mannitol and trehalose. Their study emphasizes the importance of carefully selecting mixed excipient systems to stabilize lysozyme more effectively than single excipients [167].

Pulmonary vaccines are being tested as an alternative non-invasive route to induce mucosal and systemic immunity [57]. This alternative route is considered to have more advantages than

parenteral vaccination since the antigen (macromolecules, peptides or proteins) can be administered directly to the site of infection giving better immune response. Compared to traditional liquid formulations, dry powder pulmonary vaccine formulations have better chemical and physical stability and eliminates the need for cold chain storage. Spray-drying is considered to be a viable technique to produce dry powder vaccines formulation as it enables easy incorporation of excipients which help preserve integrity of antigenic macromolecules. Spray-dried influenza vaccine was found to be biochemically and physically stable for up to 3 years at 20ºC with inulin as a stabilizer [168]. Also, various viral respiratory infectious diseases are treated by pulmonary delivery of short interfering RNA (siRNA) that act by inhibiting specific gene expression, degrading viral mRNA and preventing viral replication [169, 170]. Chow et al., produced dry powder formulation of naked siRNA (2% ((w/w))) without delivery vectors using spray-drying technique. L-leucine was added as an excipient and helped improve aerosol performance by surface-enrichment [171]. Liang et al., showed that dry powder formulation of siRNA co-spray-dried with pH responsive peptides such as LAH (histidine-rich peptides) and LADap (2,3-diaminopropionic acid (Dap)-rich peptides) and excipient mannitol not only protected siRNA from enzymatic degradation but enhanced local delivery for treatment of influenza [170]. The spray-dried powders containing siRNA/peptides/mannitol was found to be stable in the crystalline form for up to 5 months storage due to low moisture absorption tendency.

Excipient selection for spray-dried biologics should have a dual property of enhancing aerosol performance as well as stabilizing the biologics. Sou et al., studied in detail the impact of leucine as an excipient on morphology and solid-state properties of multi-component spray-dried

formulations for pulmonary delivery of biomolecules [172]. Leucine as an excipient is well studied to improve the aerosol performance of spray-dried powders by surface-enrichment [173, 174]. However, their results suggest that leucine not only enriches on the surface of the multi-component spray-dried formulations but also inhibits crystallization of components within the formulation thereby preserving amorphous environment for biomolecules to remain stable [172].

2.6.3 Spray freeze drying

Spray freeze drying (SFD) presents an alternative drying method to spray-drying for the heat labile therapeutics. Usually larger surface area and porous particles can be obtained by spray-freeze drying [99], which is suitable for inhalation delivery. Lyophilization or freeze drying has been the gold standard method for protein dry powder formulation administered through the injectable route [140, 175, 176]. However, spray freeze drying has advantages over lyophilization because particle size, surface area and powder density can be controlled by altering process parameters such as atomization and freeze-drying conditions [177, 178]. Such advantages are particularly important for pulmonary drug delivery since the powders can be engineered with desired aerodynamic properties to ensure delivery to deep lungs [179].

Spray freeze drying consist of two steps involving atomizing the feedstock into a freezing medium followed by lyophilization to remove the ice via sublimation, thereby leaving behind a powder [112]. The freeze-drying part involves three essential steps: (1) freezing, (2) primary drying, and (3) secondary drying [180]. Relatively low temperature drying conditions provided by lyophilization is a safe and effective approach for maintaining stability of many biopharmaceutical products [181]. Spray freeze drying has the advantages of preserving integrity of biotherapeutics, providing a very high production yield with light and porous particles which

is beneficial to the inhalation formulations. Attempts have been made to produce inhalable biological powder formulations using spray freeze drying including insulin, bovine serum albumin, trypsinogen [182], small interfering RNA (siRNA) [183] as well influenza vaccine [112].

As mentioned above, though spray freeze drying has many advantages for producing protein particles, the limitation seems to be extensive damage to some protein molecules due to excessive stress during atomizing, in addition to freezing and drying [184, 185]. The proteins are exposed to shear stress and air-water interface during atomization, which increase the likelihood of protein unfolding and aggregation [186]. Excipients such as sugars, polyols, surfactants and buffers are commonly added to stabilize proteins [187, 188]. Webb et al., demonstrated that adsorption of recombinant human interferon-gamma (rhIFN-γ) at the air-liquid interface during atomization step of spray freeze drying resulted in loss of protein native structure [189]. But the adsorption was significantly reduced by addition of surfactant like polysorbate 20 by reducing the amount of protein molecules on the surface and improving stability [189].

Also, it has been shown that stability of spray freeze dried powders can be improved by annealing the frozen particles prior to drying which would result in decrease in specific surface area [163]. Audouy et al., have shown that whole inactivated virus influenza vaccine (WIV) can be spray freeze dried in the presence of inulin without loss of its bioactivity [98]. The resultant powder was found to be suitable for pulmonary administration [98]. Murugappan et al., further investigated the physical and immunogenic stability of spray freeze dried whole inactivated virus with inulin, dextran and a mixture of dextran and trehalose as protectants. They noted that the

spray freeze dried whole inactivated virus was stable when stored at 30ºC for 3 months with these different protectants. However, they found that the WIV incorporated in dextran alone was even stable at temperatures as high as 40ºC for 3 months with no change in particle size and specific surface area [190]. Higher storage temperature of 40ºC reduces particle size and specific surface area of SFD inulin and SFD dextran/trehalose. The initial glass transition temperature (Tg) for SFD dextran (220ºC) was higher compared to SFD inulin and SFD dextran/trehalose (154ºC) and thus the residual moisture associated with the spray freeze dried dextran could not decrease the Tg sufficiently at 40 ºC to cause a change in physical stability [191].

However, on the flip side, the inhalation performance of large porous powders produced by SFD could be impaired as the porous particles with large surface always tends to absorb moisture [192]. Sooner et al., showed in their study that SFD trehalose powder stored at 33% RH/25°C was found to be more hygroscopic than spray-dried trehalose powders due to the highly porous nature and resulting high specific surface area [182]. Also, Leung et al., in their study spray freeze dried trehalose-mannitol-leucine in different ratios (F1 = 60:20:20 and F2 = 40:40:20 respectively) with phage as the active ingredient and observed that with high concentration of hygroscopic trehalose (F1) the SFD particles lost their porous structure [193]. They also compared it to spray-dried formulation which did not have a porous structure and observed that the degree of recrystallization of trehalose in the spray freeze dried was stronger due to increase moisture uptake by the porous particles with increased specific surface area [193]. The SFD-F1 had a lower FPF as compared to SFD-F2 since the porous structure was lost. Otake et al., studied various properties of SFD powders of 7 hydrophobic amino acids on storage at 75% RH for four

weeks [192]. They found that the SFD powder composed of L-leucine, L-isoleucine, or L-phenylalanine exhibit high inhalation performance as well as high anti-hygroscopicity [192].

Physico-chemical properties such as residual moisture and crystallinity of spray freeze dried formulation are important apart from aerodynamic properties and biological activities for developing a suitable pharmaceutical product. Liang et al., used two-fluid nozzle to produce better spray freeze dried powder of small interfering RNA (siRNA) [194]. The formulations were prepared with 2% ((w/w)) siRNA and with mannitol as bulking excipient. Spray freeze dried siRNA powders were produced at various atomization gas flow rates which showed to have an impact on the particle size. PXRD study showed that mannitol was crystalline in all spray freeze dried formulations but exhibited different polymorphs (delta form for the spray freeze dried formulations) to the beta form for the raw mannitol [194]. The polymorphic transition in mannitol was attributed to the change in the atomization gas flow rate but could also be due to freezing process during spray freeze drying [195]. Since mannitol was in the crystalline state it confers formulation stability for this particular formulation however this might not be the case with other amorphous excipients and suitability of SFD needs to be determined in future when using other carriers such as sucrose, lactose etc. for DPIs [194].

2.6.4 Supercritical fluid technology

Super critical fluid technology (SCF) is a relatively new technology which has been exploited for production of DPIs as crystalline particles with desired physico-chemical properties (e.g. size, shape etc.) can be obtained without use of extreme temperatures [69, 77, 196-198]. It makes use of supercritical fluid such as ethanol, ethylene, and CO_2 (instead of a drying gas) above their critical temperature and pressure [188]. By altering pressure and temperature of the process,

density of the SCF and solubility of the solute can be altered. SCF technology has been successfully used in producing inhaled APIs like ipratropium [109], budesonide [110], salbutamol, salmeterol [107] and terbutaline [108]. Though the conventional processes like crushing/milling and crystallization/precipitation are still commonly used but the supercritical fluid is a unique technology with very different mechanism to form particles. Supercritical processes generate micron- or even nano- sized particles with relatively narrow size distributions with the advantages of single step process, enhanced purity of the products, control of crystal polymorphism and being environment friendly. Most of these advantages are due to the use of carbon dioxide (CO_2), a substitute to organic solvents and its low critical temperature (T_c = 31.1°C) [140]. Green,
sustainable, safe and environment friendly characteristics of this technology has made SCF one of the new-generation particle production technologies [199].

Supercritical methods can be broadly divided into two types: (1) the SCF is used as a solvent (Rapid expansion of supercritical solution) where the solute is dissolved in a SCF such as CO_2 and made to precipitate mechanically by rapid expansion [200, 201]; however, most pharmaceuticals have poor solubility in supercritical CO_2 and hence a process known as solution enhanced dispersion by supercritical fluids (SEDS) is employed in which co-solvents such as methanol are added to enhance solubility [202] (2) is used as an antisolvent (Gas Anti-solvent or supercritical antisolvent process) where the SCF is responsible for precipitation [188, 203] and the solute is forced to crystallize due to low solubility in the SCF such as CO_2. The anti-solvent method employs organic solvents such as dimethylsulfoxide or methylene chloride which is not environment friendly and can also disrupt native protein structure [202]. Apart from these

methods, Sellers et al., has developed a method which involves an emulsion formation between supercritical CO_2 and an aqueous solution of water-soluble drug and excipient called supercritical CO_2-assisted nebulization and dehydration of aqueous solutions for producing dry powders for inhalation [202, 204].

Different polymorphic forms of the same drug with different surface properties can be obtained using the SCF technology. This method can thus help improve flow and dispersion by altering particle surface energy [205]. The controlled crystallization of drug from supercritical CO_2 (as an antisolvent) can overcome the drawback of producing amorphous regions on the particle surface after traditional jet-milling [112]. Young et al., compared the properties of salbutamol sulfate produced by either micronized or solution enhanced dispersion by supercritical fluids (SEDS). Particles produced by the two techniques demonstrated the different influence of humidity on aerosolization. There was a decrease in aerosol performance when humidity was raised (>63%) for the micronized salbutamol sulfate due to re-crystallization of amorphous content present on the surface, but not for the powders produced by SCF technology [121]. This study highlights the significance of powder production methods on physical and aerosol stability of DPI formulations.

Supercritical fluid (SCF) drying has achieved limited successes with proteins; however, protein aggregation seemed to be a key concern for this process. There is limited published data on stability of protein materials dried through SCF. More systemic investigations are warranted since proteins dried by SCF processes are exposed to the stress conditions which are different from freeze-drying. Sellers et al., produced amorphous and crystalline dry (Lysozyme and lactate

dehydrogenase as API) powders for inhalation by using excipients such as 10% ((w/w)) mannitol; 10% ((w/w)) sucrose and 10% ((w/w)) sucrose with 0.01% ((w/w)) Tween 20 [202]. They observed that amorphous sucrose prevented unfolding of lysozyme better than crystalline mannitol during CO_2 associated nebulization and drying. Whereas in case of lactate dehydrogenase sucrose along with tween 20 helped in preserving the native structure [202]. Surfactants such as tween 20 could prevent aggregation by saturating hydrophobic sites on the protein surface. Jovanović et al., attempted to produce stable, sugar-containing lysozyme formulations with sucrose and trehalose as stabilizers using supercritical fluid (SCF) drying with CO_2 and ethanol mixture or CO_2 alone as SCF [206]. Lysozyme-sucrose and lysozyme-trehalose formulations in 1:10 ((w/w)) ratio crystallized with one and three months respectively of storage at 4 ºC due to higher Tg of the latter. However, crystallization was not observed with 1:1 or 1:4 ((w/w)) protein-sugar formulation possibly due to sufficient hydrogen bonding. On the other hand, Lysozyme was found to be structurally stable in the 1:4 and 1:10 ((w/w)) ratio but reversible protein aggregation was observed for the pure protein with no excipient and 1:1 ((w/w)) lysozyme-sugar formulation as amount of sugar was not sufficient to prevent protein-protein interaction. Thus, this study highlighted that an optimum protein-sugar ratio is essential to prevent crystallization of excipient and aggregation of protein and thereby enhance stability of particles prepared by SCF drying. They also reported that eliminating ethanol during SCF drying would help obtain stable formulation with high Tg as ethanol acts as a plasticizer and could also cause crystallization of amorphous excipient by lowering Tg [206]. In another study [207], insulin were dissolved in organic solvents such as DMSO or DMFA and dried by spraying in SCF. Such SCF dried insulin powders showed increased ß-sheet content and concomitant loss of α- helicity, as detected by a shift of the amide I band in Raman and FTIR spectra. Higher

operating temperatures and pressures led to more extensive β-sheet-mediated intermolecular interactions in the precipitates; the drying process itself probably contributes to such conformational changes [207].

2.6.5 Novel particle engineering techniques

Pulmosphere™ is a novel particle engineering technique to produce phospholipid-based small porous particles for inhalation of high-dose drugs [208]. The liquid feed consists of an oil-in-water emulsion which is stabilized by distearoylphosphotidylcholine (DSPC) and $CaCl_2$ in 2:1 molar ratio [143]. Miller et al., showed in their study (using the PulmoSphere™ process) that the porous and hydrophobic layer controls inter particle forces and enhances fluidization and dispersion of powders. The phospholipid shell improves dispersion by minimizing the contact between the drug particles, reducing the particle density, and decreasing the surface energy [209]. Drug incorporation into the small porous Pulmosphere particle can be achieved by either solution-based format in which drug is dissolved in the continuous phase of the emulsion or suspension-based format in which drug is micronized by jet milling and then suspended within the emulsion prior to spray-drying [143].

Tobramycin Inhalation Powder (TIP) produced by this technique are marketed as TOBI Podhaler for treatment of cystic fibrosis [210]. In this particular formulation, Tobramycin exist in the amorphous form. In general, amorphous materials are unstable and could recrystallize upon storage posing challenges in terms of physical and chemical stability. Such physical instability is avoided by adding suitable excipients to the formulation which will either hydrogen bond with the active ingredient or if they have sufficient high Tg and low residual moisture such phase transition can be prevented. It is very crucial to understand the relationship between moisture

content and glass transition temperature (Tg) while predicting long-term physical stability of amorphous formulations. Miller et al., studied physical stability of TIP upon exposure to a broad range of relative humidity (RH) and temperature conditions [209]. No re-crystallization of amorphous TIP was observed when they were exposed to the relative humidity ranging from 0% to 60% at 25ºC. Such physical stability was attributed to the enrichment of excipient such as Distearoylphosphatidylcholine (DSPC) on the surface of TIP particles thereby impeding crystallization of tobramycin sulfate [210] . It is worth nothing that unpackaged bulk powder underwent viscoelastic collapse during storage at RH values at and above 60% at 25ºC and 48% at 40ºC. Such change in solid-state properties of unpackaged drug under the extreme conditions resulted from decrease in glass transition temperature (Tg) below the storage temperature as a result of plasticizing effect of water [209]. Miller et al., have further integrated the results obtained from different characterization techniques to predict long-term stability of TIP under a broad range of temperature and water content (Figure 3). They have predicted that a storage temperature of 30ºC would be well below Tg even if water content is as high as 10% (w/w); hence TIP can remain stable in amorphous form for as long as 3 years when powders were well packaged in the aluminum foil blister to maintain the water content in the package below 10% (w/w) (Figure 2-10).

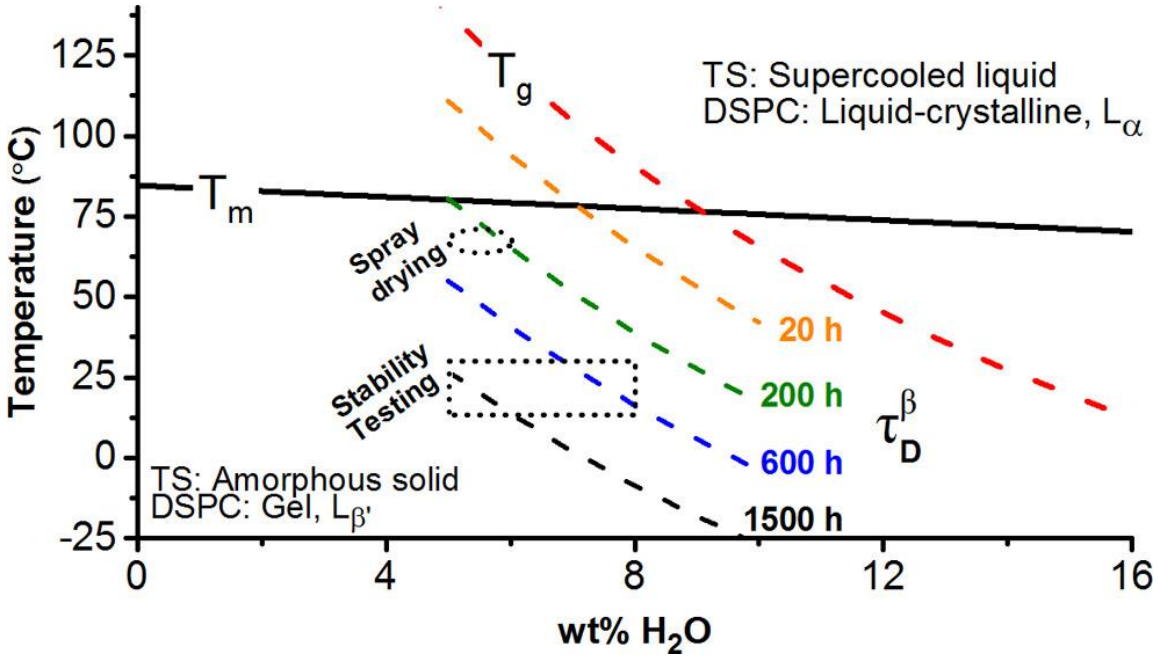


Figure 2-10: Solid state behavior of TIP as a function of water content and temperature. (TS = tobramycin sulfate, DSPC=distearoylphosphatidylcholine). The short, dashed curves represent iso-relaxation time contours (τDβ) of amorphous TS (Reprinted with permission from Molecular Pharmaceutics 2017 14 (6), 1950-1960. Copyright (2017) American Chemical Society) [209].

The manufacture of Tobramycin Inhalation Powder using solution-based PulmoSphere™ method produces amorphous drug products since the drug is fully dissolved and drying occurs very rapidly providing insufficient time for crystal growth and nucleation. Thus, amorphous powders obtained by solution-based spray-drying involves development risk and require addition characterization to maintain the drug in the amorphous form [143]. This can be avoided by forming crystalline drug particles during spray-drying by employing suspension-based spray-drying. Maintains of drug in the crystalline form using suspension-based spray-drying enables better physical and chemical stability of the drug product. McShane et al., reviewed Ciprofloxacin DPI which incorporated suspension-based PulmoSphere technology™ to ensure consistent aerosol performance, particle size, density, and aerodynamic properties allow efficient delivery to the lower respiratory tract [211]. In this technique the drug Ciprofloxacin is in its

neutral zwitterionic form which is poorly water soluble and thus the crystalline form was maintained during spray-drying [211].

Another novel particle engineering method employs a combination of microfluidics with spray-drying to produce dry powders for inhalation [212-215]. Saboti et al., developed budesonide fine particles (fine crystalline powders) using a microfluidic reactor coupled with ultrasonic spray freeze drying (Figure 2-11) [213]. No stabilizers were needed for this technique because of the crystalline nature of the fine particles as reflected by PXRD patterns. Similarly, DVS results confirmed minimal moisture uptake by the Budesonide (BDS) particles even at 90% RH with no indication of crystallization. It appears that microfluidics can emerge as a potential alternative particle production technique for dry powder inhalation formulation [213].

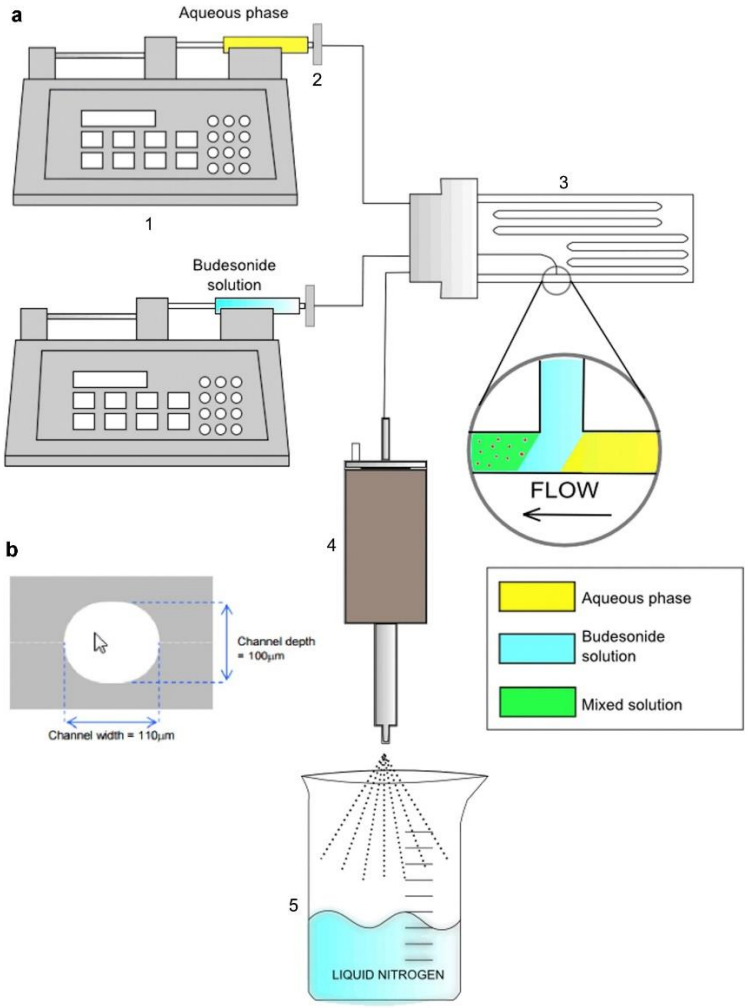


Figure 2-11: Schematic diagram of microfluidic reactor coupled with ultrasonic spray freeze drying [213]

Novel particle engineering method such as particle replication in non-wetting template (PRINT) technology could be suitable for inhaled biologics [216, 217]. This technique claims complete control on particle morphology with no impact on protein structure or function. PRINT technology was shown to produce protein dry powders for inhalation using bovine serum albumin (BSA) and lysozyme 80% ((w/w)) and generated a high aerosol performance of 78.6% ± 0.3 and 84.6% ± 4.3 respectively while preserving native structure and function of the model proteins such as BSA and lysozyme [216, 217].

2.7 Conclusion

Diverse pharmaceutical operations aimed at size reduction such as milling, blending, drying, lyophilization, spray-drying etc. definitely improve dissolution and bioavailability of the formulation but may impact long-term stability of the drug product. These methods could be used successfully to produce drug particles suitable for dry powder inhalation formulations. However, many of these processes involve mechanical stress which may lead to change in physical state, physico-chemical properties and phase transitions which have an impact on the stability of the drug particles particularly for use in DPI. Certain precautions and methods such as surface coating, using novel excipient or particle engineering methods can be used to preserve or retain particle size, shape, density, polymorphism, surface morphology and hygroscopicity of the drug particles so that the stability of the DPI formulation could be maintained, and respiratory infections can be successfully treated. However, making use of advanced characterization technique is crucial as it would help in better understanding of powder behavior in the early stages of DPI formulation development.

CHAPTER 3. EFFECTS OF MOISTURE INDUCED CRYSTALLIZATION ON THE AEROSOL PERFORMANCE OF SPRAY-DRIED AMORPHOUS CIPROFLOXACIN POWDER FORMULATIONS*

*Published as- **Shetty, N.;** Zeng, L.; Mangal, S.; Nie, H.; Rowles, M. R.; Guo, R.; Han, Y.; Park, J. H.; Zhou, Q. T. Effects of Moisture-Induced Crystallization on the Aerosol Performance of Spray-dried Amorphous Ciprofloxacin Powder Formulations

3.1 Abstract

This study aims to investigate the influence of different storage humidity conditions on crystallization and aerosol performance of inhalable spray-dried amorphous powder formulations (Ciprofloxacin hydrochloride as the model drug). The spray-dried samples were stored at 20%, 55% and 75% relative humidity (RH). Crystallinity was monitored by Powder X-ray diffraction (PXRD), and particle morphology was measured by scanning electron microscopy (SEM) and atomic force microscopy (AFM). Aerosol performance was evaluated using a multi-stage liquid impinger (MSLI). PXRD diffractograms showed the spray-dried Ciprofloxacin stored at 20% RH for three weeks were amorphous; whereas those stored at 55% RH and 75% RH started crystallizing after one hour. Fine particle fraction (FPF) of the particles was improved from 28% to 42% after storage at 55% RH for three days. Such improvement was attributed to the crystallization of amorphous powders, which led to increased particle roughness and reduced particulate contact area, as visualized by SEM and quantified by AFM. A linear relationship was observed between degree of crystallinity/crystallite size and FPF (R^2 = 0.94 and R^2=0.96, respectively). However, deterioration in aerosol performance was observed after storage at 75% RH due to formation of inter-particulate liquid/solid bridges, as confirmed by SEM. This study provides a fundamental understanding in moisture-induced physical and aerosol instability of the spray-dried powder formulations.

3.2 Introduction

Pulmonary drug delivery systems have become popular for treatment of respiratory diseases because direct delivery of drugs to the disease sites offers quick drug action, high drug concentration in the lungs and lower systemic drug exposure [218, 219]. Dry powder inhalers (DPIs) has attracted increasing attention among all inhaler devices as they are easy to carry and use, and most drugs are more stable in the solid form [46, 47].

However, there are a few limitations associated with dry powder inhalers (DPIs). These include inconsistent flow rate and variable dose delivery which are mainly due to dependence of some passive DPIs on inspiration rate of patients [220] and dispersion properties of the drug powders in the inhalation formulations [114]. Dispersion behavior of inhalable powders are affected by powder properties such particle size distribution, surface morphology, surface energy, hygroscopicity and electrostatic properties of the drug/carrier particles [221-224]. Surface morphology affects particle cohesion/adhesion by increasing or reducing contact area. Hence, modifying surface morphological properties may be an effective way to alter cohesion/adhesion and control the aerosol performance [225]. Higher roughness of the drug/carrier is shown to reduce the particle-particle contact area and cohesiveness leading to better aerosol performance [226]. Crystalline properties, particularly particle surface crystallinity of both drug and carriers may also have significant influence on aerosol performance and stability of DPI formulations [7].

Though different particle engineering technologies have been used for producing the drug particles for use in DPIs, spray-drying technology has attracted increasing attentions [227, 228]. Spray-drying involves atomizing drug solutions/suspensions into small droplets and evaporating the solvents using hot and dry gas [229, 230]. In most cases, particularly for small-molecules,

spray-drying produces amorphous powders due to rapid drying [231]. It has been observed that spray-dried amorphous powder of different material tends to crystallize at varying rates depending on the relative humidity (RH) and molecular weight [11]. Linna Wu et al. reported that the spray-dried amorphous lactose particles, a carrier for DPI formulations, tend to recrystallize when stored at the humidity > 32% [12]. Costantino et al. observed a drastic decrease in aerosol performance of a pharmaceutical protein rhuMabE25 (Recombinant humanized anti-IgE monoclonal anti body) when the co-spray-dried excipient of mannitol powder was crystallized form the amorphous form upon storage [13]. However, there is a lack of studies that systematically examine the impact of crystallization of spray-dried drug particles on aerosol performance upon storage at various humidity conditions. Such significant changes in aerosol performance of DPIs upon storage may cause critical quality concerns in product stability. Therefore, there is a strong need to understand the mechanisms of such impact of crystallization on aerosol performance to ensure the quality and stability of the DPI products.

Here our study has systematically examined the impact of crystallization of spray-dried drug particles on aerosol performance upon storage at different RHs. Correlations between crystallinity/crystallite size, surface roughness and aerosol performance were established for the first time. Ciprofloxacin, a broad spectrum antibacterial drug often used for the treatment of lower respiratory infections was chosen as the model compound for our current study as it showed a tendency of crystallization upon storage post spray-drying in the literature [14, 15] and in our preliminary studies. Additionally, Ciprofloxacin is being developed as a dry powder for inhalation product [232]. Effects of change in solid-state properties and surface morphology of the spray-dried Ciprofloxacin particles on in-vitro DPI performance upon exposure to various

humidity conditions were evaluated. Outcomes of the present study are critical for understanding the physical instability associated with amorphous spray-dried particles and are useful for developing an effective DPI formulation with consistent quality.

3.3 Materials and Methods

3.3.1 Chemicals

Ciprofloxacin hydrochloride monohydrate (abbreviated as Ciprofloxacin or Cipro in the text) was purchased from ßetaPharma® (Shanghai) Co., Ltd (Wujiang City, JiangSu Province, China). High performance liquid chromatography (HPLC) grade acetonitrile was supplied by Fischer scientific (Fair Lawn, NJ, USA). Magnesium nitrate and sodium chloride were purchased from fisher scientific (Fair Lawn, NJ, USA) to prepare saturated salt solutions for generating corresponding equilibrium RHs.

3.3.2 Production and Storage of Powder Formulations

A spray-drying feed solution (16 mg/ml) was prepared by dissolving Ciprofloxacin in MilliQ water. The drug solution was spray-dried using a BUCHI B-290 mini spray dryer, - (BUCHI Labortechnik AG, Flawil, Switzerland) at a feed rate of 2 mL/min. The spray dryer was operated under the following conditions: inlet air temperature (T_{in}) 120 ± 2ºC, aspirator at 35 m^3/h and airflow of 700 L/h. These conditions resulted in an outlet temperature (T_{out}) of 60 ± 2 °C. The spray-dried powder was divided into 3 equal parts and stored at 20 ± 2 °C in: (1) a desiccator containing silica gel to maintain 20 ± 2% RH; (2) a humidity chamber containing saturated magnesium nitrate solution to maintain 55 ± 2% RH; (3) a humidity chamber containing saturated sodium chloride solution to maintain 75 ± 2% RH.

3.3.3 Powder X-ray Diffraction (PXRD)

The X-ray diffraction patterns of the powder samples were collected on a Rigaku Smartlab™ diffractometer (Rigaku Americas, Texas, USA) with a Cu-Kα radiation source operated at 40 kV and 44 mA and a highly sensitive D/tex ultra-detector. All the measurements were conducted at ambient conditions in Bragg−Brentano geometry with 2θ ranging from 5 to 40° 2θ at a step size of 0.02°and a scan rate of 4°/min [233].

3.3.4 Particle Morphology

A field emission scanning electron microscope (NOVA nanoSEM, FEI Company, Hillsboro, Oregon, USA) was employed to examine morphology of formulations. Samples were added on a rectangular carbon tape attached to metal stubs and excess powders were removed using compressed air. The samples were then placed in a sputter coater operated at 40 mA for 1 minute (208 HR, Cressington Sputter Coater, England, UK) to obtain platinum coating at 40 mA for 1 min. The images were captured at 5 kV.

3.3.5 Particle Size

Particle size distributions of the powder formulations were measured using an in-built software with SEM micrographs. The diameters at 10% (d_{10}), 50% (d_{50}) and 90% (d_{90}) undersize were calculated in approximately 100 particles for each formulation.

3.3.6 Differential Scanning Calorimetry

The DSC measurement was carried out using a DSC Q2000 (TA instruments, Delaware, USA). Samples were transferred into perforated aluminum sample pans and the mass was measured. Each sample was heated from 20 to 370ºC at 10ºC/min with purging of nitrogen gas at a flow rate of 50 mL/min.

3.3.7 Dynamic Vapor Sorption

Moisture sorption behavior for the spray-dried Ciprofloxacin formulation was determined using dynamic vapor sorption (DVS-Intrinsic, Surface Measurement Systems Ltd., London, UK). Approximately 5 - 10 mg of the spray-dried powder was weighed into a pan and the pan was placed inside the measuring chamber. The powder sample was equilibrated at 0% RH to provide a baseline. The sorption mass change was measured at RH ranging from 0-90% (10 % RH steps) at 25ºC and desorption mass change was measured at RH ranging from 90-0%.

3.3.8 Surface Roughness Quantification

The surface topography and roughness of spray-dried Ciprofloxacin particles stored at 20% and 55% RH were evaluated using an atomic force microscopy (AFM; Veeco DI3100, Veeco Ltd (Current Bruker AFM), Santa Barbara, CA). AFM scanning was carried out using the tapping mode with a super sharp tip cantilever (TESP-SS-10, NanoAndMore Ltd, Watsonville, CA, USA) oscillating at 287 ± 2 kHz, with 5% offset frequency of first bending resonance. The images were captured at a scan rate of 1 Hz for a scan size of 1~2 μm and analyzed using Gwyddion software (Czech Metrology Institute, Brno, Czechia) extensively used for scanning probe microscopy data analysis [234]. Six images for each formulation were analyzed for the spray-dried Ciprofloxacin particles stored at 20% RH and 55% RH. Root mean square (RMS) was calculated based on the AFM images as the indicator of surface roughness. Higher RMS represents rougher surfaces.

3.3.9 Drug Quantification

The concentration of Ciprofloxacin in water was determined by high performance liquid chromatography (HPLC) using 76% v/v of 30 mM solution of sodium sulfate (adjusted to pH 2.5 with H_3PO_4) and 24% v/v acetonitrile as mobile phase resulting in isocratic elution of the sample

for 7 min with a flow rate of 1.0 mL/min. Briefly, the HPLC system consisted of G1311C (1260 Quat Pump VL) pump, G1330B (1290 Thermostate) thermostate, G1329B (1260 ALS) autosampler, G1316A (1260 TCC) thermostated column compartment, G1314F (1260 VWD) variable wavelength detector (Agilant, Waldbronn, Germany), and an Agilant Eclipse Plus, 5 μm C18 150 × 4.60 mm column (Agilant, Waldbronn, Germany). The calibration curve for Ciprofloxacin hydrochloride was linear (R^2> 0.999) over the concentration range of approximately 0.25 to 0.01 mg/mL.

3.3.10 In-vitro Aerosol Performance

Aerosolization performance of the powder formulations was measured using a Multi-Stage Liquid Impinger (MSLI) (Copley Scientific Limited, Nottingham, UK) with a USP induction port (USP throat). Prior to each run, Stage 1 to Stage 4 were filled with 20 mL of water and a 0.2 μm glass microfiber filter was placed at the bottom of the impinger base. The formulation (10 ± 1 mg) was weighed into a Size 3 hydroxypropyl methylcellulose capsule (Qualicaps, Whitsett, NC, USA). A standard dispersion procedure (USP 38) was carried out by passing 4L of air through the inhaler at an airflow of 100 L/min for 2.4 s, with a pressure drop of <4 kPa (approximately 3.2 kPa at 100 L/min) across a RS01 DPI device (Plastiape S.p.A., Osnago, Italy). The RS01 DPI device has a similar design and characteristics to the Osmohaler. Stages 1–4 of the liquid impinger at 100 L/min had cutoff diameters of 10.4, 4.9, 2.4, and 1.2 μm, respectively [235]. For the powder formulations stored at each relative humidity condition, four replicated experiments were carried out and results are averaged.

After the dispersion, the drug particles remained in the capsule and deposited in the inhaler device, USP throat, Stage 1-4 and filter paper were collected using MilliQ water. Drug contents

were analyzed using a validated HPLC method described above. The emitted dose was determined as the drug released from the capsule and device over the recovered dose; whereas the fine particle fraction was defined as particles with an aerodynamic size below 4.9 μm (cut-off diameter of Stage 2) relative to the total recovered drug.

3.3.11 Statistical Analysis

Statistical analysis was performed by one-way analysis of variance (ANOVA) with Tukey-Kramer post hoc tests using a GraphPad Prism Software (GraphPad Software, Inc., La Jolla, CA). Probability values of less than 0.05 were considered as a statistically significant difference and "NS" represents not significant ($p > 0.05$).

3.4 Results and Discussion

3.4.1 Spray-dried Formulations upon Storage at Various RHs

3.4.1.1 In-vitro Aerosol Performance

Aerosol deposition profiles of the spray-dried Ciprofloxacin formulations stored under 20% RH, 55% RH and 75% RH for one day are shown in Figure 3-1a. The corresponding emitted dose (ED) and fine particle fraction (FPF) values of spray-dried Ciprofloxacin particles stored under three different relative humidity conditions are summarized in Figure 3-1b. At 75% RH, emitted dose of the spray-dried Ciprofloxacin was found to be 87.9 ± 5.8 %, which was significantly higher than that stored at 55% RH (72.5 ± 1.7 %) and at 20% RH (69.9 ± 1.1 %). There was no statistical difference in the emitted dose for spray-dried Ciprofloxacin formulations stored at 55% RH and 20% RH (Figure 3-1b, $p > 0.05$). The FPF increased significantly from 28.0 ± 3.2 % at 20% RH to 35.6 ± 1.7 % upon storage at 55% RH for one day ($p < 0.05$). Additionally, there was a statistically significant decrease ($p < 0.01$) in the FPF when the samples were stored

at 75% RH (21.0 ± 5.8%) for one day as compared to those stored at 55% RH (FPF: 35.6 ± 1.7 %).

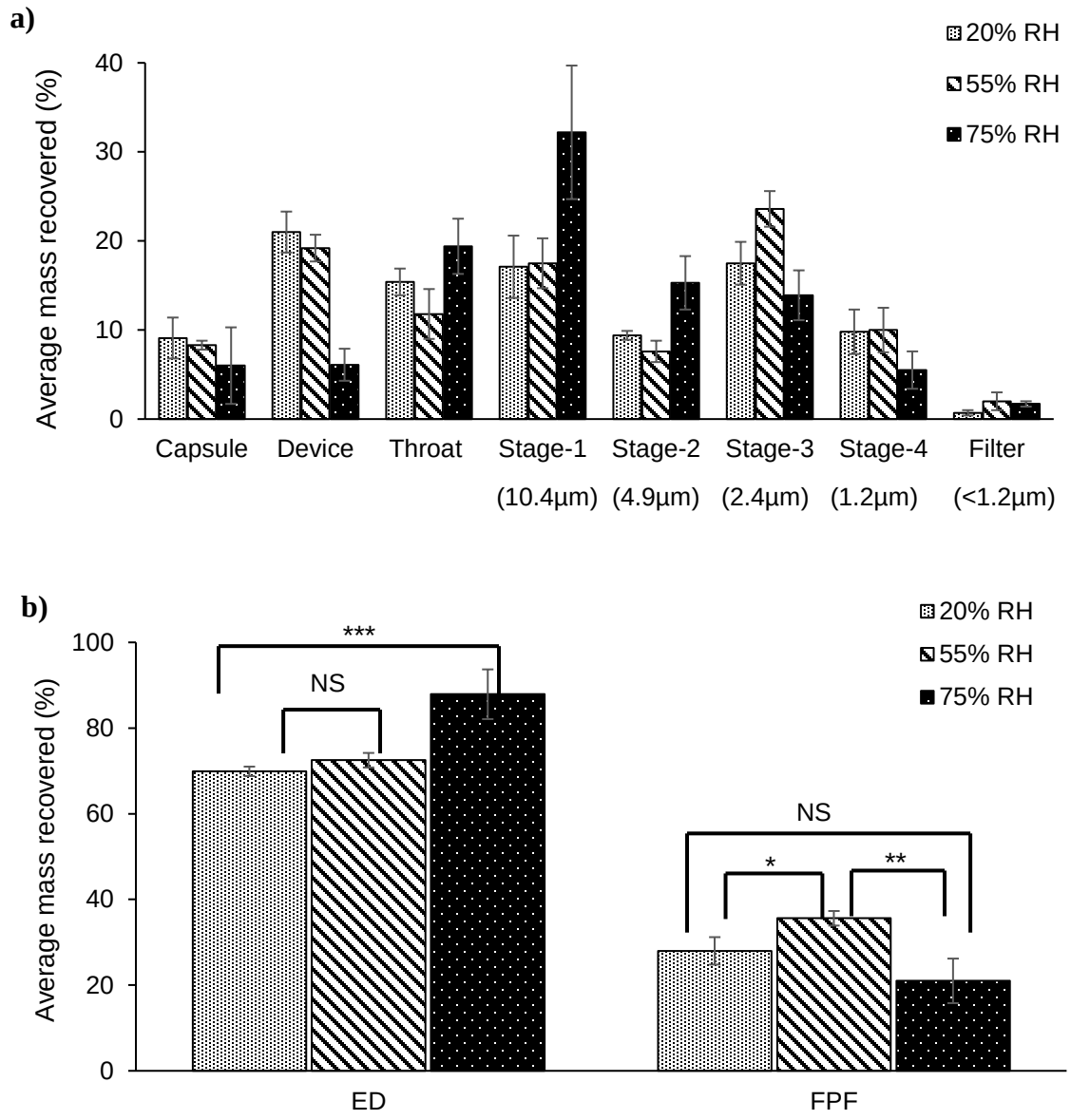


Figure 3-1**:** (a) Deposition profiles and (b) aerosol performance of the spray-dried Ciprofloxacin formulations as reflected by ED and FPF at different RHs (mean ± SD, n=4; *, p <0.05; **, p <0.01; ***, p < 0.001; NS, no significant difference)

The increase in FPF at 55% RH was attributed to increased deposition of drug particles on the stage 3, stage 4 and filter whereas the decrease in FPF at 75% RH was attributed to higher deposition of particles in the USP throat, stage 1 and stage 2 (Figure 3-1a). The instability in aerosol performance upon storage at different humidity conditions could be of great concern in terms of clinical efficacy and safety. Thus, it is crucial to understand the physicochemical behavior of amorphous spray-dried powders at an early formulation development stage as it could have significant impacts on manufacturability, stability and drug delivery performance [236]. Since the physical size of inhalable particles may have a significant influence on aerosol deposition [237], it was measured for the spray-dried Ciprofloxacin powders stored at different RHs.

Table 3-1: Particle size distributions of the spray-dried Ciprofloxacin powders (n = 100).

Formulation	**D_{10} (µm)**	**D_{50} (µm)**	**D_{90} (µm)**
SD Ciprofloxacin (20% RH)	0.91	1.56	2.39
SD Ciprofloxacin (55% RH)	1.20	1.73	2.54

Table 3-1 shows the physical particle size distributions of spray-dried Ciprofloxacin powders stored at 20% and 55% RH. The particle size distribution of spray-dried Ciprofloxacin powder stored at 75% RH could not be measured because the particles fused and formed coarse lumps. Agglomeration or increased particle size enabled easy flow of the powders from the capsule and device upon storage at 75% RH. Thus, the emitted dose for spray-dried Ciprofloxacin powders stored at 75% RH was significantly higher as compared to 20% and 55% storage humidity

(Figure 3-1a). No significant difference in particle size between 20 % RH and 55% RH was observed and the changes in aerosol performance were not due to altered physical particle size distributions.

3.4.1.2 Particle Morphology

Scanning electron micrographs (SEM) showed that the spray-dried Ciprofloxacin powder stored under 20% RH exhibited a near-spherical shape with relatively smooth surfaces and some concaveties (Figure 3-2a). However, those powders stored at 55% RH had much rougher surfaces (Figure 3-2b) compared to 20% RH samples (Figure 3-2a). On the other hand, the samples stored at 75% RH were fused into irregular lumps (Figure 3-2c). As suggested by Visser et al, at elevated relative humidity (≥ 65%) the particles are likely to be held together by capillary force due to presence of excess moisture thus resulting in fusion of particles [151]. Similar observations were made by Zhou et al. where the spray-dried formulations of Colistin were hygroscopic and the particles fused together upon storage at 90% RH [224].

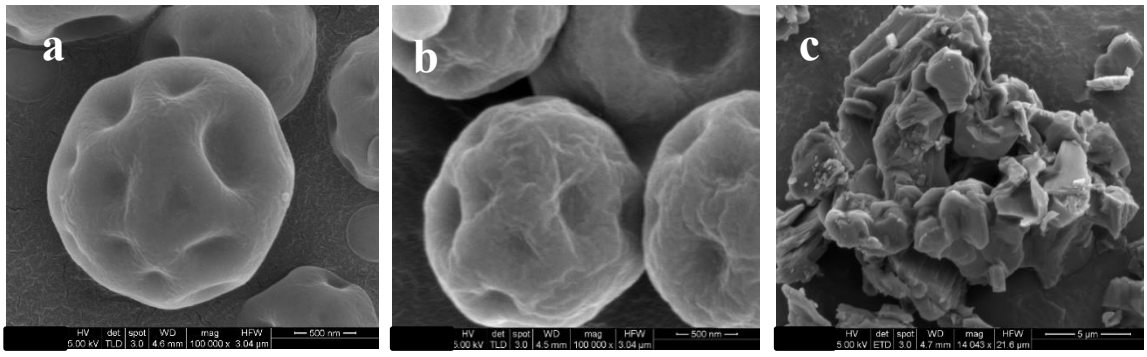


Figure 3-2: SEM micrographs of the spray-dried Ciprofloxacin powders that were stored at (a) 20% RH (b) 55% RH and (c) 75% RH for one day.

Chew and Chan demonstrated that increasing the surface roughness of the inhalable particles could lower the area of contact, and thus reduce the powder cohesiveness [226]. In our study, the

change in morphology for spray-dried Ciprofloxacin particles stored at 55% RH and 75% RH is most likely responsible for the change in aerosol performance.

3.4.1.3 Dynamic Vapor Sorption

Moisture sorption isotherm for spray-dried Ciprofloxacin formulation is shown in Figure 3-3. The sorption behavior of spray-dried Ciprofloxacin formulation was found to increase with increasing relative humidity (8.1% ((w/w)) moisture uptake) up to approximately 60% RH beyond which a decrease in mass was observed, indicating loss of moisture and crystallization. As relative humidity was further increased from 70% to 90% a small amount of moisture was absorbed presumably on the surface of the crystalline Ciprofloxacin [15]. The increase in weight from the starting point of the sorption run to the end of the desorption run indicates moisture-induced crystallization of amorphous spray-dried Ciprofloxacin powders.

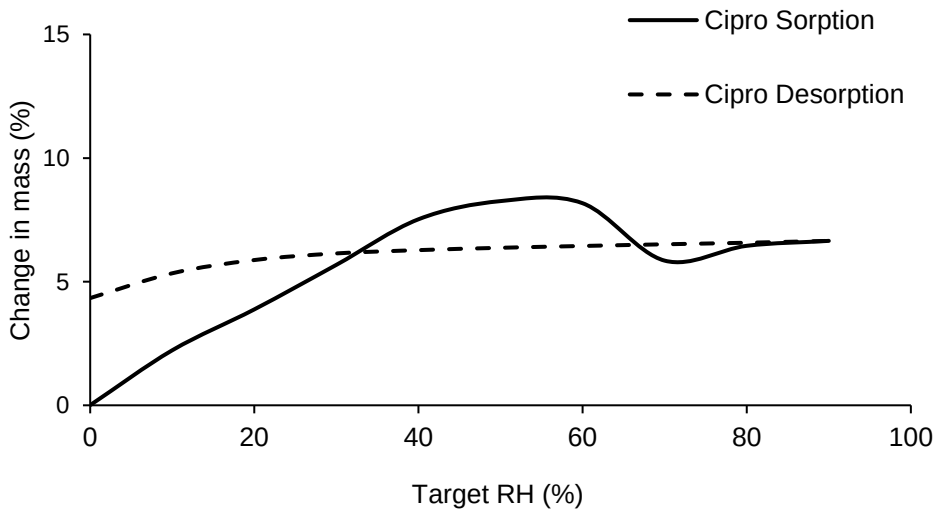


Figure 3-3: Moisture sorption isotherm for spray-dried Ciprofloxacin formulation

3.4.1.4 Solid-state Characterization

To understand the underlying cause for the changes in surface morphology of spray-dried powder formulations, we examined crystallinity of Ciprofloxacin particles stored at three different humidity conditions using PXRD. Sharp peaks were observed for the spray-dried Ciprofloxacin formulations stored at 55% RH and 75% RH for one day (Figure 3-4). Whereas, no crystallinity was detected for the samples stored at 20% RH, indicating the amorphous nature (Figure 3-4). Degree of crystallization was much higher for the spray-dried Ciprofloxacin powders stored at 75% RH as opposed to the powders stored at 55% RH for one day. PXRD patterns for the samples stored at 75% RH were similar to the raw Ciprofloxacin hydrochloride monohydrate. However, PXRD pattern for spray-dried Ciprofloxacin stored at 55% RH exhibited presence of a novel metastable polymorphic form with a distinct peak at 5.8º 2 theta.

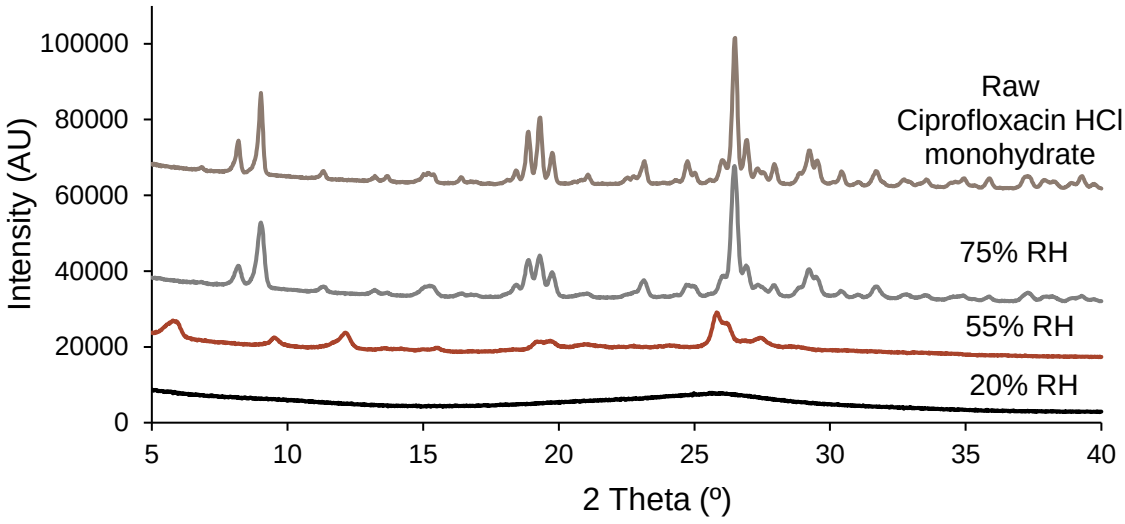


Figure 3-4: X-ray powder diffraction (PXRD) patterns of the spray-dried Ciprofloxacin formulations stored at 20% RH, 55% RH and 75% RH for one day and raw Ciprofloxacin.

DSC thermograms of the raw and spray-dried Ciprofloxacin samples stored at different relative humidity conditions after one day are shown in Figure 3-5. For the raw Ciprofloxacin powder,

first endothermic peak around 158ºC could be attributed to dehydration (Ciprofloxacin hydrocloride monohydrate was used here) [238]. Second endothermic peak between 305°C to 320ºC corresponded to the characteristic melting peak of Ciprofloxacin hydrochloride [238]. For the spray-dried Ciprofloxacin samples stored at 20% RH, an exothermic peak (heat of crystallization, ΔH=40.36 J/g) was observed around 198ºC indicative of a phase transition of amorphous Ciprofloxacin samples; whereas, the endothermic peak around 321ºC represents melting of the sample. Similarly, exothermic peaks were observed around 198ºC for the spray-dried Ciprofloxacin samples stored at 55% RH and 75% RH with heat of crystallization corresponding to 29.84 J/g and 23.43 J/g, respectively. The heat of crystallization decreased from 40.36 J/g for amorphous spray-dried Ciprofloxacin at 20% RH to 23.43 J/g for the recrystallized samples stored at 75% RH, indicating a decrease in amorphous content/polymorphic transition upon storage at the high humidity.

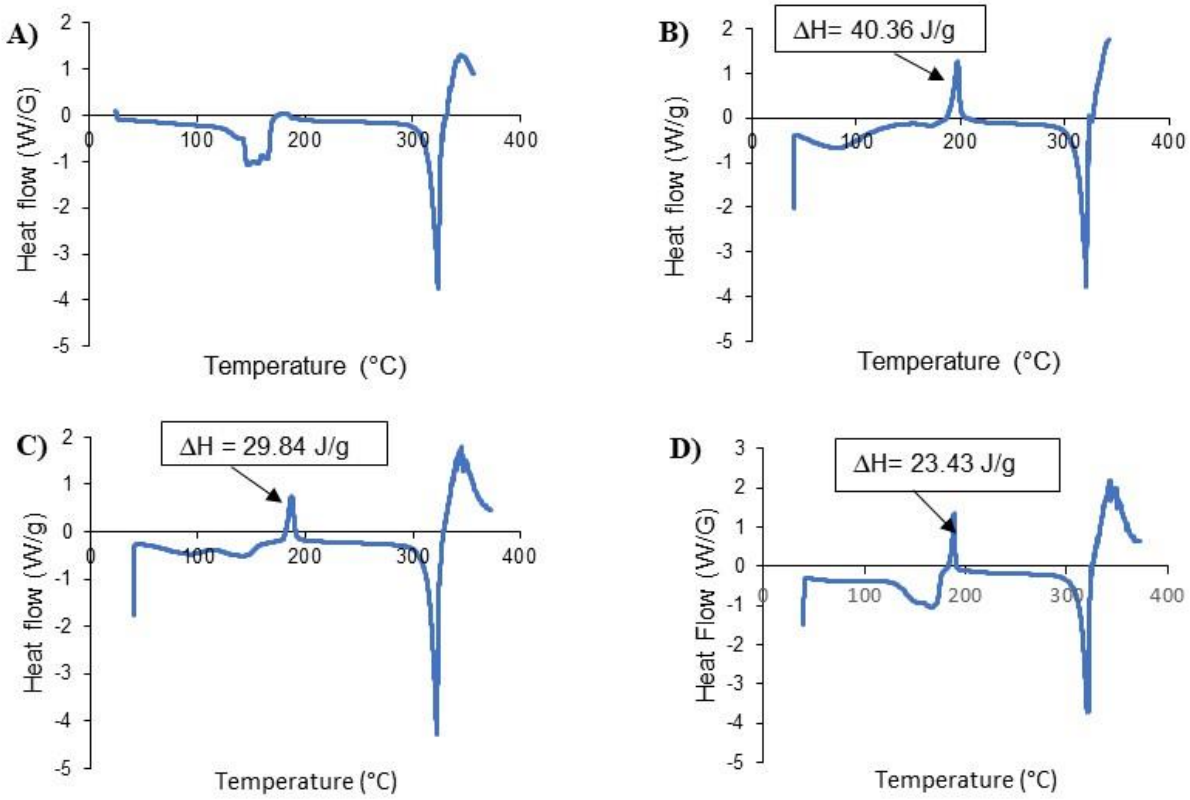


Figure 3-5: DSC thermograms of A) raw Ciprofloxacin; B) SD Ciprofloxacin at 20% RH; C) SD Ciprofloxacin at 55% RH; D) SD Ciprofloxacin at 75% RH for one day. ΔH is the heat of crystallization.

3.4.2 Mechanisms of Moisture-induced Crystallization on Aerosol Performance

Physical instability is a common drawback associated with spray-dried amorphous particles [11]. Depending upon moisture, temperature and time of storage, these metastable amorphous spray-dried particles may tend to convert to its more stable crystalline form [11]. Presence of water may promote the crystallization of spray-dried samples as water molecules increase the mobility of the drug molecule [239]. Crystallization of amorphous powders upon storage could cause severe stability issues. There have been studies showing water-induced crystallization of different spray-dried powders upon exposure to higher relative humidity [11] but impact of

crystallization on in-vitro aerosol performance has not been systematically studied. Our work has demonstrated the substantial impacts of moisture-induced crystallization on aerosol performance of the spray-dried drug powders such as Ciprofloxacin (Figure 3-6).

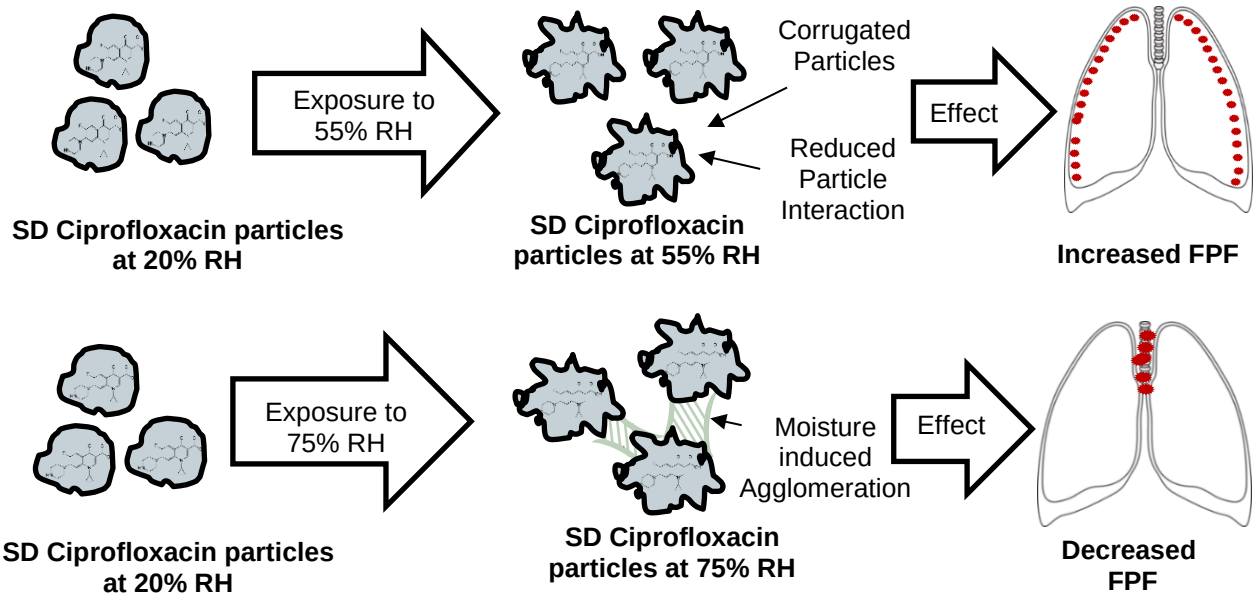


Figure 3-6: Schematic Diagrams showing different effects of moisture-induced crystallization on particle morphology and aerosol performance when spray-dried amorphous Ciprofloxacin particles were stored at various RHs for 24 hours.

Our hypothesis is that crystallization of amorphous spray-dried Ciprofloxacin on exposure to 55% RH is responsible for the increased surface roughness and decreased particle-particle interactions. The cohesive forces between particles with corrugated surfaces are lower thereby reducing the contact areas between particles and improving the aerosolization performance (Figure 3-6). However, storage of the spray-dried Ciprofloxacin powders at 75% RH lead to fusion of particles along with crystallization due to presence of excess moisture resulting in decreased aerosol performance. Since the spray-dried Ciprofloxacin stored at 55% RH did not crystallize fully as compared to spray-dried Ciprofloxacin powders stored at 75% RH (Figure 3-4

& 3-5), the crystallization of spray-dried Ciprofloxacin formulations at 55% RH was further examined for up to 3 weeks to study longer-term stability.

3.4.3 Correlations between Crystallinity and Aerosol Performance at 55% RH

The amorphous nature of the spray-dried Ciprofloxacin formulations stored at 20% RH was well maintained for up to 3 weeks; In contrast, a progressive increase in the crystallinity was observed for the spray-dried Ciprofloxacin samples stored at 55% RH (Figure 3-7). Impact of increasing degree of crystallinity on aerosol performance was further investigated.

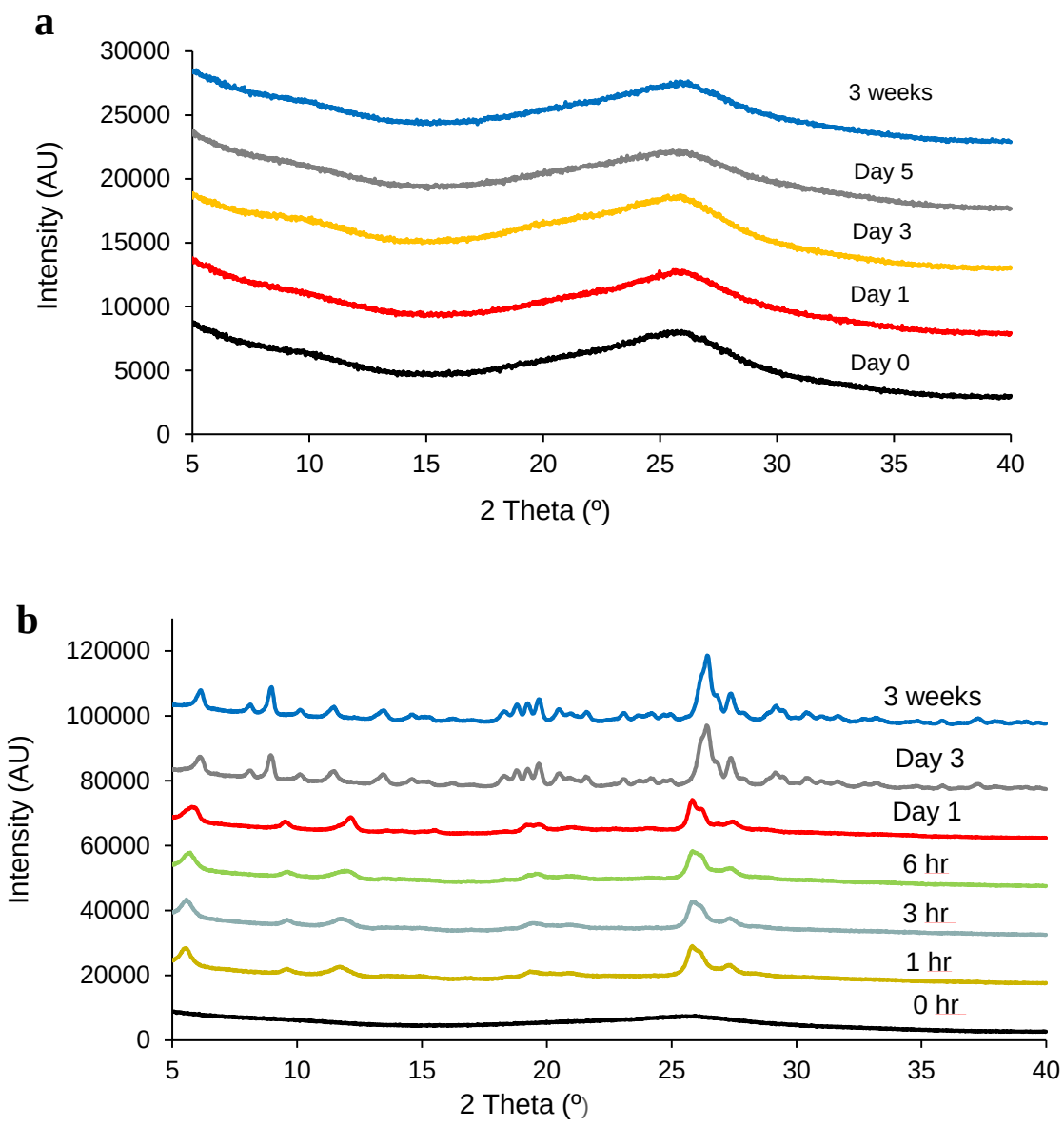


Figure 3-7: PXRD patterns of the spray-dried Ciprofloxacin formulations at (a) 20% RH and (b) 55% RH over the period of 3 weeks. The percentage values in (b) are estimated crystallinity.

Aerosol deposition profiles of the spray-dried Ciprofloxacin formulations stored at 55% RH at specific time points (Day 1, Day 3 and 3 weeks) and at 20% RH for one day are shown in Figure 3-8a. The corresponding ED and FPF values are summarized in Figure 3-8b. ED of the spray-dried Ciprofloxacin particles increased significantly from Day 1 (72.5 ± 1.7%) to Day 3 (86.5 ±

2.0%) upon storage at 55% RH (p <0.0001). FPF of the spray-dried Ciprofloxacin formulation stored at 55% RH increased significantly from 35.5 ± 1.7% at Day 1 to 42.3 ± 0.9% at Day 3 (p <0.01). The increase in FPF of spray-dried Ciprofloxacin particles at 55% RH could be attributed to much higher deposition in stage 4 at Day 3 in comparison to day 1 (Figure 3-8a). Thus, the powders showed improved flowability along with improved aerosol performance. However, the FPF did not increase any further after Day 3 and was plateaued until 3 weeks (40.2 ± 1.2%).

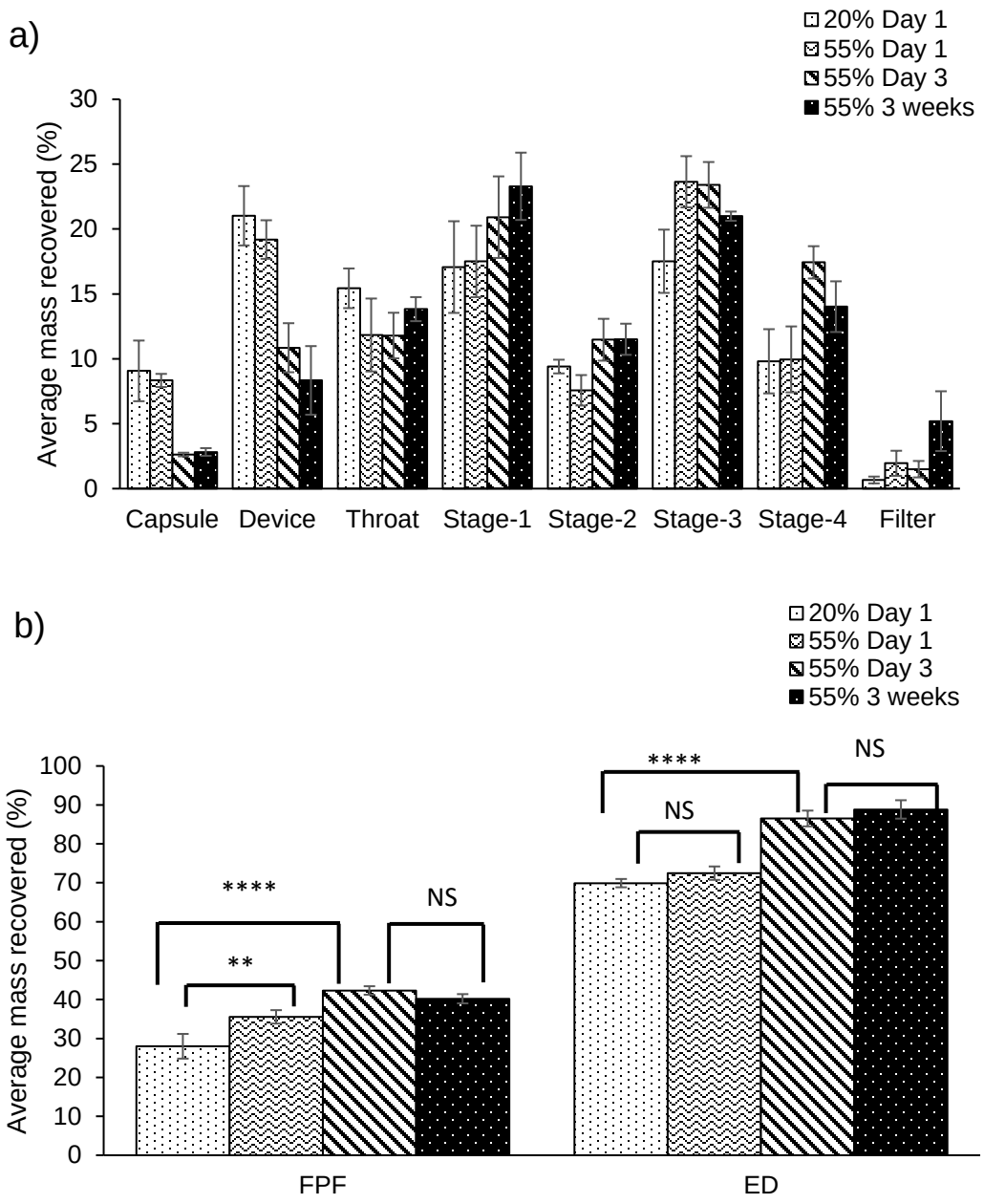


Figure 3-8: (a) Aerosol deposition profiles (b) aerosol performance of the spray-dried formulations at 55% RH at specific days (mean ± SD, n=4; *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$; NS, no significant difference)

Further, a progressive change in surface morphology of the spray-dried Ciprofloxacin particles from smooth to rough was observed upon storage at 55% RH for 3 weeks (Figure 3-9). The increase in surface roughness was attributed to progressive increase in crystallization of the

spray-dried Ciprofloxacin particles upon exposure to humidity. The increase in surface roughness for spray-dried Ciprofloxacin particles stored at 55% RH compared to 20% RH quantified using AFM is shown in Figure 3-10. From the AFM images it is visually evident that the spray-dried Ciprofloxacin particles stored at 55% RH (crystallized) are rougher in comparison to the spray-dried Ciprofloxacin particles stored at 20% RH (amorphous) (Figure 3-10). Quantitatively, RMS value for the spray-dried Ciprofloxacin particle at 55% RH (24.7 ± 4.3 nm) was significantly higher than that of the amorphous Ciprofloxacin particles stored at 20 % RH (9.7± 3.2 nm). Increased percent crystallinity led to proportional increase in surface roughness and reduction in particle-particle interaction thereby improving aerosol performance. This is in agreement with the previous findings that rougher surfaces resulted in less particle-particle contact area and therefore weaker interpaticulate interactions [226, 240].

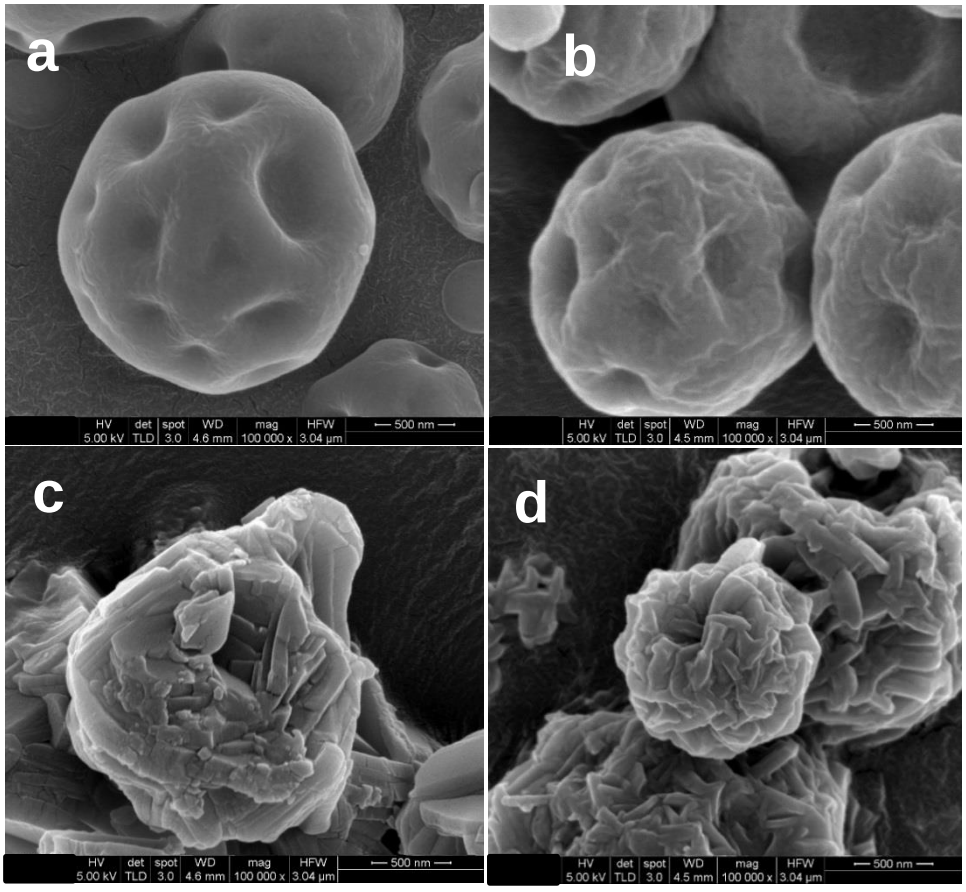


Figure 3-9: SEM micrographs showed changes in surface roughness of: (a) amorphous spray-dried Ciprofloxacin particles stored at 20% RH, and the crystallized spray-dried Ciprofloxacin particles stored at 55% RH for: (b) 1 day; (c) 3 days; and (d) 3 weeks.

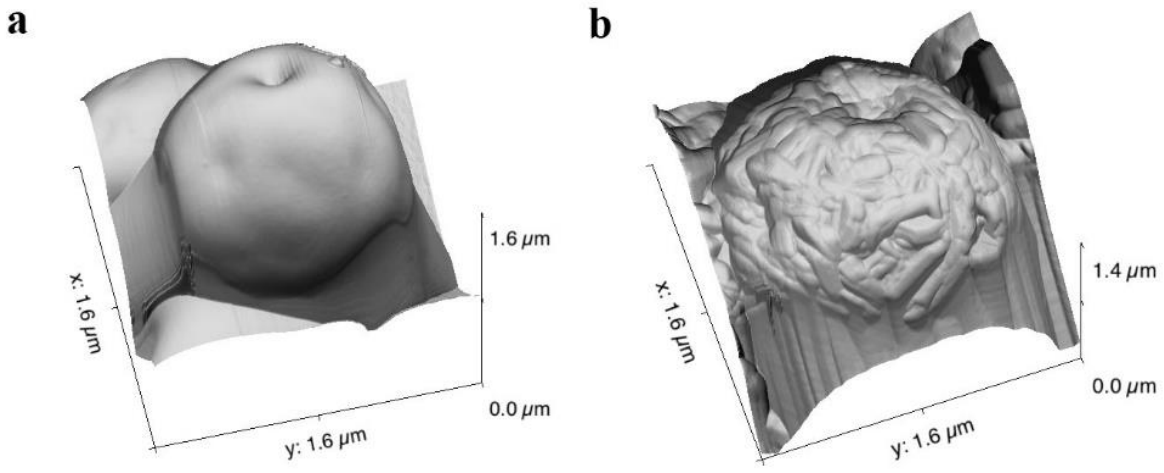


Figure 3-10: Representative AFM 3D-surface topography of the spray-dried Ciprofloxacin particles stored at (a) 20% RH (amorphous) and (b) 55% RH (recrystallized).

The AFM data support our hypothesis that crystallinity has an influence on surface roughness and aerosol performance. Surface roughness increased with degree of crystallinity and thereby impacted aerosol performance for the spray-dried Ciprofloxacin particles stored at 55% RH.

3.5 Conclusions

The present study provides fundamental understanding in physical instability of spray-dried drug particles and the impact on aerosol performance. Changes in aerosol performance of the spray-dried Ciprofloxacin powder formulations upon storage were attributed to crystallization that altered surface morphology. Improvement in aerosol performance for the spray-dried Ciprofloxacin formulation stored at 55% RH was due to moisture-induced crystallization thereby causing an increase in surface corrugation and decrease in particle-particle interactions. The decrease in aerosol performance at 75% RH was the consequence of particle fusion in presence of excess moisture. The degree of crystallinity was found to increase with time and resulted in a proportional increase in surface roughness and aerosol performance for the spray-dried

Ciprofloxacin formulations stored at 55% RH. The results of this study emphasize the importance of storage humidity on physical stability and aerosol performance of the spray-dried powder formulations. For the amorphous DPI formulations that were physically unstable when they are exposed to moisture, strict packaging or moisture protection strategies such as particle coating [67] should be considered to maintain the quality of the products.

CHAPTER 4. INFLUENCE OF EXCIPIENTS ON PHYSICAL AND AEROSOLIZATION STABILITY OF SPRAY-DRIED HIGH DOSE POWDER FORMULATION FOR INHALATION*

*Published by- **Shetty, N.;** Park, H.; Zemlyanov, D.; Mangal, S.; Bhujbal. S.; Zhou, Q. T. Influence of excipients on physical and aerosolization stability of spray-dried high-dose powder formulations for inhalation

4.1 Abstract

The aim of this study is to investigate the influence of excipients on physical and aerosolization stability of spray-dried amorphous dry powder inhaler formulations. The model drug, Ciprofloxacin hydrochloride, was co-spray-dried with excipients such as disaccharides (sucrose, lactose, trehalose), mannitol and L-leucine. The spray-dried samples were stored at two different relative humidity (RH) conditions of: (1) 20% and (2) 55% RH at 25ºC. Ciprofloxacin co-spray-dried with disaccharides and L-leucine in the mass ratio (1:1) demonstrated an increase in fine particle fraction (FPF) as compared with the spray-dried Ciprofloxacin alone when stored at 20% RH. However, deterioration in FPF of Ciprofloxacin co-spray-dried with disaccharide and mannitol was observed upon storage at 55% RH as compared to the corresponding formulations stored at 20% RH due to particle agglomeration. Whereas, 10% and 50% (w/w) L-leucine in the formulation showed no change in aerosol performance (FPF of 71.1 ± 3.5%. and 79.5 ± 3.1%, respectively) when stored at 55% RH for 10 days as compared to 20% RH (FPF of 68.1 ± 0.3% and 73.6 ± 7.1% respectively). L-leucine demonstrated short-term aerosolization stability by inhibiting crystallization of Ciprofloxacin to some extent and preventing significant change in particle morphology. L-leucine is well-recognized as aerosolization enhancer; our study has shown L-leucine is also a physical and aerosolization stabilizer for spray-dried amorphous DPI formulations. Such stability enhancing activities were attributed to the enrichment of L-leucine

on the particle surface as confirmed by XPS data, and intermolecular interactions between L-leucine and Ciprofloxacin as measured by FT-IR.

4.2 Introduction

Dry powder inhalers (DPIs) are popular for pulmonary drug administrations as they are portable, easy to operate and cost effective [218, 219]. DPIs have been used for treating asthma and chronic obstructive pulmonary disease (COPD) for decades, and more recently they have been developed for treating lung infections [5, 241]. DPIs consist of a powder formulation and a device. The drug powder is usually micronized and aerosolized into fine particles through an inhaler device. Direct delivery of antibiotics into the lungs through DPIs will lead to substantially higher drug concentrations at the site of infection, which can maximize therapeutic efficacy and minimize systemic toxicity against lung infections [242, 243].

DPI powder formulation essentially consist of micronized drug either with or without a carrier in the dry powder form. No propellants are used in case of DPIs [43] but for passive inhalers the entrainment of powders from the device and aerosolization may depend on patients inspiration capability, which could result in inconsistent flow rate and variable dose delivery for patients with compromised lung functions [48]. Deposition of these fine powders in the lower respiratory tracts depends upon the interactions between particles, lung deposition mechanisms and powder properties such as particle size distribution, surface morphology, crystallinity, hygroscopicity and surface energy etc. [5]. Therefore, DPI performance can be significantly impacted by modification of the physicochemical properties of powders. The physicochemical properties of powders can be altered by particle-engineering technologies such as spray-drying. Very recently, spray-drying has been intensively explored to engineer drug particles because micronized drug

particles produced by the milling process are extremely cohesive and prone to agglomeration [69, 80, 81].

However, using spray-drying technique to develop a DPI formulation with satisfactory aerosol performance while maintaining physical stability could be challenging [241, 244]. For example, spray-dried technology has been widely used to produce DPI formulations [83] but many spray-dried compounds are amorphous in nature and physically unstable [244, 245]. Results from our earlier study have shown that the spray-dried amorphous Ciprofloxacin powder crystallized when stored at 55% RH, which led to increased surface roughness and enhanced aerosolization [75]. Furthermore, these amorphous powders often can absorb significant amounts of moisture under humid conditions [15, 67], which likely result in deterioration in aerosolization due to increased inter-particulate capillary forces [246, 247]. There is a need to formulate DPIs which are physically stable and have consistent aerosol performance when they are stored at varying conditions [48].

Excipients such as lactose monohydrate have been used as a coarse carrier for low-dose DPIs [248]; other excipients such as mannitol, trehalose, and L-leucine have been added in the spray-dried DPI formulation with a main purpose to improve aerosolization performance or act as fillers. Lactose is the most common and frequently used carrier in DPIs formulations as it is nontoxic, stable, and is compatible with most of the active drugs [239, 249]; use of lactose as a carrier in DPI formulations is well established [126, 250]. But for high-dose antibiotics, use of coarse carriers is limited to minimize the powder volume [5]. Therefore, here we are focusing on excipients for spray-dried DPI formulations, not as coarse carriers.

Mannitol, a hexahydric alcohol, has been frequently examined as a spray-drying component for DPI formulations [251]. Mannitol was found to provide satisfactory aerosol performance and physical stability when added at a concentration of 50% in the co-spray-dried Ciprofloxacin-mannitol formulation [252]. Further, inhaled mannitol may help to remove mucous in patients with cystic fibrosis thereby improving penetration into the mucus and therapeutic efficiency of antibiotics [253]. Mannitol in its amorphous form was shown to maintain stability of proteins such as salmon calcitonin due to hydrogen bond formation and thus can be used as a stabilizing agent with protein DPI formulations at low relative humidity [245].

Trehalose is a disaccharide similar to lactose but it differs from lactose as it is a non-reducing sugar. Unlike lactose, trehalose does not have Maillard reactions with polypeptides or protein-type drugs. Trehalose has been used as a carrier for DPI formulation of albuterol sulfate, ipratropium bromide monohydrate, disodium cromoglycate and fluticasone propionate etc. [254, 255]. Inhalable trehalose microparticulate powders with low water content were produced by spray-drying their methanolic solution or by using other more innovative drying techniques such as spray freeze drying and supercritical fluid drying. [256, 257]. Cline et al. reported better aerosol performance of drugs such as albuterol and ipratropium when co-spray-dried with trehalose as compared to lactose or mannitol [254].

Some amino acids like L-leucine and tri-leucine are also known to enhance the dispersion performance of powders for inhalation [76, 258-260]; Its capability to protect spray-dried inhalation powders against moisture is also reported [154, 261]. It has been demonstrated that co-spray-drying active ingredients with leucine will result in enrichment of leucine on particle

surface and changes in surface morphology of spray-dried particles [154, 262, 263], which lead to reduced surface energy and improved aerosol performance [25]. However, the effects of excipients, particularly L-leucine on the physical and aerosolization stability of spray-dried amorphous DPI formulations have not been systemically examined.

The aim of present study was to evaluate the influence of excipients such as lactose, sucrose, trehalose, mannitol and L-leucine on physical and aerosolization stability of spray-dried Ciprofloxacin HCl on storage at different humid conditions. Ciprofloxacin was specifically chosen as a model drug as it is one of potent antibacterial drugs, effective against a wide range of Gram-positive and Gram-negative pathogens, including Pseudomonas aeruginosa that is associated with respiratory tract infections. Secondly based on our preliminary findings, spray-dried amorphous Ciprofloxacin powder tended to crystallize on storage at relative humidity (RH) > 55%, which led to a significant change in aerosol performance [75]. It is important to examine the effects of excipients on physical stability and aerosolization of spray-dried DPI formulations with a purpose to ensure quality.

4.3 Materials and Methods

4.3.1 Chemicals

Ciprofloxacin hydrochloride monohydrate was purchased from Betapharma (Shangai) Co. Ltd. (Wujiang, China). Lactose was purchased from Avantor Performance Materials, Inc. (Center Valley, PA, USA). D-Mannitol was supplied by Dot scientific inc (Burton, MI, USA). Trehalose was purchased from Spectrum Chemical Mfg. Corp. (Gardena, CA, USA). L-leucine was supplied

by Sigma-Aldrich (St. Louis, Missouri, USA). High performance liquid chromatography (HPLC)

grade acetonitrile and sucrose was supplied by Fischer scientific (Fair Lawn, NJ, USA).

4.3.2 Spray-drying

A BUCHI B-290 mini spray dryer (BUCHI Labortechnik AG, Flawil, Switzerland) was used to prepare formulations of Ciprofloxacin with each of the following excipients (i.e. sucrose, lactose, trehalose, mannitol and L-leucine) in the mass ratio (1:1) using water as the solvent. Feed solution was pumped at the rate of 2 mL/min. Spray-drying parameters were kept constant for each formulation: inlet air temperature (T_{in}) 120 ± 2ºC, aspirator at 35 m^3/h and airflow of 700 L/h. These conditions resulted in an outlet temperature (T_{out}) of 60 ± 2 °C. The spray-dried powders were divided into 2 equal parts; one half was stored in desiccators containing silica gel maintaining RH of 20 ± 2% and the other half in a humidity chamber maintaining RH of 55 ± 2% RH using the saturated magnesium nitrate solution, which were selected based on the previous work [75].

4.3.3 X-ray powder diffraction (PXRD)

Rigaku Smartlab™ diffractometer (Rigaku Americas, Texas, USA) was used to determine powder crystallinity. Powders were scanned in the range of 5 to 40° 2θ at a step size of 0.02°and a scan rate of 4°/min. Cu-Kα radiation source and a highly sensitive D/tex ultra-detector was used. A voltage of 40kV and current of 44 mA was used for the experimental operation.

4.3.4 Modulated DSC

A Q2000 DSC (TA Instruments, New Castle, DE) equipped with a refrigeration cooling system (RCS) was used to determine glass transition temperature (Tg) using reversible heat flow and the Universal Analysis 2000 software (TA Instruments, New Castle, DE). Prior to sample analysis, the enthalpic response was calibrated using indium, and the temperature scale was calibrated using

indium and tin. Dry nitrogen at 50 mL/min was used as the purge gas. 3-5 mg of sample powder was placed in TZero™ non-hermetic aluminum pans (TA Instruments, New Castle, DE). The samples were equilibrated at 0°C, and then to 200°C with heating rate of 2°C/min and a modulation of 1°C every 60s.

4.3.5 Scanning electron microscopy (SEM)

Particle morphology was assessed by scanning electron microscopy (NOVA nanoSEM, FEI Company, Hillsboro, Oregon, USA). Samples were smeared on a rectangular carbon tape and coated with platinum (208 HR, Cressington Sputter Coater, England, UK) at 40 mA for 60 seconds. The platinum coated samples were observed under SEM and the images were captured at 5 kV.

4.3.6 Particle size

Particle sizes of the powder formulations were measured using an Image J software based on SEM images. Image J is a java based, open source image processing analysis program developed by the National Institute of Health [264]. The diameters at 10% (d_{10}), 50% (d_{50}) and 90% (d_{90}) undersize were calculated for approximately 100 particles, which were sufficient for size analysis [75].

4.3.7 Dynamic vapor sorption (DVS)

Moisture sorption behavior for the spray-dried formulations were determined using dynamic vapor sorption (DVS-Intrinsic, Surface Measurement Systems Ltd., London, UK). Each formulation was equilibrated at 0% RH at the beginning to provide a baseline and then exposed to different RH. The sorption mass change was measured at RH ranging from 0 − 90% at 10 % RH increments at 25ºC and desorption mass change was measured at RH ranging from 90 − 0%.

At each testing RH moisture content was determined by a dm/dt of 0.002% per minute.

4.3.8 Solid state Fourier transform infrared spectroscopy (FTIR)

A Cary 600 series IR spectrophotometer (Agilent Technologies, Santa Clara, California, USA) equipped with an attenuated total reflectance (ATR) sample stage was used to analyze potential solid-state properties for co-spray-dried formulations of Ciprofloxacin-L-leucine in the mass ratios of 1:1 and 9:1. Samples were analyzed at a resolution of 4 cm^{-1} in the range on 400 – 4000 cm^{-1}. To minimize the interferences of water and CO_2 signals, background scans were collected before collecting the sample spectra [265].

4.3.9 X-ray photoelectron spectroscopy (XPS)

Surface composition was quantified using X-ray photoelectron spectroscopy (XPS) (AXIS Ultra DLD spectrometer, Kratos Analytical Inc., Manchester, UK) with monochromic Al Kα radiation (1486.6 eV) at pass energy (PE) of 20 and 160 eV for high-resolution and survey spectra, respectively. A commercial Kratos charge neutralizer was used to avoid non-homogeneous electric charge of non-conducting powder (in this case, the powders were conducting) and to achieve better resolution. Typical instrument resolution for PE of 20 eV is ~0.35 eV. Binding energy (BE) values refer to the Fermi edge and the energy scale was calibrated using Au 4f7/2 at 84.0 eV and Cu 2p3/2 at 932.67 eV. Powder samples were placed on a stainless-steel sample holder bar using a double-sided sticking Cu tape. XPS data were analyzed with CasaXPS software version 2313 Dev64. Prior to data analysis, the C-C component of the C 1s peak was set to a binding energy of 284.8 eV to correct for charge on each sample. Curve-fitting was performed following a Shirley background subtraction using model peaks obtained from pure compounds. The atomic concentrations of the elements in the near-surface region were estimated after a Shirley background subtraction taking into account the corresponding Scofield atomic

sensitivity factors and inelastic mean free path of

photoelectrons using standard procedures in the CasaXPS software assuming homogeneous mixture of the elements within the information depths (~10 nm).

4.3.10 Drug quantification

76% v/v of 30mM solution of sodium sulfate (adjusted to pH 2.5 with H_3PO_4) and 24% v/v acetonitrile was used as the mobile phase to determine the concentration of Ciprofloxacin hydrochloride in water using high performance liquid chromatography (HPLC). Isocratic elution of the sample was carried out for 3 min at a flow rate of 1.0 mL/min with a retention time of 2.2 mins for Ciprofloxacin. The calibration curve for Ciprofloxacin hydrochloride was linear ($r^2 >$ 0.99) over the concentration range of approximately 0.201 to 0.006 mg/ml. Briefly, the HPLC system consisted of G1311C (1260 Quat Pump VL) pump, G1330B (1290 Thermostate) thermostate, G1329B (1260 ALS) autosampler, G1316A (1260 TCC) thermostated column compartment, G1314F (1260 VWD) variable wavelength detector (Agilant, Waldbronn, Germany), and an Agilant Eclipse Plus, 5 µm C18 150 × 4.60 mm column (Agilant, Waldbronn, Germany).

4.3.11 In-vitro aerosol performance

A Multi-Stage Liquid Impinger (MSLI) (Copley Scientific Limited, Nottingham, UK) with a USP induction port (USP throat) was used to determine aerosolization performance of the powder formulations. 20 mL of water was added in each of the stages (1-4), and a 0.2 µm glass microfiber filter was placed at the bottom of the device base. The formulation (10 ± 1 mg) was weighed into a size 3 hydroxypropyl methylcellulose capsule (Qualicaps, Whitsett, NC, USA). 4L of air was passed through the inhaler at an airflow of 100 L/min for 2.4 s, with a pressure

drop of approximately 4 kPa across a RS01 DPI device. Stages 1 – 4 of the liquid impinger at 100 L/min had cutoff diameters of 10.4, 4.9, 2.4, and 1.2 μm, respectively [235]. For the powder formulations stored at 20% and 55% relative humidity conditions, four replicated dispersions were carried out and each experiment comprised sequential dispersion of two filled capsules. MilliQ water was used to collect the drug particles remaining in the capsule, inhaler device, USP throat, Stage 1-4 and the filter paper in the impactor base. Drug contents were analyzed using a validated high-performance liquid chromatography (HPLC) method described above. The emitted dose was determined as the drug released from the capsule and device over the total recovered drug; whereas the fine particle fraction was defined as particles with an aerodynamic size below 4.9 μm relative to the total recovered drug.

4.3.12 Statistical analysis

One-way analysis of variance (ANOVA) with Tukey-Kramer post hoc tests were used for statistical analysis using a GraphPad Prism Software (GraphPad Software, Inc., La Jolla, CA). Samples were considered to be significantly different if probability value (p) was less than 0.05 and not significant (NS) if $p > 0.05$.

4.4 Results

4.4.1 Effects of excipients on physical and aerosol stability upon storage at 20% RH

4.4.1.1 PXRD

Co-spray-dried formulations of Ciprofloxacin with sucrose, lactose and trehalose were amorphous at 20% RH for 1 day; the formulation with mannitol was crystalline. The crystalline peaks of mannitol formulation corresponded to the spray-dried mannitol, not to Ciprofloxacin. Also, the co-spray-dried Ciprofloxacin-L-leucine formulation was found to be crystalline and

peaks corresponded to the spray-dried L-leucine (Figure 4-1). Thus, in all formulations stored at 20% RH, Ciprofloxacin was in the amorphous form.

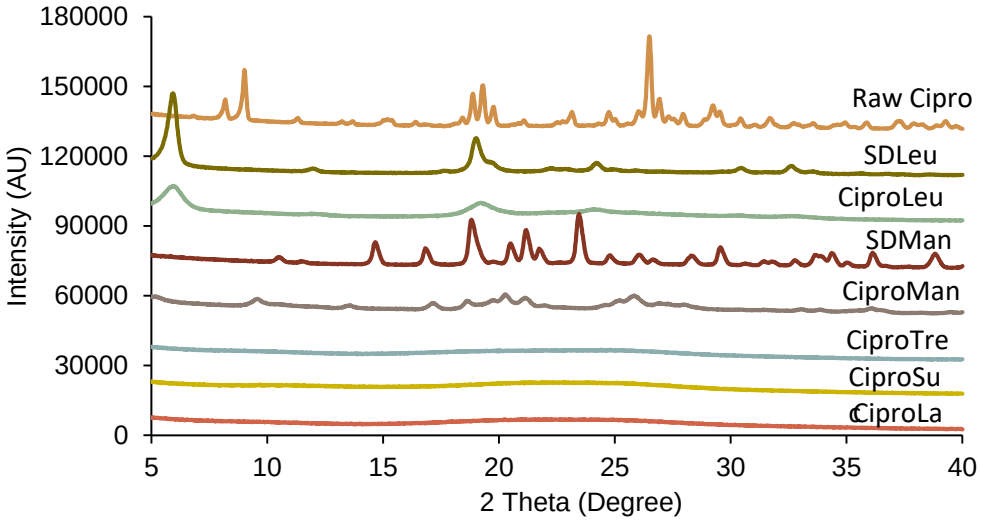


Figure 4-1: PXRD patterns for the co-spray-dried formulations of Ciprofloxacin with excipents as stored at 20% RH for 1 day and the raw Ciprofloxacin.

4.4.1.2 Modulated DSC

As shown in table 4-1, glass transition temperature of Ciprofloxacin was found to be 168ºC which was lowered in presence of sugars like lactose, sucrose and trehalose. The glass transition temperature of Ciprofloxacin co-spray-dried with mannitol and leucine could not be determined as the samples were crystalline.

Table 4-1: Glass transition temperature (Tg) for the co-spray-dried formulations of Ciprofloxacin with excipients stored at 20% RH

Formulations	Tg (°C)
Cipro	168
CiproLac	124
CiproSuc	91
CiproTre	122
CiproMan	Crystallized
CiproLeu	Crystallized

4.4.1.3 SEM

Figure 4-2 depicts morphological characteristics for the spray-dried Ciprofloxacin alone and the co-spray-dried formulations of Ciprofloxacin with different excipients as stored at 20% RH for 1 day. Spray-dried Ciprofloxacin alone powders have a spherical shape with a dimpled surface (Figure 4-2a). Co-spray-dried formulations of Ciprofloxacin with disaccharides (i.e. lactose, sucrose and trehalose) had a spherical shape with a rough surface (Figure 4-2b-d). However, co-spray-dried formulation of Ciprofloxacin with mannitol was fused (Figure 4-2e). Co-spray-dried Ciprofloxacin-L-leucine powders were found to have an irregaular and corrugated shape with a scaly surface (Figure 4-2f).

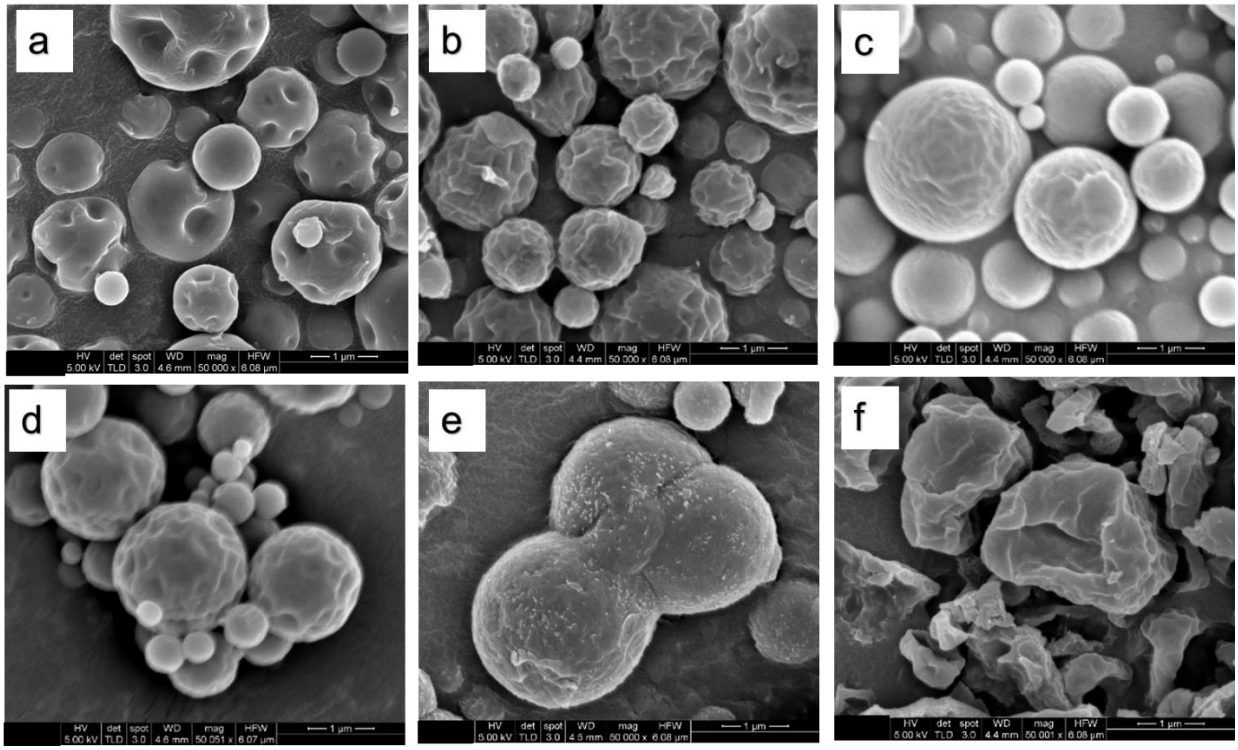


Figure 4-2: SEM micrographs of (a) the spray-dried Ciprofloxacin powder and the co-spray-dried formulations of Ciprofloxacin with (b) lactose, (c) sucrose, (d) trehalose, (e) mannitol and (f) L-leucine stored at 20% RH for 24 hours.

4.4.1.4 Physical particle size

Table 4-2 shows the physical particle sizes of the co-spray-dried Ciprofloxacin formulations with sucrose, lactose, trehalose, Mannitol and L-leucine in the mass ratio of 1:1 as stored at 20% RH for 1 day. All drug particles from the 5 different formulations were shown to have D_{90} smaller than 3 µm.

Table 4-2: Particle sizes for the co-spray-dried formulations in the mass ratio of 1:1 as stored at 20 % RH for 1 day.

Formulation	D_{10} (µm)	D_{50} (µm)	D_{90} (µm)
CiproLac	0.518	0.913	1.844
CiproSuc	0.502	0.842	1.707
CiproTre	0.512	0.919	1.763
CiproMan	0.409	0.828	1.660
CiproLeu	0.832	1.407	2.497

4.4.1.5 In-vitro Aerosol Performance

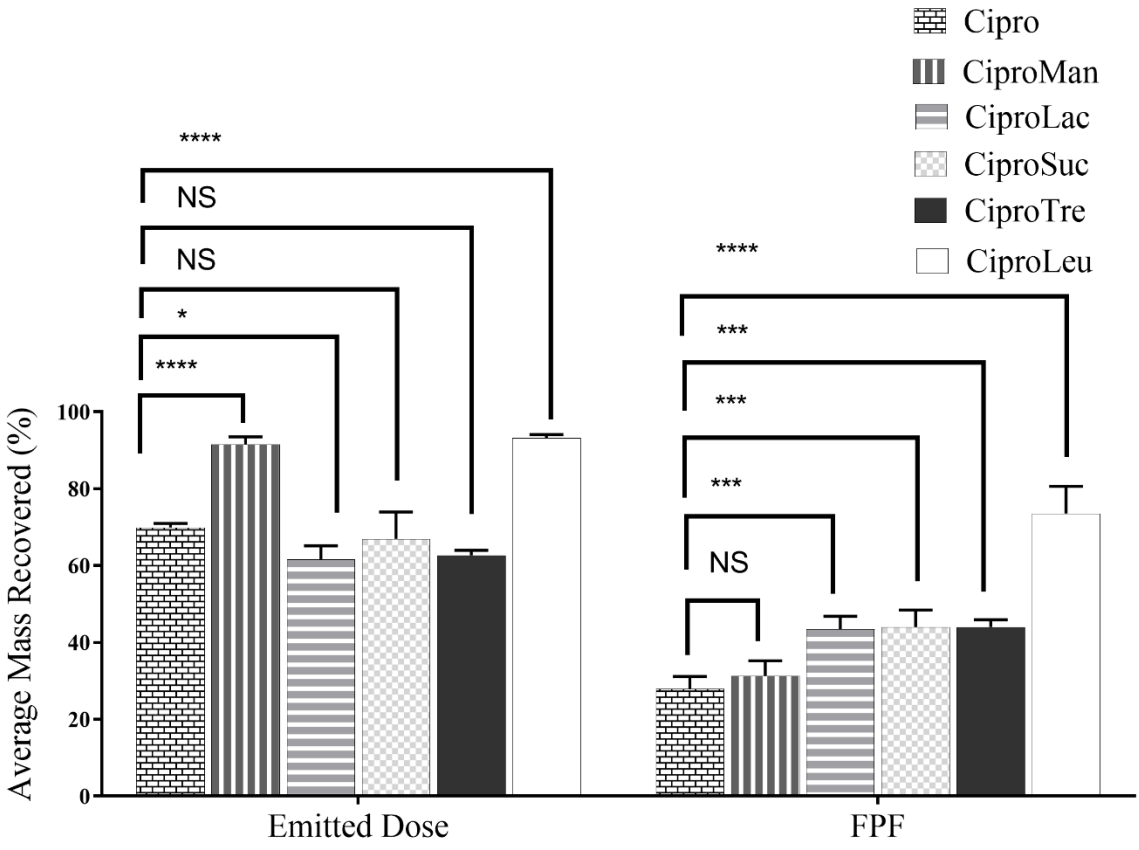


Figure 4-3: Aerosol performance of the co-spray-dried Ciprofloxacin-excipient formulations stored at 20% storage humidity for 1 day (mean ± SD, n=4; *, p <0.05; **, p <0.01; ***, p < 0.001; **** p <0.0001; NS, no significant difference).

No notable change (p >0.05) was observed between FPF of the spray-dried Ciprofloxacin alone and co-spray-dried Ciprofloxacin-mannitol formulations (Figure 4-3). However, a significant increase was observed in ED of the co-spray-dried Ciprofloxacin-mannitol formulation compared to the spray-dried Ciprofloxacin formulation alone ($p < 0.0001$). The significant increase in ED could be attributed to lower deposition of the Ciprofloxacin-mannitol co-spray-dried formulation in the capsule and device as compared to the spray-dried Ciprofloxacin formulation (Appendix Figure A1). Thus, mannitol improves ED of Ciprofloxacin but not dispersibility due to partial particle agglomeration/fusion as observed from SEM images (Figure 4-2e). On the other hand, significant increase in FPF was observed for the co-spray-dried formulation of Ciprofloxacin with lactose (43.5 ± 3.3%), sucrose (44.0 ± 4.3%), trehalose (44.0 ± 1.9%) and L-leucine (73.5 ± 7.1%) as compared to the spray-dried Ciprofloxacin alone formulation (28.0 ± 3.2%). However, the ED for Ciprofloxacin co-spray-dried with sucrose and trehalose did not differ significantly in comparison to the spray-dried Ciprofloxacin alone formulation ($p > 0.05$) (Figure 4-3).

Co-spray-drying Ciprofloxacin with disaccharides caused more drug deposition in the capsule as compared to spray-dried Ciprofloxacin formulation (Appendix Figure A1) [266]. Also, higher deposition was observed in the throat and Stage 1 for the spray-dried Ciprofloxacin alone powders in comparison to co-spray-dried Ciprofloxacin-disaccharide formulation (Appendix Figure A1). Co-spray-drying Ciprofloxacin with disaccharides helps to improve deposition of the powders in Stage 3, Stage 4 and filter as compared to spray-dried Ciprofloxacin alone (Appendix Figure A1). The increase in FPF could be attributed to formation of rougher particles upon co-spray-drying Ciprofloxacin with disaccharides (Figure 4-2). Both FPF and ED for the co-spray-dried Ciprofloxacin-L-leucine formulation (ED 93.2 ± 0.9%, FPF 73.6 ± 7.1%) were found to be

significantly higher as compared to the spray-dried Ciprofloxacin alone formulation (ED 69.9 ± 1.1%, FPF 28.0 ± 3.2%) ($p < 0.0001$). L-leucine formulation was shown to have the highest ED and FPF of Ciprofloxacin among all tested formulations.

4.4.2 Effects of excipients on physical stability and aerosol performance upon storage at 55% RH

4.4.2.1 PXRD

Figure 4-4 represents PXRD patterns of the raw Ciprofloxacin, spray-dried Ciprofloxacin formulation stored at 55% RH for 3 days and co-spray-dried formulations of Ciprofloxacin with various excipients stored at 55% RH for up to 10 days. The co-spray-dried formulation of Ciprofloxacin with sucrose were found to cake within one day of storage at 55% RH. On the other hand, caking was observed eventually for the co-spray-dried formulation of Ciprofloxacin with lactose and trehalose after 10 days. Both the co-spray-dried lactose and trehalose formulations remained amorphous up to one day and the drug Ciprofloxacin began to crystallize after 3 days of storage at 55% RH (Figure 4-4a & 4-4b). Ciprofloxacin co-spray-dried with mannitol crystallized within one day of storage at 55% RH while Ciprofloxacin-L-leucine co-spray-dried powder formulation began to crystallize after Day 3 (Figure 4-4c & 4-4d respectively). From our previous study we have observed that spray-dried Ciprofloxacin stored at 55% RH crystallizes within one hour. Qualitatively, the degree of crystallization for the co-spray-dried Ciprofloxacin-L-leucine formulation after 10 days of storage at 55% RH was much lesser as compared to the spray-dried Ciprofloxacin alone formulation stored at 55% RH for 3 days. Thus, it is evident that 50% (w/w) L-leucine inhibits the crystallization of spray-dried Ciprofloxacin.

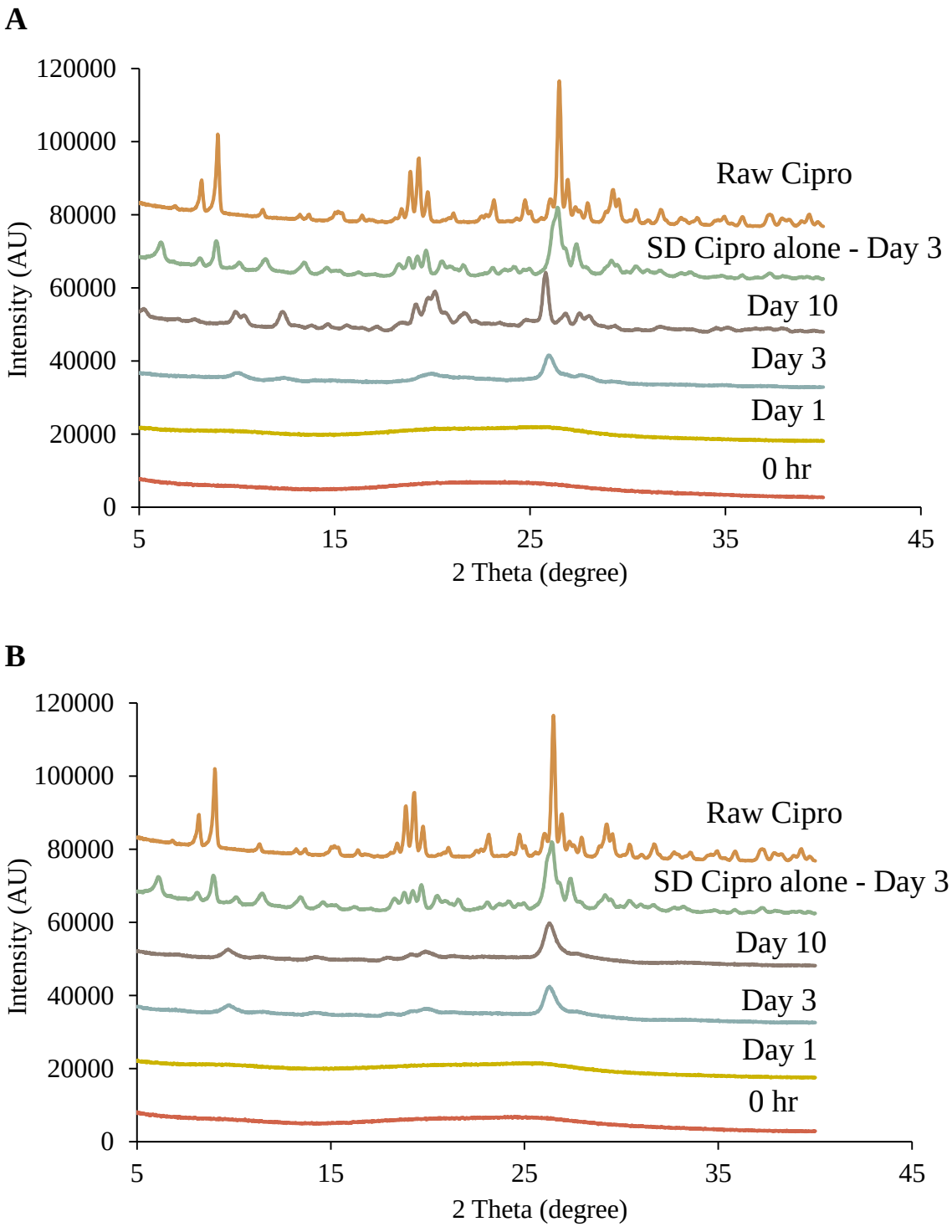
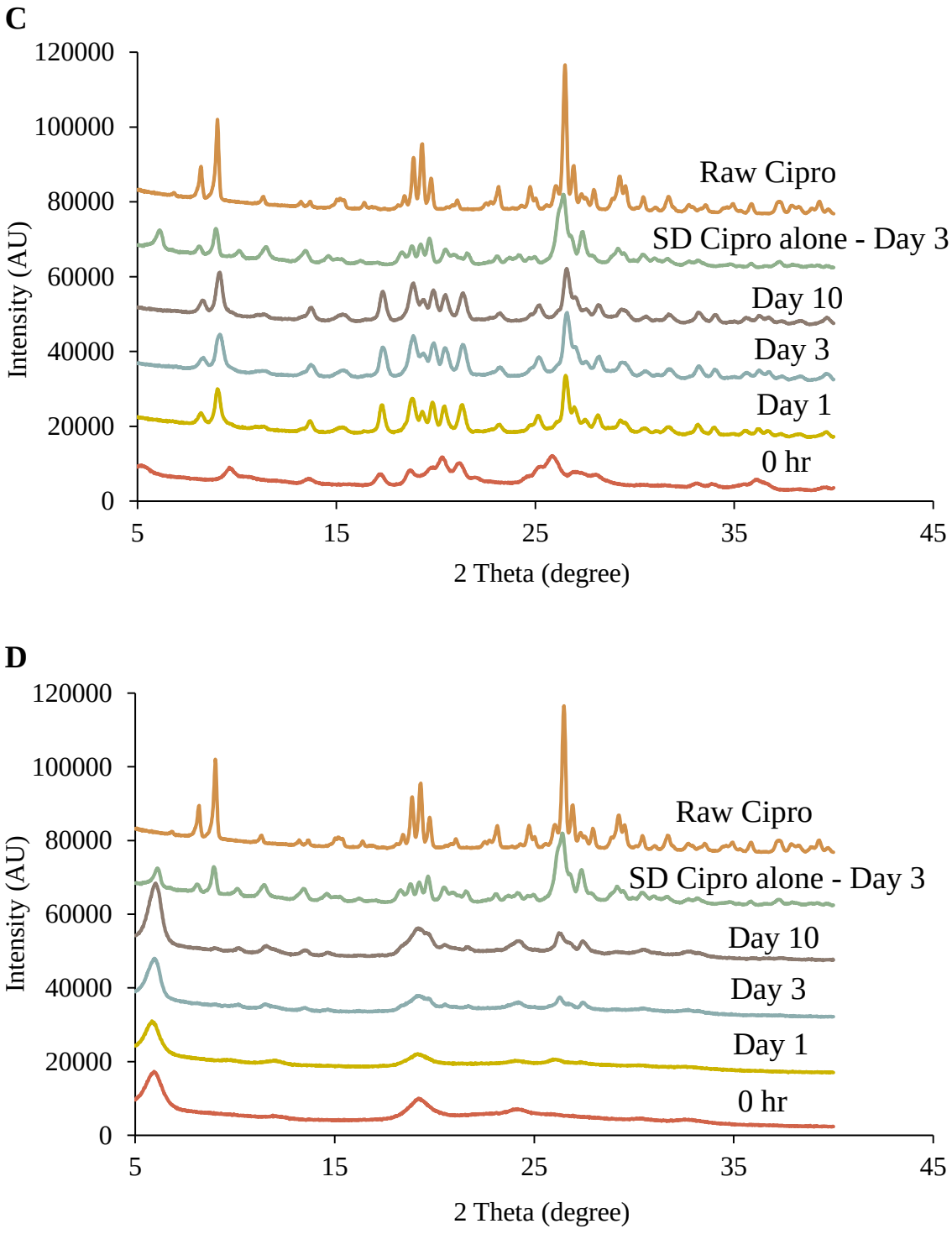


Figure 4-4**:** PXRD patterns for the co-spray-dried formulations of (A) Ciprofloxacin-lactose, (B) Ciprofloxacin-trehalose, (C) Ciprofloxacin-mannitol, and (D) Ciprofloxacin-L-leucine stored at 55% RH for 10 days.

Figure 4-4 continued



4.4.2.2 DVS

Moisture sorption/desorption isotherms are shown in Figure 4-5. Moisture-induced crystallization of the Ciprofloxacin-sucrose formulation began at around 60% RH as indicated by a decrease in mass and completed at around 80% RH [267]. These powders resumed their sorption behavior above 80% RH as indicated by increase in mass which could be attributed to dissolution of the sucrose crystals in the water being absorbed and formation of a saturated solution [268, 269]. An irrevesible change in the Ciprofloxacin-sucrose powder formulation occurred during the sorption phase since the desorption isotherm did not return back to its original value. In case of the co-spray-dried Ciprofloxacin-lactose formulation a rapid increase in moisture uptake was observed from 0 % to 50% RH beyond which the samples crystallize as indicated by loss in mass due to expulsion of water [236, 270]. The sorption isotherm of the Ciprofloxacin-trehalose formualtion shows a rapide increase in moisture uptake from 0% to 40% RH. Crystallization event is not very prominent but a slight decrease in mass was observed between 40% and 55% RH beyond which the samples absorbed more moisture. Excess water is retained in the sample as the desoprtion isotherm does not return to its initial value (0% (w/w)).

The co-spray-dried Ciprofloxacin-mannitol formulation absorbed 4.48% ((w/w)) water between 0% and 60% RH; however beyond 60% RH, a decrease in mass was observed indicative of crystallization. The desorption isotherm contained hysterisis and did not return to 0% (w/w) value due to retention of water in the crystalline powder [15]. The co-spray-dried Ciprofloxacin-L-leucine formulation absorbed 6.09% ((w/w)) water up to 70% RH. A decrese in mass from 6.09% (w/w) to 3.50% (w/w) was observed from 70% to 90% RH due to loss of water and phase transition from amorphous to crystalline state. The water was retained in the crystal lattice and hence the

desoprtion isotherm did not return to 0% (w/w). Unlike spray-dried Ciprofloxacin alone, the event of crystalization was delayed in the co-spray-dried Ciprofloxacin-L-leucine formulation.

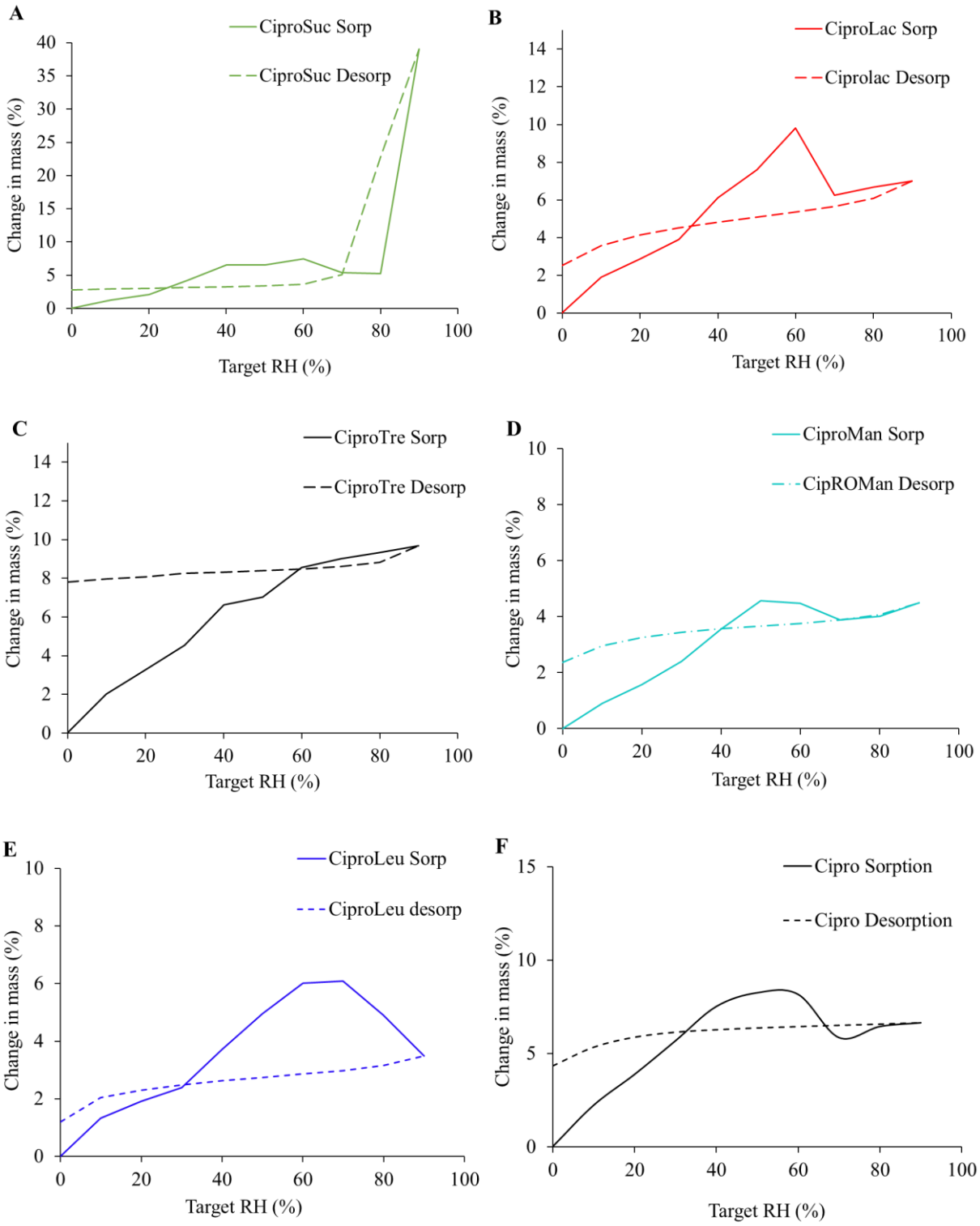


Figure 4-5: Moisture sorption behavior for the co-spray-dried formulations of (A) Ciprofloxacin-sucrose, (B) Ciprofloxacin-lactose, (C) Ciprofloxacin-trehalose, (D) Ciprofloxacin-mannitol, (E) Ciprofloxacin-L-leucine and (F) SD Ciprofloxacin alone.

4.4.2.3 SEM

Figure 4-6 depicts morphological characteristics for the Ciprofloxacin powder and the co-spray-dried formulations of Ciprofloxacin with different excipients stored at 55% RH. The spray-dried Ciprofloxacin formulation showed increased surface roughness upon storage at 55% RH (Figure 4-6a). Co-spray-dried formulation of Ciprofloxacin with lactose, sucrose, trehalose and mannitol was fused upon storage at 55% RH (Figure 4-6b-d). Presence of sugars in the spray-dried formulation has a tendency to increase powder stickiness. The sticky behaviour is associated low glass transition temperature of low molecular weight sugars such as sucrose, lactose etc. Sucrose has a Tg of 62ºC and lactose has a Tg of 101ºC. Thus, if the spray-drying temperature is +20ºC higher than their Tg, it would result in sticking powders because the sugar molecules tend to have high molecular mobility [266, 271]. However, no change was observed in the surface morphology of the co-spray-dried Ciprofloxacin-L-leucine formulation upon storage at 55% RH (Figure 4-6f) for 10 days as compared to the powders stored at 20% RH (Figure 4-2f).

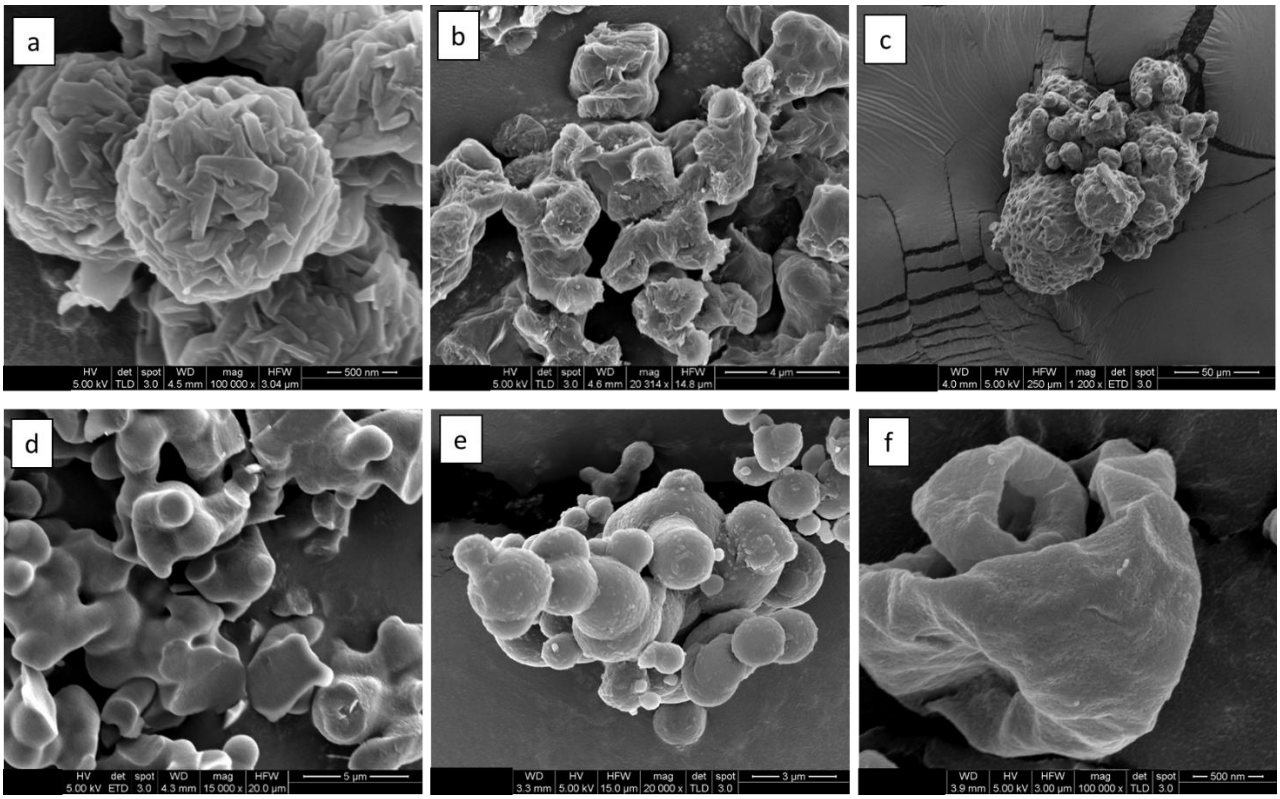


Figure 4-6: SEM micrographs of the (a) spray-dried Ciprofloxacin powder and the co-spray-dried formulations of Ciprofloxacin with (b) lactose, (c) sucrose, (d) trehalose, (e) mannitol and (f) L-leucine stored at 55% RH for 10 days. (*Ciprofloxacin-sucrose was stored for only 1 day and began to cake/fused).

4.4.2.4 Physical particle size

Physical particle size determination could not be performed for Ciprofloxacin co-spray-dried with lactose, sucrose, trehalose and mannitol stored at 55% RH due to caking of the powders. However, CiproLeu formulation had fine physical particle sizes (D_{10} 0.885 µm, D_{50} 1.561 µm, and D_{90} 2.662 µm) after storage at RH of 55% for 10 days.

4.4.2.5 In-vitro aerosol performance

Figure 4-7 shows the changes in ED and FPF after storage of the co-spray-dried formulations at 55% RH for 10 days. A significant decrease (p<0.0001) was observed with the FPF when the co-

spray-dried formulation with lactose was stored at 55% RH (Figure 4-7A). This was due to significant deposition of the co-spray-dried Ciprofloxacin-lactose powders stored at 55%RH in Stage 1 compared to the formulation stored at 20% RH (Appendix Figure A2A). No significant difference was observed in the emitted dose for the co-spray-dried Ciprofloxacin-lactose formulation stored at 20% RH and 55% RH (Figure 4-7A). Similarly, a statistically significant difference was observed with the FPF when co-spray-dried formulation of Ciprofloxacin with trehalose was stored at 55% RH (Figure 4-7B). This was attributed to higher deposition of the formulation stored at 55% RH in Stage 1 compared to the powders stored at 20% RH (Appendix Figure A2B). Dispersion for co-spray-dried formulation of Ciprofloxacin-sucrose stored at 55% RH could not be determined as the powders had caked within 24 hours.

For the co-spray-dried Ciprofloxacin-mannitol formulation, no change in emitted dose was measured upon storage at 55% RH. However, a significant decrease in FPF was noted for the co-spray-dried formulation of Ciprofloxacin with mannitol upon storage at 55% RH (Figure 4-7C). The FPF for the co-spray-dried Ciprofloxacin-mannitol formulation decresed from 31.4 ± 3.9 % when stored at 20% RH to 17.7 ± 1.2 % upon storage at 55% RH.

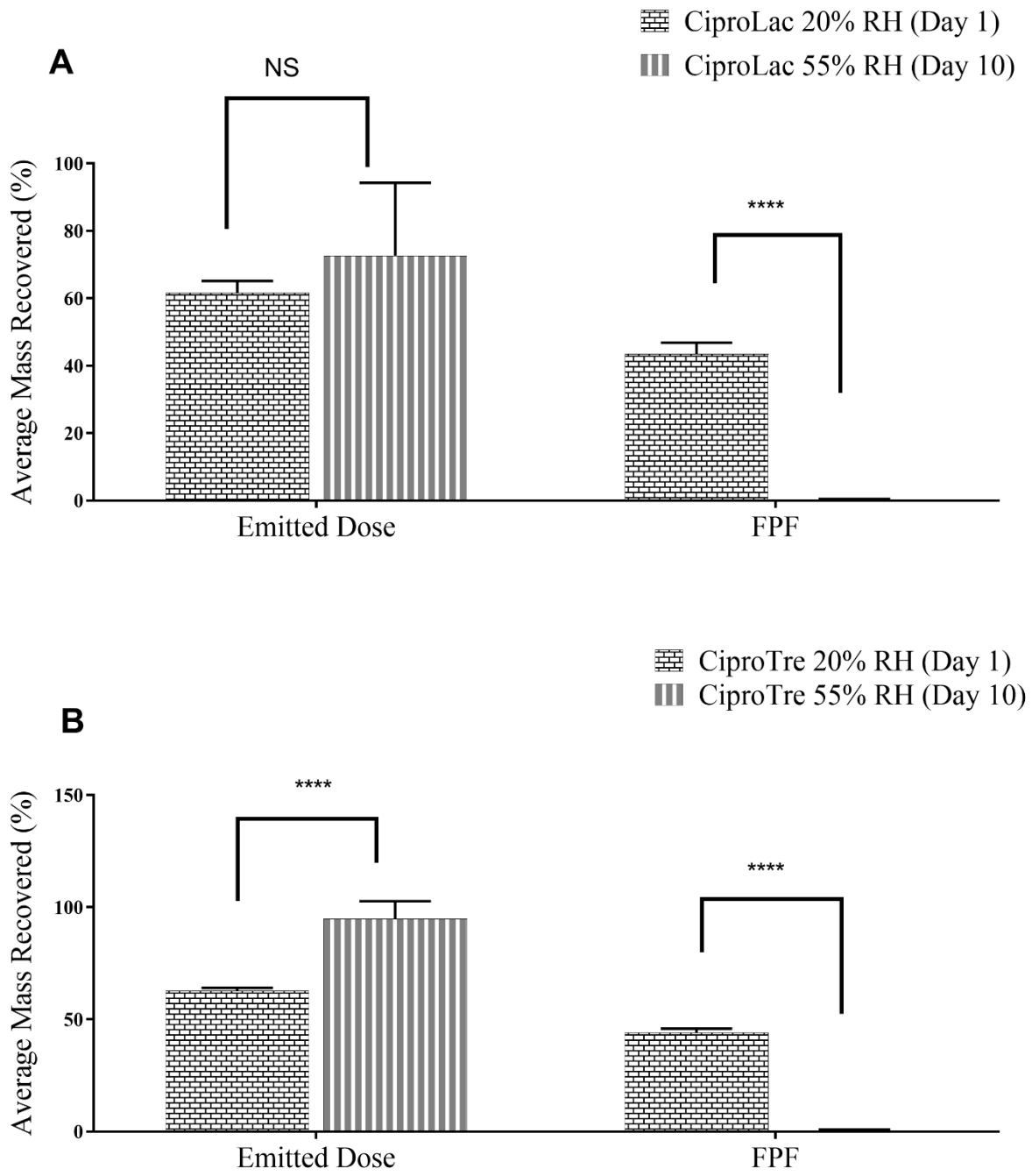
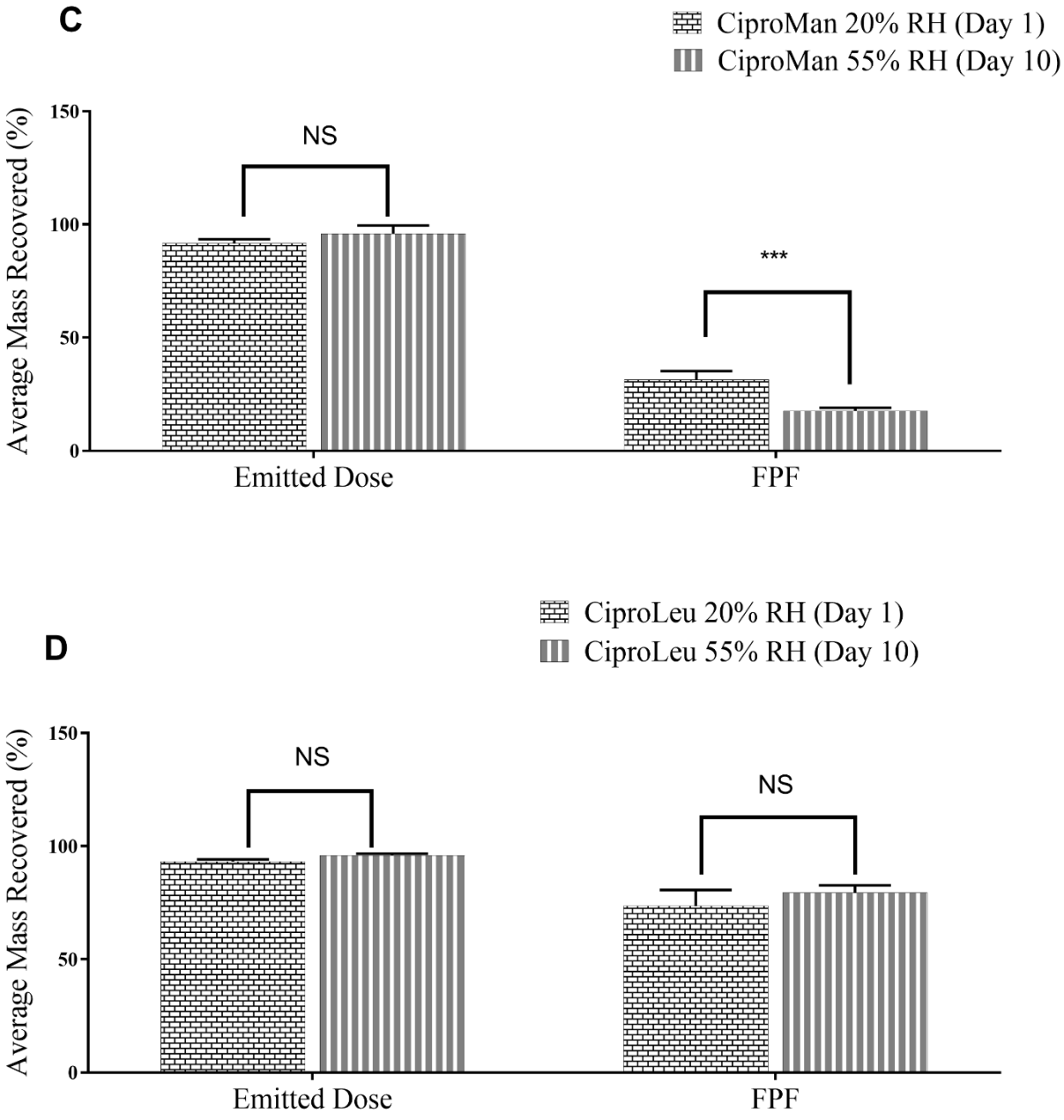


Figure 4-7: Aerosol performance of the co-spray-dried (A) Ciprofloxacin-lactose, (B) Ciprofloxacin-trehalose, (C) Ciprofloxacin-mannitol and (D) Ciprofloxacin-L-leucine formulations as reflected by ED and FPF at 20% and 55% storage humidity (mean ± SD, n=4; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; **** $p < 0.0001$; NS, no significant difference)

Figure 4-7 continued



However, L-leucine as an excipient did not show any significant difference (p>0.05) in ED and FPF between the co-spray-dried formulation of Ciprofloxacin-L-leucine in the mass ratio (1:1) stored at 20% RH and 55%RH (Figure 4-7D). The FPF for the co-spray-dried Ciprofloxacin-L-leucine formulation was 73.6 ± 7.1% when stored at 20% RH and was 79.5 ± 3.1% upon storage at 55% RH. Higher deposition of the co-spray-dried Ciprofloxacin-L-leucine powders were found in Stage 3, Stage 4 and filter at both 20% RH and 55% RH (Appendix Figure A2D). Unlike the spray-dried Ciprofloxacin alone, the co-spray-dried Ciprofloxacin-L-leucine formulation showed no change in particle morphology (Figure 4-6f) and thus no change in aerosol performance leading to more stable formulation. In the following studies, we further investigated the effects of lower concentration of L-leucine (10%) on stability and aerosol performance of spray-dried Ciprofloxacin, with a purpose to reduce the total powder mass for this high-dose DPIs.

4.4.3 Effects of low concentration (10% (w/w)) of L-leucine on physical stability and aerosol performance

4.4.3.1 PXRD

Figure 4-8 represents PXRD patterns of raw Ciprofloxacin, spray-dried Ciprofloxacin alone formulation stored at 55% RH for 3 days and the co-spray-dried formulation of Ciprofloxacin with 10% (w/w) L-leucine stored at 55% RH for up to 10 days. PXRD data suggest that co-spray-drying Ciprofloxacin with 10% L-leucine resulted in crystallization of the drug on Day 1 (Figure 4-8). However, it is evident that the degree of crystallization for the co-spray-dried Ciprofloxacin-L-leucine formulation in the mass ratio (9:1) at day 10 was lower as compared to crystallization of spray-dried Ciprofloxacin alone at Day 3 upon storage at 55% RH. Thus, L-leucine even at a low concentration of 10% (w/w) was found to alleviate the degree of

crystallization for Ciprofloxacin.

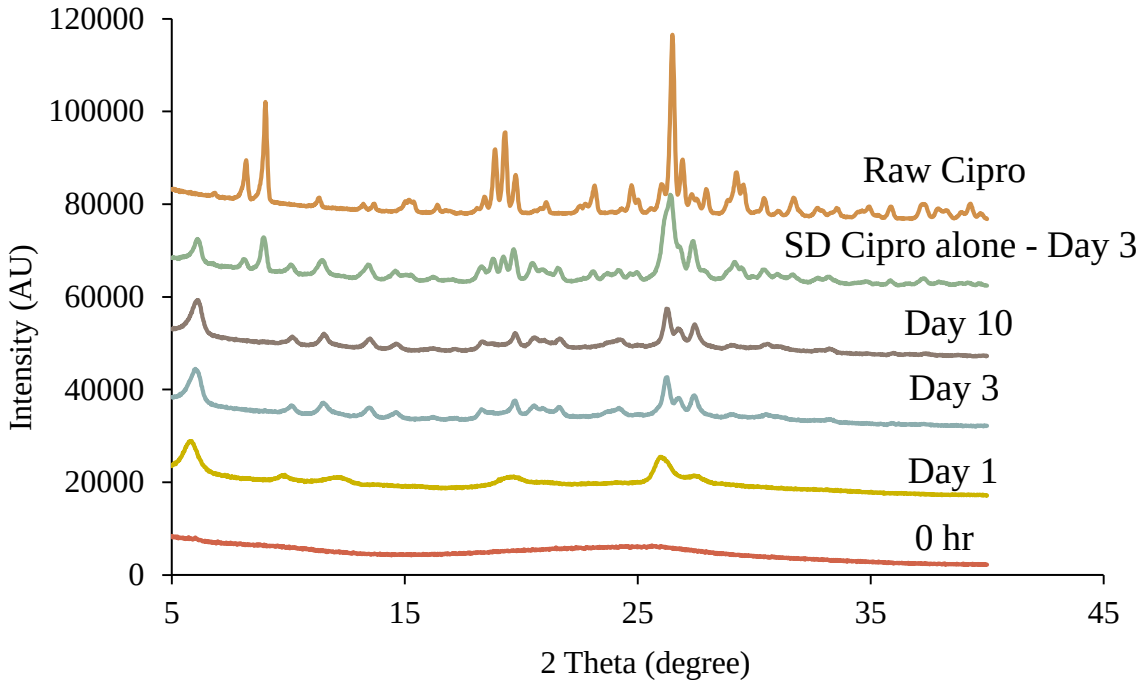


Figure 4-8: PXRD patterns for the co-spray-dried formulation of Ciprofloxacin-L-leucine in the mass ratio (9:1) stored at 55% RH for 10 days and Ciprofloxacin only formulation at 55% RH for 3 days.

4.4.3.2 DVS

Co-spray-dried Ciprofloxacin-L-leucine formulation in the mass ratio (9:1) was found to absorb 7.5% moisture up to 55% RH. Beyond 55% RH a significant decrease in mass was observed as indicative of crystallization (Figure 4-9).

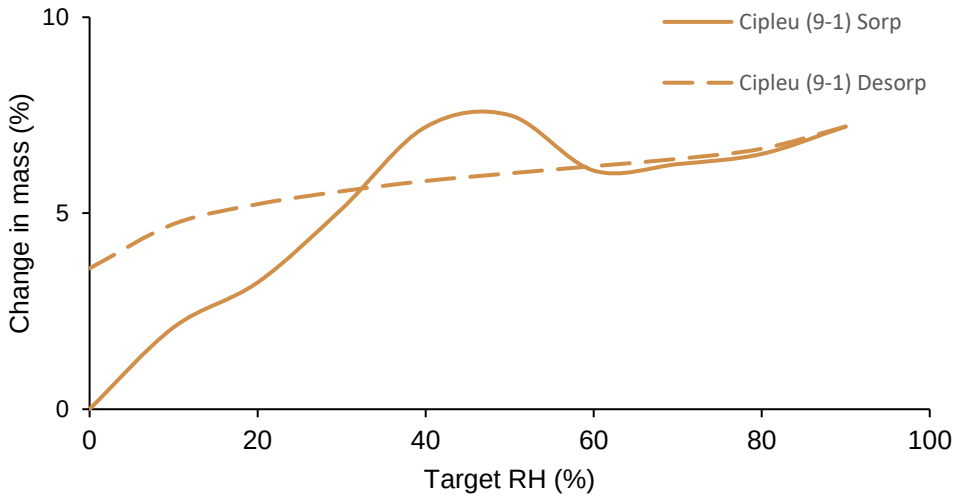


Figure 4-9: Moisture sorption behavior for the co-spray-dried formulation of Ciprofloxacin-l-L-leucine in the mass ratio (9:1).

4.4.3.3 SEM

Co-spray-dried Ciprofloxacin-L-leucine powders in the mass ratio (9:1) appeared to be spherical in shape with a rough surface. Upon storage at 55% RH for 10 days, no major change in surface morphology was observed for the co-spray-dried Ciprofloxacin-L-leucine formulation in the mass ratio (9:1) (Figure 4-10).

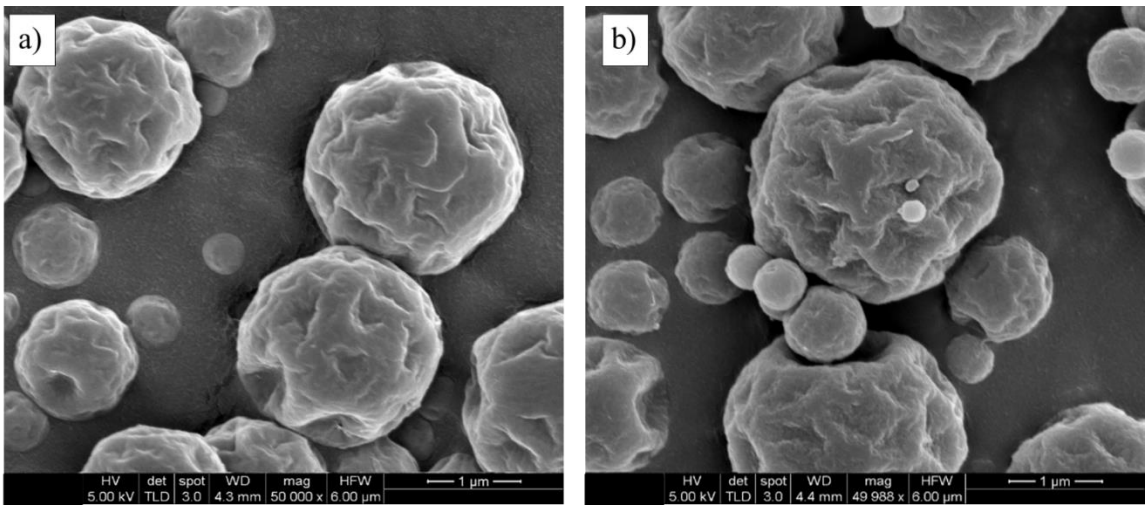


Figure 4-10: SEM micrographs of the co-spray-dried Ciprofloxacin-L-leucine formulation in the mass ratio (9:1) stored at (a) 20% RH for 1 day and (b) 55% RH for 10 days.

4.4.3.4 In-vitro aerosol performance

10% (w/w) L-leucine in the co-spray-dried Ciprofloxacin-L-leucine formulation showed no significant difference (p>0.05) in the ED and FPF between powders stored at 20% RH and 55% RH (Figure 4-11). The FPF for the co-spray-dried Ciprofloxacin-L-leucine formulation in the mass ratio (9:1) was 68.1 ± 0.3% upon storage at 20% RH for 1 day. At 55% RH for 10 days, FPF for the co-spray-dried Ciprofloxacin-L-leucine formulation in the mass ratio (9:1) was 71.1 ± 3.5%. Thus, even at a low L-leucine concentration of 10% (w/w), storage at 55% RH led to no change in morphology and aerosol performance for the co-spray-dried Ciprofloxacin-L-leucine formulation.

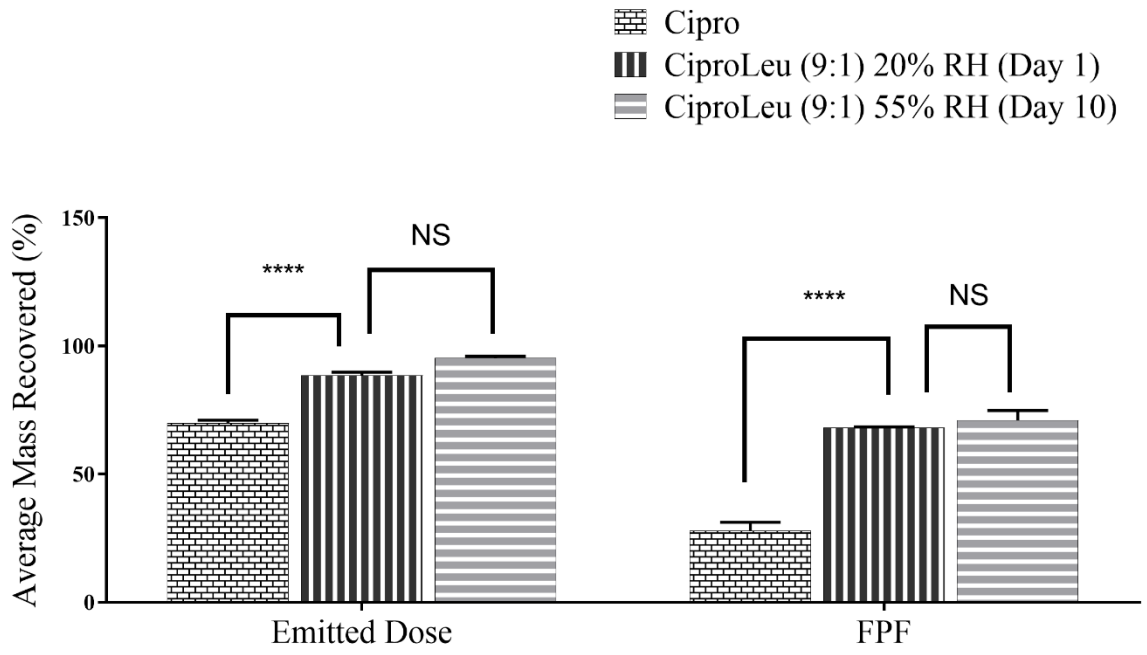


Figure 4-11: Aerosol performance of the co-spray-dried Ciprofloxacin-L-leucine (9:1) formulations as reflected by ED and FPF at 20% for 1 day and 55% storage humidity for 10 days (mean ± SD, n=4; *, p <0.05; **, p <0.01; ***, p < 0.001; **** p <0.0001; NS, no significant difference)

4.4.3.5 FTIR

FTIR was used to determine any potential interactions between L-leucine and ciprofloxacin at the molecular level in the co-spray dried ciprofloxacin-L-leucine formulation. In Figure 4-12, the raw L-leucine demonstrates two distinct peaks at 1510 and 1577 cm^{-1}, which can be assigned to the symmetric deformation of NH_3^+ and asymmetric stretching of COO^- [272]. It was reported that the carbonyl oxygen contributes to the intermolecular hydrogen bonding with one of the hydrogen atoms of the NH_3^+ moiety. Shifting of these peaks towards higher wavenumbers could suggest breaking of such intermolecular hydrogen bonding [273] There is a shift in the NH_3^+ peak from 1510 cm^{-1} for raw L-leucine to the higher wavenumber of 1514 cm^{-1} for the SD L-leucine which is likely indicative of the amorphous nature due to lack of regular molecular arrangement caused by hydrogen bonding.

There are likely new intermolecular interactions formed during the process of co-spray drying ciprofloxacin with L-leucine since there is a slight shift in the COO^- and NH_3^+ peaks to the higher wavenumbers [274] (Figure 12). By breaking the intermolecular interactions between L-leucine discussed above, the carbonyl oxygen of the carboxylic acid group or hydrogen of amine group can work as a strong hydrogen bond acceptor or donor, respectively, to interact with the hydrogen bond donating or accepting group of ciprofloxacin [275]. However, it is very challenging to confirm the interaction center on ciprofloxacin due to its complex chemical structure and the limited spectral information. Further investigation of the molecular interactions in the ciprofloxacin-L-leucine complex will be warranted.

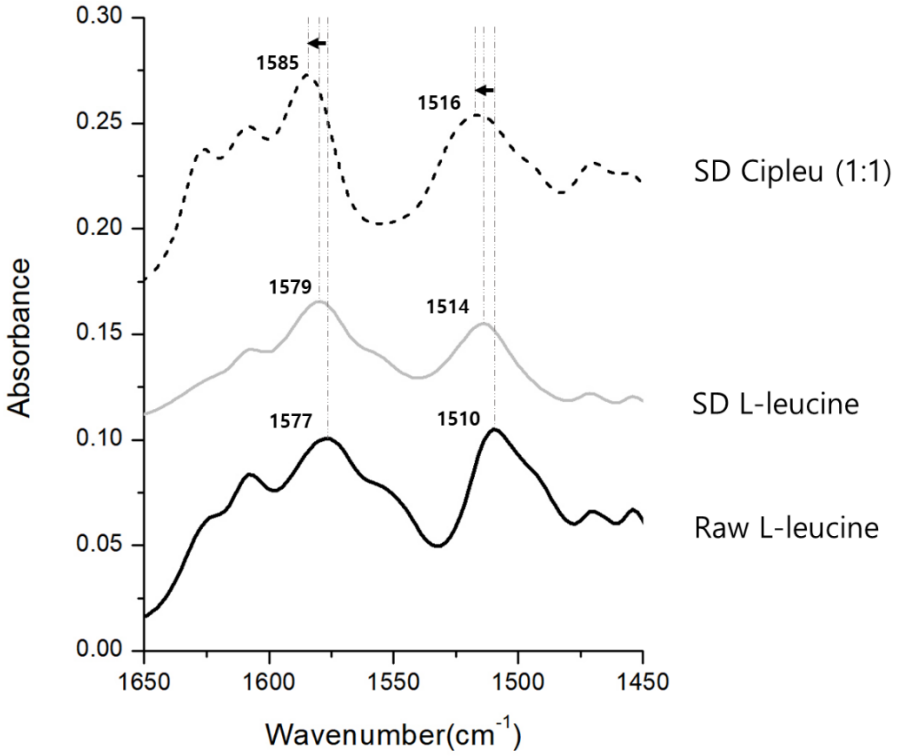


Figure 4-12: FTIR spectra for the raw L-leucine, spray-dried leucine alone and Cipleu formulations.

4.4.3.6 XPS

The XPS data demonstrated that the measured concentration of L-leucine on the particle surface was significantly higher than the theoretical value (Table 4-3). The theoretical concentration of L-leucine was 10% in the co-spray-dried Ciprofloxacin-L-leucine formulation in the mass ratio (9:1); while the measured surface concentration was 42% (calculated using C1s curve-fits and mass ratio based on number of carbon atoms). Likewise, theoretical concentration of L-leucine was 50% in the co-spray-dried Ciprofloxacin-L-leucine formulation in the mass ratio (1:1), whereas calculated surface composition was 70% indicating surface enrichment by L-leucine in the co-spray-dried Ciprofloxacin-L-leucine formulation.

Table 4-3: Theoretical and measured surface compositions by XPS based on number of carbon atoms for the co-spray-dried Ciprofloxacin-L-leucine formulations in different mass ratios.

Formulations	% Surface Composition (Theoretical)		% Surface Composition (Measured)	
	L-leucine	Ciprofloxacin	L-leucine	Ciprofloxacin
CiproLeu_9:1	10	90	42	58
CiproLeu_1:1	50	50	70	30

4.5 Discussion

For inhalation drug delivery systems such as dry powder inhalation, amorphous powders obtained by spray-drying process may have poor physical stability affecting their aerosol performance [75, 244]. Excipients such as sugars or amino acids can be added as stabilizers to improve the physical and aerosol stability. In the present study effects of various excipients on moisture induced-crystallization of spray-dried Ciprofloxacin were studied. At 20% RH, the formulations of Ciprofloxacin spray-dried with the sucrose, lactose and trehalose were amorphous and showed a significant increase in aerosol performance as compared to the spray-dried Ciprofloxacin alone. The increase in aerosol performance by addition of disaccharides could be attributed to increase in surface roughness of these particles in comparison to spray-dried Ciprofloxacin alone [276, 277]. Ciprofloxacin co-spray-dried with mannitol did not show significant change in aerosol performance and the powders appeared to agglomerate at 20% RH. On the other hand, Ciprofloxacin-L-leucine formulation had an irregular shape and a significant increase in aerosol performance as compared to the spray-dried Ciprofloxacin alone. At 20% RH, the drug Ciprofloxacin was in its amorphous form when co-spray-dried with different excipients. However,

at 55% RH despite the presence of various excipients, the drug crystallized over time in the different formulations.

Ciprofloxacin co-spray-dried with mannitol crystallized within one day of storage at 55% RH while caking was observed eventually for the co-spray-dried formulation of Ciprofloxacin with sucrose, lactose and trehalose after 10 days. Ciprofloxacin-mannitol formulation retained unchanged ED and did not cake at 55% RH; however, a decrease in FPF was observed compared to that formulation stored at 20% RH. Co-spray-dried formulations of Ciprofloxacin with lactose, sucrose, and trehalose fused upon storage at 55% RH, making it a concern in physical stability. Similar observations were made by Naini et al. (1998) who compared the stability and moisture uptake of crystalline fractions for spray-dried powders of lactose, sucrose, mannitol and trehalose on storage at different RH% for 30 days. These sugars recrystallized as sintered masses and became undispersible at > 52% RH [278].

Co-spray-drying with L-leucine significantly increased the aerosol performance of Ciprofloxacin and enhanced physical and aerosol stability for the DPI formulation with no change in FPF upon storage at 55% RH. It is well recognized that co-spray-drying drugs with amino acids such as L-leucine can enhance aerosol performance, and such improvements depend on the enrichment of L-leucine on the particle surface [275]. The XPS and ToF-SIMS data have proved such enrichment of L-leucine on the particle surface, which led to the corrugated particle shape and improved aerosol performance compared with the spray-dried Ciprofloxacin alone. It is further interesting to note that unlike the spray-dried Ciprofloxacin alone powders, the co-spray-dried

Ciprofloxacin-L-leucine formulation showed no major change in particle morphology and alleviated crystallization of Ciprofloxacin when stored at 55% RH. These effects of L-leucine on physical stability were measured even at a low L-leucine concentration of 10% (w/w). To understand the underlying mechanisms of enhanced physical stability by co-spray-drying with L-leucine, we have conducted FT-IR study to examine the molecular interactions between Ciprofloxacin and L-leucine. FT-IR data suggested that intermolecular interactions including hydrogen bonding between L-leucine and Ciprofloxacin likely led to the inhibition on the crystallization of amorphous ciprofloxacin. Future studies are warranted to use other solid-state technologies such as solid-state NMR to investigate such interactions.

4.6 Conclusions

The present study has shown various excipients had different effects on the physical and aerosolization stability of spray-dried amorphous DPI formulations. It is an interesting finding that among all tested excipients, co-spray-drying with L-leucine not only enhanced the aerosolization performance of Ciprofloxacin, but also improved the physical and aerosolization stability upon storage at a mild condition of RH 55%. It is well-recognized that co-spray-drying with surface-active L-leucine will reduce surface energy and form corrugated particles, which lead to enhanced aerosolization. Here we have proved enrichment of L-leucine on the spray-dried particle surface by XPS and ToF-SIMS data. FT-IR results indicated that inhibitory effects of L-leucine on crystallization of amorphous Ciprofloxacin are attributed to the intermolecular interactions between L-leucine and Ciprofloxacin. This study provides some insights in physical and aerosolization stability of spray-dried DPI formulations for high-dose medications, which are critical for the product quality.

CHAPTER 5. IMPROVED PHYSICAL AND AERSOL STABILITY OF INHALABLE AMORPHOUS CIPROFLOXACIN POWDER FORMULATIONS BY INCORPORATING SYNERGISTIC COLISTIN*

*Published by- **Shetty, N.;** Ahn, P.; Park, H.; Bhujbal. S.; Zemlyanov, D.; Cavallaro. A.; Mangal, S.; Li, J.; Zhou, Q. Improved physical stability and aerosolization of inhalable amorphous Ciprofloxacin powder formulations by incorporating synergistic Colistin**.**

5.1 Abstract

This study aimed to develop dry powder inhaler (DPI) combination formulations of Ciprofloxacin and Colistin for use in respiratory infections. Effects of Colistin on physical stability and aerosolization of spray-dried Ciprofloxacin were examined. The combination DPI formulations were produced by co-spray-drying Colistin and Ciprofloxacin in mass ratios of 1:1, 1:3 and 1:9. Colistin and Ciprofloxacin were also co-sprayed with L-leucine in the mass ratio of 1:1:1. The physical and aerosolization stability of the selected co-sprayed formulations stored at 20, 55 and 75% relative humidity (RH) were examined. Formulation characterizations were carried out using Powder X-ray diffraction (PXRD) for crystallinity, scanning electron microscopy (SEM) for morphology and particle size distribution and Dynamic Vapor Sorption (DVS) for moisture sorption. Particle surface analysis was performed using X-ray Photoelectron Spectroscopy (XPS), Energy Dispersive X-ray Spectrometry (EDX) and Nano-Time-of-flight Secondary Ion Mass Spectrometry (Nano ToF-SIMS). Potential intermolecular interactions were studied using Fourier-transform infrared spectroscopy (FTIR). Aerosol performance was evaluated using a multi-stage liquid impinger (MSLI) with a RS01 Monodose inhaler device. PXRD diffractograms showed that the co-spray-dried Colistin-Ciprofloxacin formulation in the mass ratio (1:1) was amorphous at 55% RH for up to 60 days; whereas the co-spray-dried Colistin-Ciprofloxacin (1:3) and Colistin-Ciprofloxacin (1:9) crystallized after storage for 3 days

at 55% RH. However, the extent of crystallization for the combination formulations was less as compared to the spray-dried Ciprofloxacin alone formulation. Surface morphology of the co-spray-dried formulations at different concentrations did not change even after storage at 55% RH for 60 days, unlike the spray-dried Ciprofloxacin alone powder which became rougher after 3 days of storage at 55% RH. Surface analysis data indicated surface enrichment of Colistin in the co-spray-dried formulations. Increasing Colistin concentration on the composite particles surfaces improved aerosol performance of Ciprofloxacin. FTIR data demonstrated intermolecular interactions between Colistin and Ciprofloxacin, thereby delaying and/or preventing crystallization of Ciprofloxacin when co-spray-dried. Co-spray-drying Ciprofloxacin with Colistin in the mass ratio (1:1) completely prevented crystallization of Ciprofloxacin at 55% RH for up to 60 days. However, the Colistin-Ciprofloxacin formulation (1:1) began to fuse when stored at 75% RH due to moisture absorption resulting in compromised aerosol performance. However, the Colistin-Ciprofloxacin-leucine (1:1:1) formulation demonstrated no particles fusion, enabling a stable aerosol performance at 75% RH for 7 days. This study demonstrated that incorporation of Colistin in the spray-dried formulations can improve physical stability and aerosolization of amorphous Ciprofloxacin at 55% RH. At 75% RH for 7 days, further addition of L-leucine in the formulation prevents particle fusion and deterioration in aerosol performance, attributed to enrichment of non-hygroscopic L-leucine on the particle surface.

5.2 Introduction

Lower respiratory infections (or lung infections) caused by Gram-negative pathogens such as *Pseudomonas aeruginosa* are difficult to treat and associated with high risks of mortality, morbidity and increased hospitalization [279]. This is mainly due to the emergence of drug resistance against the first-line antimicrobials [280] and the insufficient drug concentrations in

the infection site in lungs via oral or parenteral administrations. Ciprofloxacin is one of the potent broad-spectrum antibacterial for respiratory tract infections including those caused by *P. aeruginosa*. However, the resistance to Ciprofloxacin is emerging rapidly in some Gram-negative bacteria [281]. High-level fluoroquinolone-resistance appears to be due to alterations in the A subunit of DNA gyrase and in a simultaneous alteration in cell membrane permeability that probably is related to loss of outer-membrane proteins [282].

Combination antibiotic therapy is a robust strategy to effectively address such emerging human health concerns. Combination of Ciprofloxacin with polypeptide antibiotic like Colistin, maximized therapeutic efficacy and minimized resistance development, in severe lung infections caused by Gram-negative pathogens [283]. Colistin, despite being an old antibiotic, has retained its excellent antibacterial activity against Gram-negative bacteria including *Pseudomonas* species [284]. However, use of high doses of intravenous Colistin to treat lung infections may cause serious adverse events such as nephrotoxicity [284, 285]. In contrast, inhaled Colistin has shown to be safe and well tolerated in animals and patients with *P. aeruginosa* infections [286-289]. PK/PD data from animal and clinical studies of inhaled Colistin have shown in-vivo advantages over systemic administration [286-289].

A combination of oral Ciprofloxacin with Colistin could successfully prevent emergence of resistance even when used continuously for 10 years as a prophylactic regimen [290]. The mechanism of the synergistic action of Ciprofloxacin with Colistin is uncertain, however, it is proposed that such effects may be attributed to the ability of Colistin to enhance the uptake of the companion antibiotic by destabilizing outer membrane of Gran-negative bacteria [291]. Combination of Ciprofloxacin with Colistin was also found to be effective against ‘difficult to tackle’ *P. aeruginosa* biofilms [292]. In such combinations, Ciprofloxacin effectively eliminates

the metabolically active pathogenic population of the biofilm while Colistin specifically kills the bacterial population with low metabolic activity [293, 294].

A combination of nebulized colistimethate sodium and oral Ciprofloxacin has been successfully used in eradicating multidrug-resistant *P. aeruginosa* associated with severe lower respiratory infections [284]. However, nebulizers including the commonly used jet nebulizers have certain disadvantages, such as bulkiness, loss of drug during the process of nebulization and inconsistent/low performance [295]. On the contrary, dry powder inhalers (DPI) are gaining popularity as they are ease to carry, more chemically stable and suitable for high-dose antibiotics [220, 296]. Inhalation products such as TOBI® Podhaler and Colobreathe are available commercially to treat lower respiratory tract infections [297].

Inhalable powders produced via jet milling are typically cohesive in nature due to high surface energy and consequently exhibit poor aerosolization performance [7, 298]. Physical properties such as particle size, shape, morphology, etc. play a critical role in the clinical efficacy of the DPIs [69, 78, 299, 300]. These properties can be altered using specialized particle engineering techniques such as spray-drying [7, 83, 301, 302]. However, spray-drying has limitations such as that most spray-dried small molecules tend to be amorphous in nature and physically unstable, which may transform into a crystalline state upon storage [75, 244]. In our recent study, we have demonstrated that a spray-dried powder of Ciprofloxacin was amorphous and crystallized on storage at the elevated humidity such as at 55% RH and 75% RH, which consequently altered the aerosol performance [75]. Thus, addressing such physical and aerosolization instability issues are critical to develop DPI formulations with superior quality. Although addition of excipients such as leucine can improve the physical stability of spray-dried amorphous Ciprofloxacin particles [145], there is a need to minimize the use of excipients for high-dose DPIs [220].

The hypothesis of current study is that co-spray-drying Ciprofloxacin with a polypeptide antibiotic, Colistin, would improve physical and aerosolization stability since Colistin was shown to remain amorphous upon storage at high humidity [303]. Here we aimed to examine the effects of Colistin on the physical stability and aerosolization of Ciprofloxacin in co-spray-dried DPI formulations.

5.3 Materials and Methods

5.3.1 Chemicals

Colistin sulfate (referred to as Colistin) and Ciprofloxacin hydrochloride monohydrate (abbreviated as Col and Cipro, respectively, in the text) were purchased from ßetaPharma® Co., Ltd (Wujiang City, JiangSu Province, China). L-leucine (abbreviated as leu in the text) was supplied by Sigma-Aldrich (St. Louis, Missouri, USA). Acetonitrile (HPLC grade) and magnesium nitrate were supplied by Fischer Scientific (Fair Lawn, NJ, USA).

5.3.2 Spray-drying

Spray-dried formulations as outlined in Table 5-1 were prepared by spray-drying aqueous solution (16 mg/mL total solutes) of Ciprofloxacin hydrochloride and/or Colistin sulfate using a BUCHI B-290 mini spray dryer (BUCHI Labortechnik AG, Flawil, Switzerland). Spray-drying was conducted at a feed rate of 2 mL/min with an inlet air temperature (T_{in}) of 120 ± 2 ºC, aspirator at 35 m^3/h and atomizing air of 700 L/h. These conditions resulted in an outlet temperature (T_{out}) of approximately 60 ± 2 °C. The spray-dried powders were divided into 2 equal parts and stored in a (1) desiccator containing silica gel to maintain 20 ± 2% RH at 20 ± 2 °C; (2) a humidity chamber containing saturated magnesium nitrate solution to maintain 55 ± 2% RH at 20 ± 2 °C.

Additionally, the co-spray-dried ColCipro (1:1) powder formulation was stored at 75% ± 2% RH at 20 ± 2 °C (desiccator containing saturated sodium chloride solution). The co-spray-dried formulations were stored at 55% RH for up to two months to determine physical and aerosolization stability and for up to 7 days at 75% RH.

Table 5-1: Compositions of the spray-dried formulations.

Formulation	**Concentration (%(w/w))**		
	Colistin sulfate ((w/w))	**Ciprofloxacin hydrochloride ((w/w))**	**L-leucine ((w/w))**
SD Cipro	0	100	0
SD Col	100	0	0
co-SD ColCipro (1:1)	50	50	0
co-SD ColCipro (1:3)	25	75	0
co-SD ColCipro (1:9)	10	90	0
co-SD ColCipLeu (1:1:1)	33	33	33

5.3.3 X-ray Powder Diffraction (PXRD)

Crystallinity of formulations was evaluated using a Rigaku Smartlab™ diffractometer (Rigaku Americas, Texas, USA). Cu-Kα radiation source and a highly sensitive D/tex ultra-detector at a voltage of 40kV and current of 44 mA were used [233]. Settings were as follows: 5 to 40° 2θ at a step size of 0.02° with a scan rate of 4°/min.

5.3.4 Scanning Electron Microscopy (SEM)

Scanning electron micrographs of the formulations were taken using a NOVA nano SEM (FEI Company, Hillsboro, Oregon, USA). The samples were platinum coated using a sputter coater (208 HR, Cressington Sputter Coater, England, UK) with a current of 40 mA for 1 min. The images were captured at 5 kV.

5.3.5 Particle Size

Particle size distribution of the powder formulation was measured using an Image J software based on SEM images [264]. The diameter at 10% (d_{10}), 50% (d_{50}) and 90% (d_{90}) undersize was calculated for approximately 100 particles.

5.3.6 Dynamic Vapor Sorption (DVS)

Moisture sorption behavior was determined using dynamic vapor sorption (DVS-Intrinsic, Surface Measurement Systems Ltd., London, UK). Each formulation was equilibrated at 0% RH to provide a baseline and subjected to a sorption and desorption cycle. In the sorption cycle, the equilibrium mass change was measured at RH ranging from 0-90% at 10 % RH increments at 25 ^{o}C; and in the desorption cycle, the mass change was measured at RH ranging from 90-0% with 10% interval. An equilibrium criterion of$dm/dt \leq 0.002\%$ per minute was specified for the system to achieve at each RH step.

5.3.7 X-ray Photoelectron Spectroscopy (XPS)

Surface composition was quantified using X-ray photoelectron spectroscopy (XPS) (AXIS Ultra DLD spectrometer, Kratos Analytical Inc., Manchester, UK) with monochromic Al Kα radiation (1486.6 eV) at pass energy (PE) of 20 and 160 eV for high-resolution and survey spectra, respectively. A commercial Kratos charge neutralizer was used to avoid non-homogeneous

electric charge of non-conducting powder (in this case, the powders were conducting) and to achieve better resolution. Typical instrument resolution for pass energy (PE) of 20 eV is ~0.35 eV. Binding energy (BE) values refer to the Fermi edge and the energy scale was calibrated using Au 4f7/2 at 84.0 eV and Cu 2p3/2 at 932.67 eV. Powder samples were placed on a stainless-steel sample holder bar using a double-sided sticking Cu tape. XPS data were analyzed with CasaXPS software version 2313 Dev64. Prior to data analysis, the C-C component of the C 1s peak was set to a binding energy of 284.8 eV to correct for charge on each sample. Curve-fitting was performed following a Shirley background subtraction using model peaks obtained from pure compounds. The atomic concentrations of the elements in the near-surface region were estimated after a Shirley background subtraction taking into account the corresponding Scofield atomic sensitivity factors and inelastic mean free path of photoelectrons using standard procedures in the CasaXPS software assuming homogeneous mixture of the elements within the information depths (~10 nm).

5.3.8 Energy Dispersive X-ray Spectrometer (EDX)

EDX was used to map the distributions of Colistin (sulphur was used as a selective molecular marker for Colistin) and Ciprofloxacin (chlorine was used as a selective molecular marker for Ciprofloxacin) in the powder formulations (ULTRA plus, Zeiss, Germany). For each measurement, the beam was focused on a single particle. Ten to fifteen EDX measurements were acquired from each sample at an accelerating voltage of 7 kV and the images were captured at a working distance of ~10 mm. The electron beam energy influences the penetration depth into the surface, which is in the range of a few micrometers and was kept constant for all measurements. The emitted X-rays were detected by x-act detector (Oxford instruments, Oxfordshire, UK,) and were analyzed using the Aztec® EDX analysis software.

5.3.9 Time-of-flight Secondary Ion Mass Spectrometry (ToF-SIMS)

The surface distributions of different components in the spray-dried composite formulations were evaluated using Time-of-flight secondary ion mass spectrometry (ToF-SIMS, TRIFT V nanoToF, Physical Electronics Inc., Chanhassen, MN, USA). The detailed descriptions were described elsewhere (25). Mass resolution for spectra was optimized by the "bunched" Au1 instrumental settings, while spatial resolution was optimized by "unbunched" Au1 instrumental settings for the Collection of images. ToF-SIMS data were collected randomly from 5 different areas (75 × 75 µm each) for each sample. Characteristic mass fragments were identified to effectively discern surface Colistin, Ciprofloxacin, and leucine signals. The unique characteristic mass fragment selected for Colistin was at m/z 86 atomic mass unit (amu) corresponding to $[C_5H_{12}N^+]$, Ciprofloxacin was at m/z 101 amu corresponding to $[C_4H_9N_2O^+]$ and l-leucine was at m/z 132 amu corresponding to $[C_6H_{14}NO_2^+]$. The spectra were integrated, and high-resolution surface composition overlays were constructed using WincadenceN software (Physical Electronics Inc., Chanhassen, MN, USA).

5.3.10 Solid State Fourier Transform Infrared Spectroscopy (FTIR)

FTIR analysis was performed for co-spray-dried formulations of Colistin-Ciprofloxacin in the mass ratios of 1:9, 1:3, and 1:1 using a Cary 600 series IR spectrophotometer (Agilent Technologies, Santa Clara, California, USA) equipped with an attenuated total reflectance (ATR) sample stage. The spectra were collected in the range of 400 – 4000 cm^{-1} with 4 cm^{-1} resolution and 64 scans. Prior to collecting sample spectra, background scans were collected by purging the detector with clean dry nitrogen gas thereby minimizing the interferences of water and CO_2 signals [265].

5.3.11 Drug Quantification

Drug concentration for Ciprofloxacin hydrochloride and Colistin sulfate was determined by high performance liquid chromatography (HPLC) using 76% (v/v) of 30 mM solution of sodium sulfate (adjusted to pH 2.5 with H_3PO_4) and 24% (v/v) acetonitrile as mobile phase resulting in isocratic elution of the sample at a flow rate of 1.0 mL/min [304]. The elution time for Ciprofloxacin was ~2 min and that of Colistin was ~3.2 and 5.2 min corresponding to Colistin A and Colistin B, the two main components of Colistin. Peak area for Colistin A and Colistin B was summed for quantification. Briefly, the HPLC system consisted of G1311C (1260 Quat Pump VL) pump, G1330B (1290 Thermostate) thermostate, G1329B (1260 ALS) autosampler, G1316A (1260 TCC) thermostated column compartment, G1314F (1260 VWD) variable wavelength detector (Agilent, Waldbronn, Germany), and an Agilent Eclipse Plus, 5 μm C18 150 × 4.60 mm column (Agilent, Waldbronn, Germany). The calibration curve for Ciprofloxacin hydrochloride was linear ($r^2 > 0.99$) over the concentration range of approximately 0.006 to 0.22 mg/mL. The calibration curve for Colistin sulfate was linear ($r^2 = 1$) over the concentration range of approximately 0.006 to 0.5 mg/mL.

5.3.12 In-vitro Aerosol Performance

A Multi-Stage Liquid Impinger (MSLI) (Copley Scientific Limited, Nottingham, UK) with a USP induction port (USP throat) was used to determine in-vitro aerosol performance of the spray-dried formulations at an ambient condition of approximately 20 °C and 40% RH. Each formulation (10 ± 1 mg) was filled into a size 3 hydroxypropyl methylcellulose capsule (Qualicaps, Whitsett, NC, USA) and dispersed through a RS01 DPI device (with a similar design to Osmohaler, Plastiape S.p.A., Osnago, Italy) [305]. The capsules were actuated at an airflow of 100 L/min for 2.4 s, allowing a pressure drop of approximately 4 kPa [306]. The cutoff diameters

for Stages 1–4 of the liquid impinger at 100 L/min were 10.4, 4.9, 2.4, and 1.2 μm, respectively [303]. Four replicate experiments were carried out for the powder formulations stored at 20%, 55%, and 75% relative humidity conditions, and each experiment comprised sequential dispersion of two filled capsules. Drug particles deposited in the capsule, inhaler device, USP throat, Stage 1-4, and the filter paper in the impactor base were collected using MilliQ water. Drug contents were analyzed using the validated high-performance liquid chromatography (HPLC) method described above [307]. The emitted dose was determined as the drug released from the capsule and device; whereas the fine particle fraction was defined as particles with an aerodynamic size below 4.9 μm (cut-off diameter of Stage 2) relative to the total recovered drug.

5.3.13 Statistical Analysis

Statistical analysis was performed by one-way analysis of variance (ANOVA) with Tukey-Kramer post hoc tests using a GraphPad Prism Software (GraphPad Software, Inc., La Jolla, CA). Probability values of less than 0.05 were considered as a statistically significant difference and NS represents not significant when *p* values were greater than 0.05.

5.4 Results

5.4.1 Physical stability and aerosol performance of co-spray-dried formulation at 55% RH

5.4.1.1 PXRD

PXRD patterns depict that the spray-dried Ciprofloxacin alone, spray-dried Colistin alone and co-spray-dried ColCipro powder formulations with different mass ratios were amorphous (Figure 5-1A) and did not show any crystallization up to 60 days when stored at 20 % RH (Figure 5-1B). However, the spray-dried Ciprofloxacin alone particles crystallized upon one-day storage at 55% RH but all other formulations were amorphous after one day storage at 55% RH (Figure 5-1C).

The co-spray-dried ColCipro formulation in the mass ratio (1:3) and (1:9) crystallized at Day 3 upon storage at 55% RH (Figure 5-1D). But, the co-spray-dried ColCipro (1:1) was amorphous for up to 60 days upon storage at 55% RH (Figure 5-1E). Crystallization of Ciprofloxacin was prevented when co-spray-dried with Colistin in the mass ratio (1:1) and stored at 55% RH for up to 60 days.

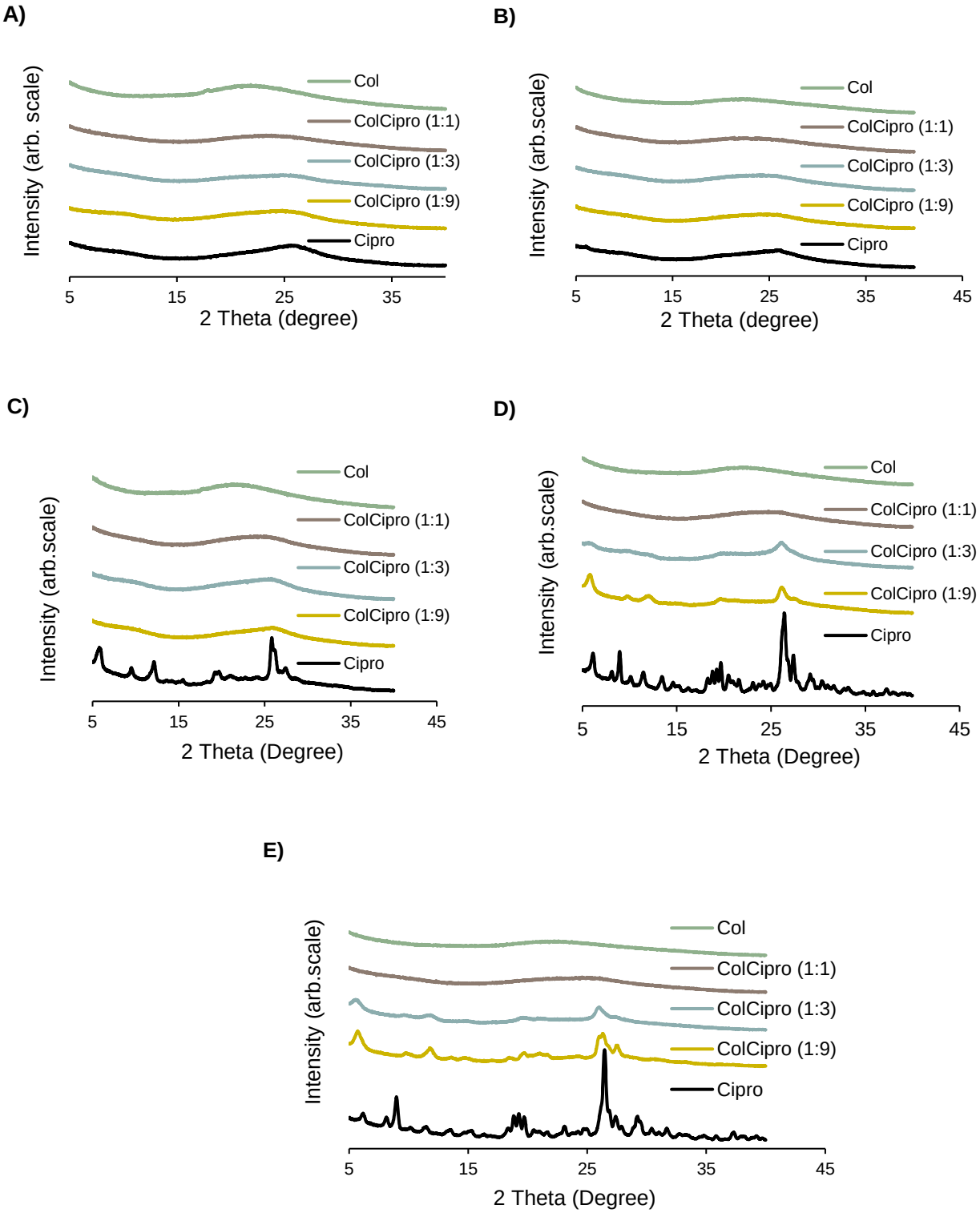


Figure 5-1: X-ray powder diffraction patterns of the drug alone and co-spray-dried powder formulations (A) immediately after spray-drying and stored at (B) 20%RH after 60 days (C) 55% RH after 1 day (D) 55% RH after 3 days (E) 55% RH after 60 days.

5.4.1.2 FTIR

The FT-IR result are shown in Figure 5-2 and Table 5-2. For the raw Colistin, two characteristic absorption bands at 1643.0 cm^{-1} and 1521.5 cm^{-1} were detected and attributed to amide I C=O stretching and amide II N-H bending vibrations, respectively [308]. There was no significant change in FT-IR spectrum after spray-drying of Colistin compared to the amorphous form of raw Colistin. Raw Ciprofloxacin showed its characteristic peaks at 1702.8 cm^{-1} due to carboxyl C=O stretching [309]. There were significant shifts to higher wavenumber at this peak for the spray-dried Ciprofloxacin which is likely indicative of the amorphous nature due to lack of regular molecular arrangement caused by hydrogen bonding.

In the co-spray-dried Ciprofloxacin and Colistin, there were significant changes in the IR spectra compared to each spray-dried pure drug. The peaks due to amide II N-H bending and amide I C=O stretching of Colistin shifted to lower and higher wavenumber, respectively, in the co-spray-dried Colistin-Ciprofloxacin (1:1) formulation. The carboxyl C=O stretching peak of Ciprofloxacin shifted to higher wavenumber as the ratio of Colistin increased. Significant changes were also observed in the range of 1500~1400 cm^{-1} of Ciprofloxacin, but it was difficult to explain clearly because there was a limitation of information due to overlapping and broadening of peaks.

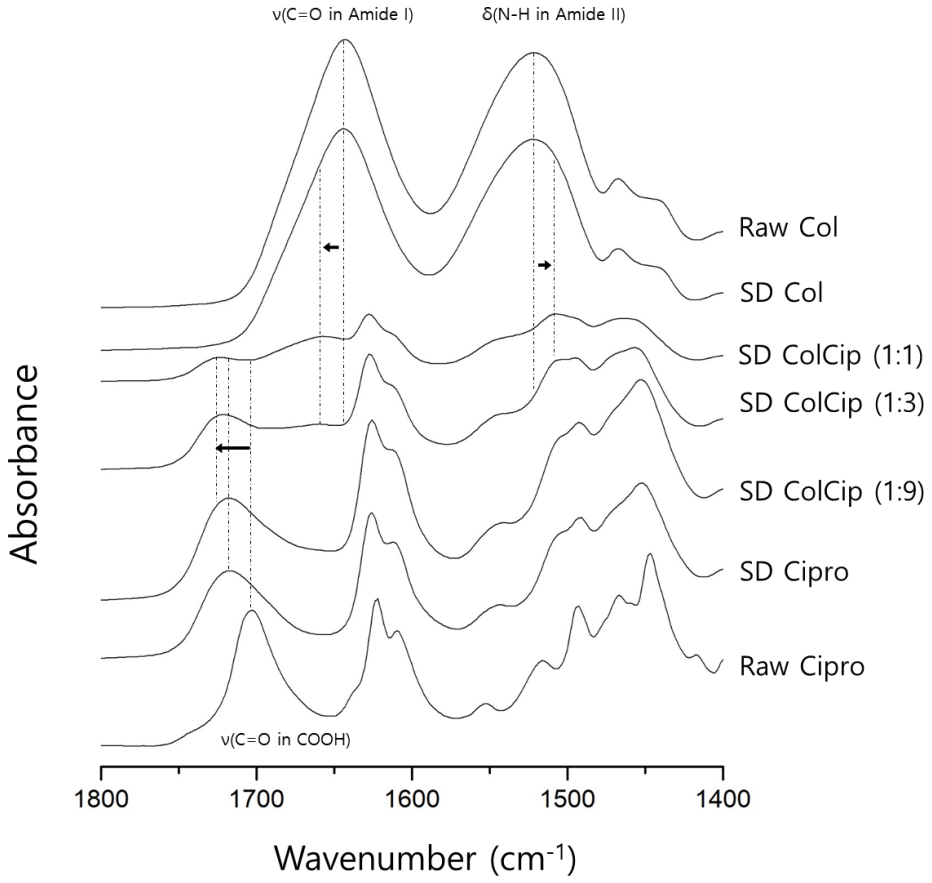


Figure 5-2: FT-IR spectra of the raw materials and spray-dried formulations in the wavenumber range of 1800~1400 cm-1 (ν: stretching, δ: bending).

Table 5-2: FT-IR band assignments for raw materials and spray-dried formulations in the wavenumber range of 1800~1400 cm-1

Formulation	**Band assignment and wavenumber (cm^{-1})**		
	Colistin		**Ciprofloxacin**
	Amide I C=O stretching	**Amide II N-H bending**	**Carboxyl C=O stretching**
Raw Colistin	1643.0	1521.5	-
SD Colistin	1643.0	1521.5	-
SD ColCipro (1:1)	1656.5	1508.0	1722.1
SD ColCipro (1:3)	-	-	1720.2
SD ColCipro (1:9)	-	-	1718.2
SD Ciprofloxacin	-	-	1716.3
Raw Ciprofloxacin	-	-	1702.8

5.4.1.3 Particle Size

Tables 5-3 and 5-4 show the physical particle size distribution. No remarkable difference in physical particle size was observed for five different formulations stored at 20% and 55% RH. All five formulations had fine physical size with D_{50} < 1.4 µm and D_{90} < 3 µm.

Table 5-3: Particle size distribution for the SD Cipro, SD Col, and co-spray-dried ColCipro formulations in the mass ratio (1:1), (1:3), and (1:9) stored at 20 % RH for 3 days.

Formulation	**D_{10} (µm)**	**D_{50} (µm)**	**D_{90} (µm)**
SD Cipro	0.4	1	2.1
ColCipro (1:1)	0.7	1.3	2.2
ColCipro (1:3)	0.6	1.2	2.1
ColCipro (1:9)	0.5	1.0	2.1
SD Col	0.8	1.3	2.4

Table 5-4: Particle size distribution for SD Cipro, SD Col, and co-spray-dried Col-Cipro formulation in the mass ratio (1:1), (1:3), and (1:9) stored at 55 % RH for 3 days.

Formulation	D_{10} (µm)	D_{50} (µm)	D_{90} (µm)
SD Cipro	0.2	1.2	2.4
ColCipro (1:1)	0.6	1.2	2.0
ColCipro (1:3)	0.6	1.2	2.1
ColCipro (1:9)	0.5	1.0	2.0
SD Col	0.8	1.4	2.3

5.4.1.4 Surface Morphology

SEM images are shown in Figure 5-3. The SD Ciprofloxacin alone formulation stored at 20% RH had a smooth dimpled surface with spherical shape; but at 55% RH for 3 days, the partices were rougher, which is in good agreement to our previous finding [75], attributed to crystallization of amorphous spray-dried Ciprofloxacin particles. The spray-dried Colistin formulation stored at 20% and 55% RH had a more wrinkled shape (Figure 5-3). No change was observed in morphology of the spray-dried Colistin sample stored at two RHs (Figure 5-3). Similarly, no change in morphology was observed for the composite formulations at different concentrations stored at two different RHs. Although, the co-spray-dried formulations in the mass ratio (1:3) and (1:9) crystallized after three days of exposure to 55% RH, no apparent change in particle morphology was observed.

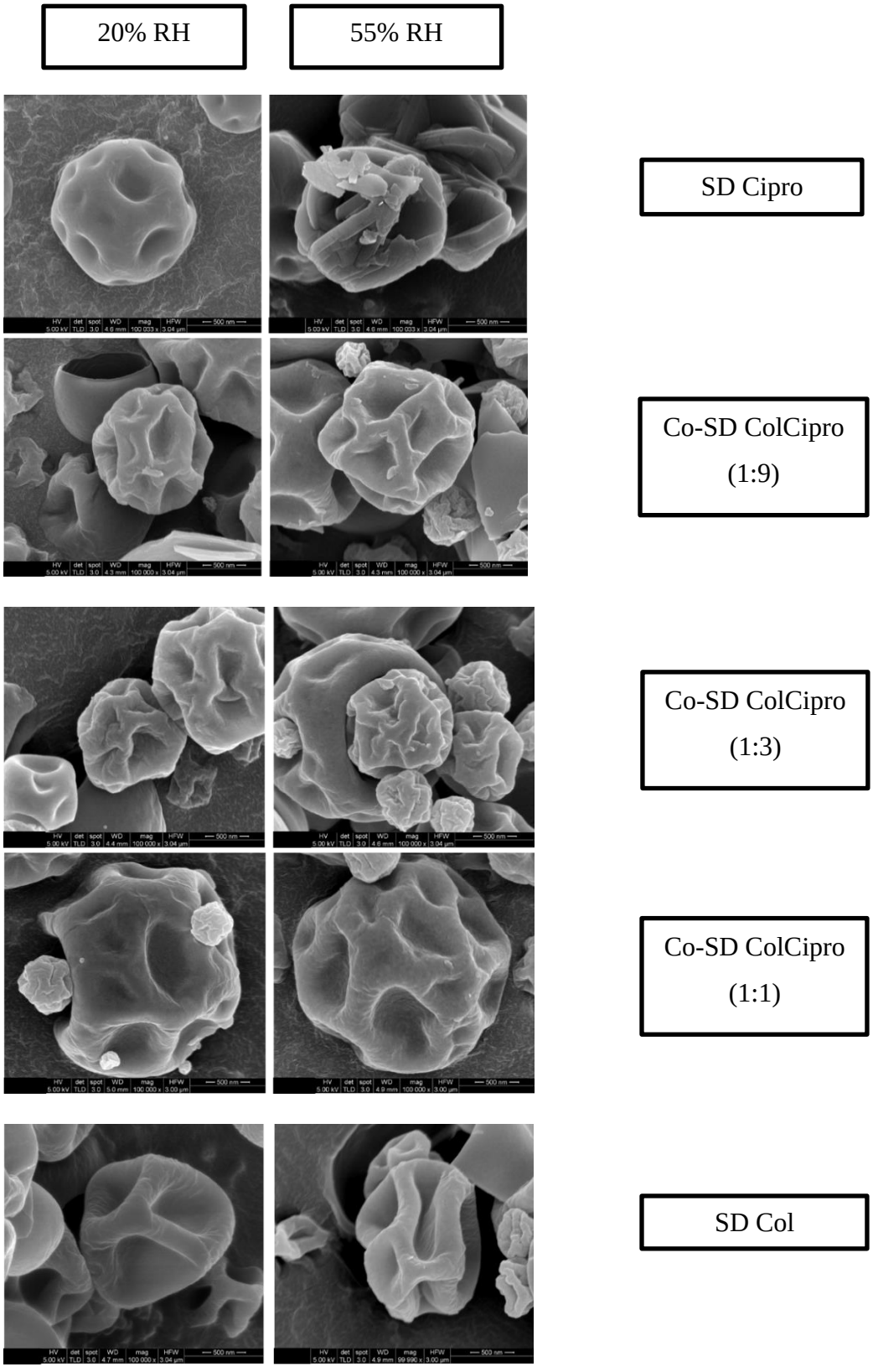


Figure 5-3: SEM images of the spray-dried drug alone and co-spray-dried Colistin-Ciprofloxacin powder formulations stored at 20% and 55% RH for 3 days.

5.4.1.5 Time-of-flight secondary ion mass spectrometry (ToF-SIMS)

Figure 5-4 represents the ToF-SIMS images. In the overlay images, the green signal represents Ciprofloxacin and the red signal represents Colistin. An increase in Colistin signal was shown with higher Colistin concentrations in the combination formulations, which was further quantified using XPS and EDX.

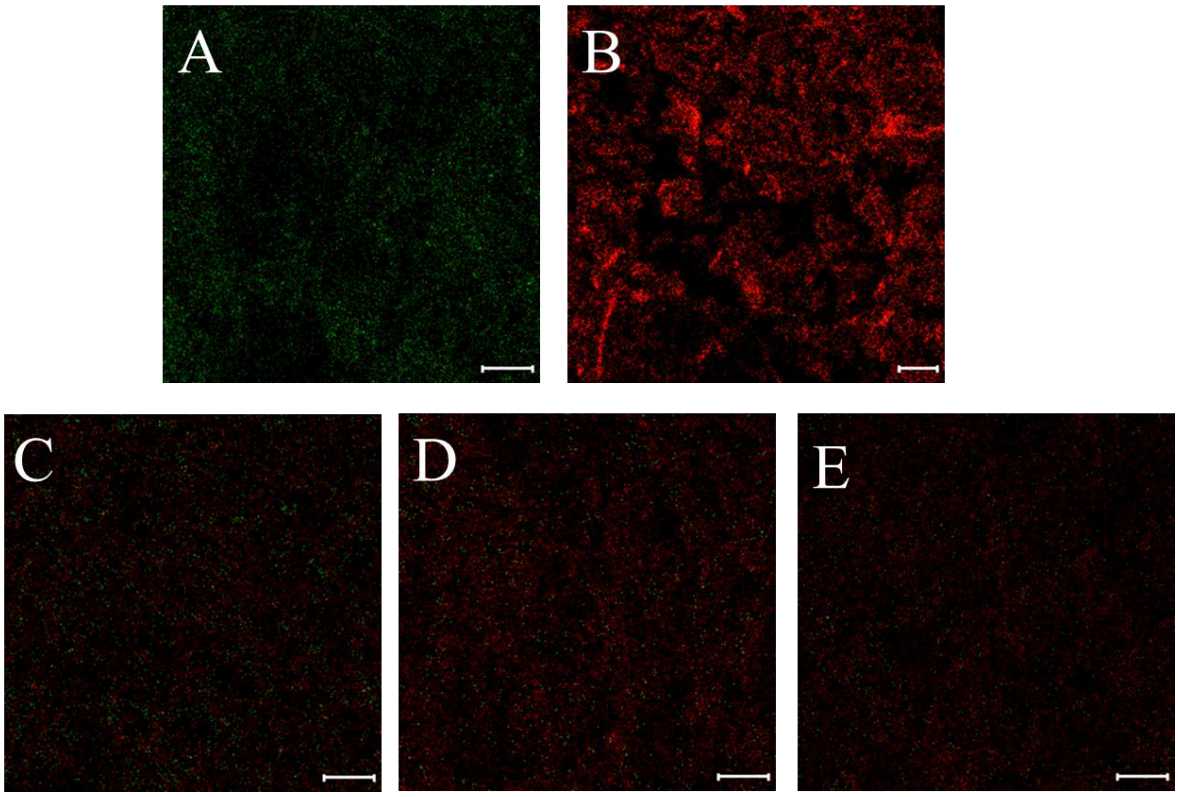


Figure 5-4: Surface composition distributions of Colistin (red) and Ciprofloxacin (green) on the surfaces of composite particles obtained by ToF-SIMS: (A) Raw Ciprofloxacin; (B) Raw Colistin; (C) Colistin-Ciprofloxacin (1:9); (D) Colistin-Ciprofloxacin (1:3) and (E) Colistin-Ciprofloxacin (1:1) (scale bar represents 10 μm).

5.4.1.6 Energy Dispersive X-ray Spectroscopy (EDX)

Figure 5-5 depicts quantitative surface composition estimates of the elements in the co-spray-dried formulations. Theoretical atomic concentration of sulfur in the co-spray-dried ColCipro formulation (1:9) was found to be 3.3% whereas the experimental atomic concentration was

found to be 12.5 ± 0.5%. Experimental sulfur (Colistin) concentration was greater than theoretical value in the co-spray-dried formulations for all different mass ratios, which is likely due to surface enrichment of Colistin in these combination formulations. However, it should be noted that such surface experimental element concentration could be underestimated as the X-ray penetration depth is approximately 1 µm and likely to penetrate into the particles. Therefore, more surface sensitive characterization techniques of XPS and ToF-SIMS were employed.

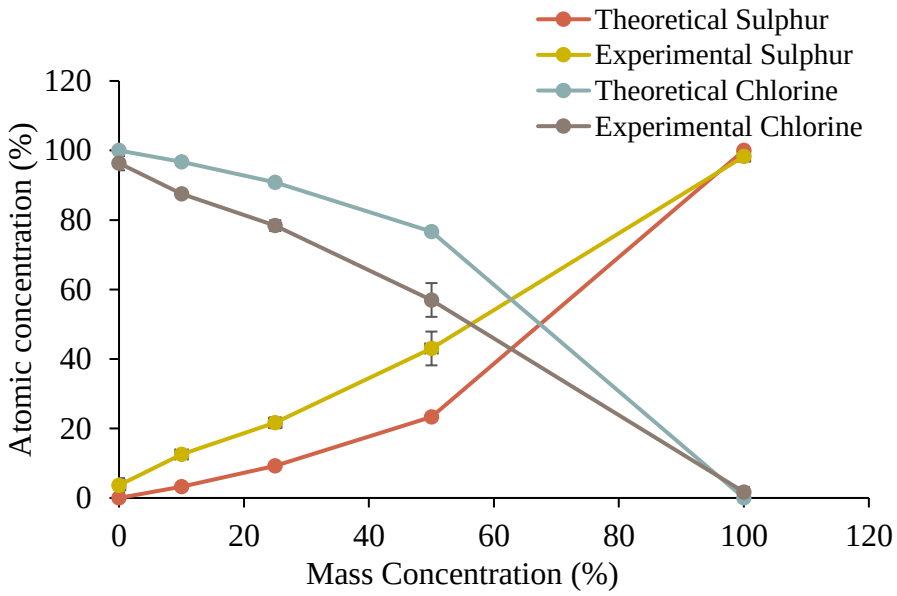


Figure 5-5: Surface concentrations based on elemental analysis of chlorine (in Ciprofloxacin hydrochloride) and sulfur (in Colistin sulfate) measured by EDX.

5.4.1.7 X-ray photoelectron spectroscopy (XPS)

Table 5-5 shows theoretical and surface compositions of Colistin and Ciprofloxacin in the composite formulations measured by XPS. As noted in Table V, surface Colistin concentration was higher than the theoretical Colistin composition for each formulation. For ColCipro (1:1), XPS detected 74% of the surfaces were Colistin compared with 50% theoretical Colistin based on carbon composition of Colistin (50%) and Ciprofloxacin (50%). Likewise, Colistin (60%) and Ciprofloxacin (40%) carbon composition from XPS differed significantly from the theoretical

carbon composition for Colistin (25%) and Ciprofloxacin (75%) in Colistin-Ciprofloxacin (1:3) co-spray-dried formulation. Even when both drugs were present at 50%, the Colistin composition from XPS was 74%. Thus, for all 3 formulations the measured XPS surface composition for Colistin was significantly higher than the corresponding theoretical values, indicating enrichment of Colistin on the particles surface in the co-spray-dried Colistin-Ciprofloxacin formulations.

Table 5-5: Theoretical and measured (by XPS) surface compositions (% mass ratio) for the co-spray-dried Colistin-Ciprofloxacin formulations.

Formulations	**Theoretical Surface Composition (%)**		**Measured Surface Composition (%) as determined by C 1s curve-fits**	
	Colistin	**Ciprofloxacin**	**Colistin**	**Ciprofloxacin**
ColCipro_1:9	10	90	35	65
ColCipro_1:3	25	75	60	40
ColCipro_1:1	50	50	74	26

5.4.1.8 Dynamic Vapor Sorption

The spray-dried Ciprofloxacin alone presented a sorption profile characteristic of water absorption and crystallization (via water expulsion). Specifically, moisture sorption 7.9% ((w/w)) increased with respect to increase in humidity up to 60% RH. Further increase in humidity (up to 70% RH) resulted in a decrease in mass, indicating loss of water and crystallization. When the material undergoes an amorphous to crystalline transition, the water sorption capacity typically decreases drastically. This results in an overall mass loss as excess water is desorbed during crystallization [310]. The increase in weight from the starting point of the first sorption run to the end of the first desorption run may be a useful approach to the

characterization of the amorphous content [270]. In our study we speculate that amorphous spray-dried Ciprofloxacin crystallizes to form a monohydrate. The molecular weight of Ciprofloxacin HCl monohydrate is 385.8, of which 4.6% is the water of crystallization. So, if it recrystallized to from the monohydrate then the weight gain after desorption (of physically adsorbed water, but not stoichiometric hydrate water) would be approximately 4-5% which is in accordance with our results for spray-dried Ciprofloxacin. The spray-dried Ciprofloxacin alone formulation has shown to absorb much less moisture in comparison to spray-dried Colistin which absorbed upto 32% moisture upon exposure to 90% RH. No crystallization corresponding to decrease in mass was observed for spray-dried Colistin which is in acordance with PXRD data[311]. The sorption and desorption profiles for Colistin, ColCipro (1:1), and ColCipro (1:3) formulations were similar with no evidence of crystallization or permanent retention of water, which are in accordance with our hypothesis that Colistin prevents crystallization of Ciprofloxacin when exposed to moisture (Figure 5-6B, 5-6D & 5-6E). However, for the ColCipro (1:9) formulation it showed a tendency to crystallize at 80% RH and retention of water in the sample at the end of the measurment of 0% RH (Figure 5-6C). This is in accordance with the PXRD results where ColCipro (1:9) crystallized at Day 3 upon storage at 55% RH (Figure 5-1D).

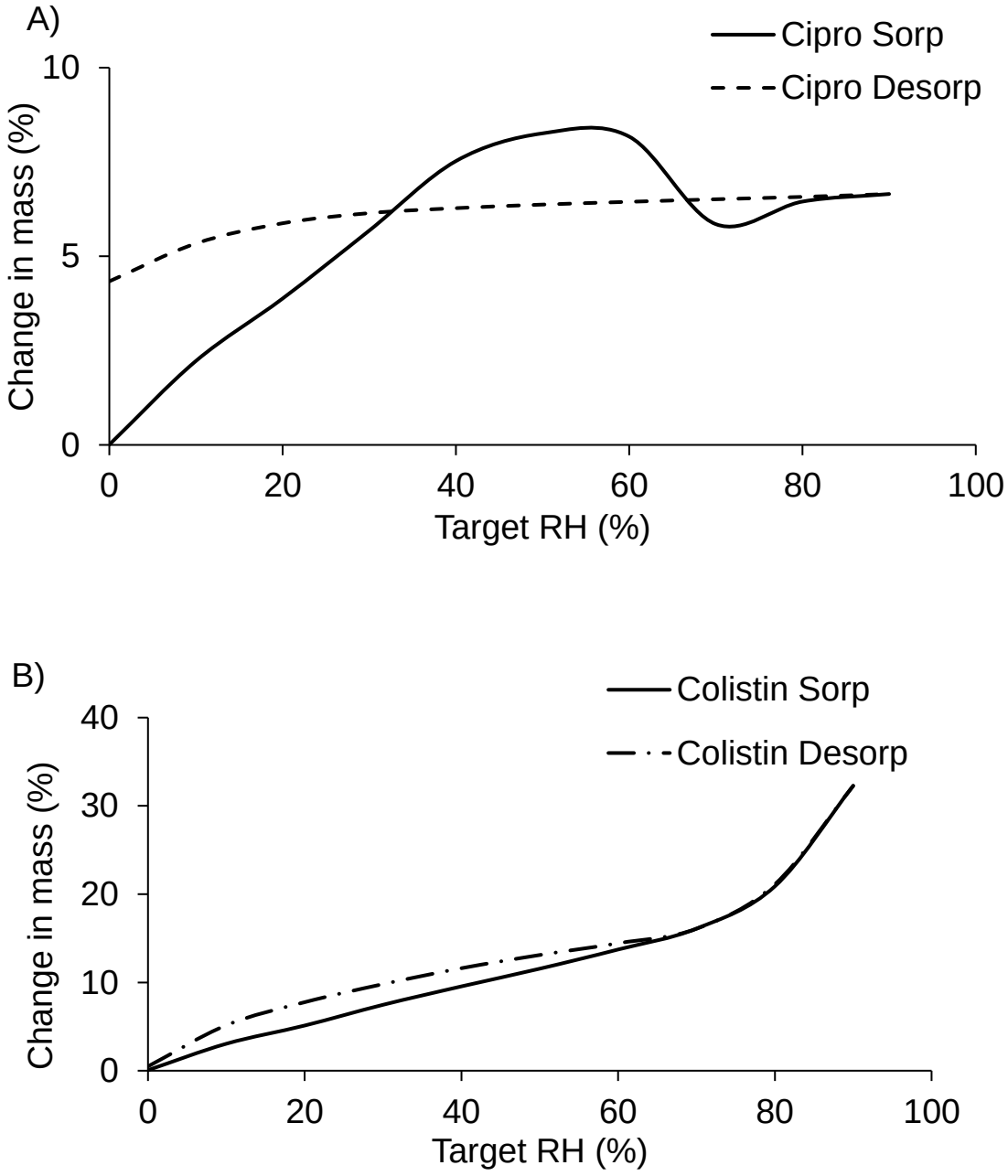
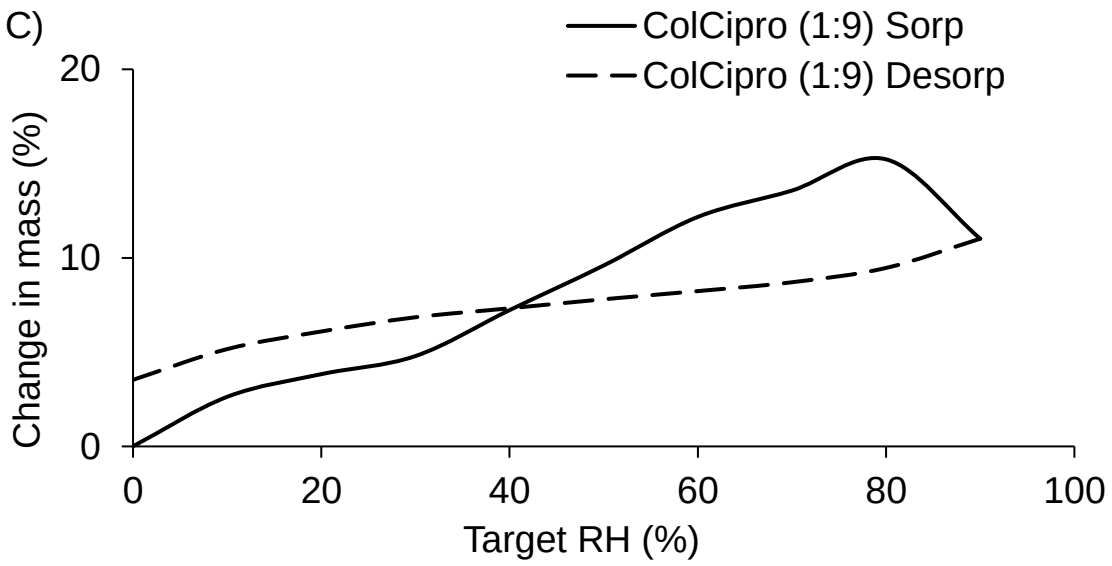


Figure 5-6**:** Moisture sorption isotherms for (A) SD Ciprofloxacin alone; (B) SD Colistin alone; (C) SD ColCipro (1:9); (D) SD ColCipro (1:3); and (E) SD ColCipro (1:1).

Figure 5-6 continued



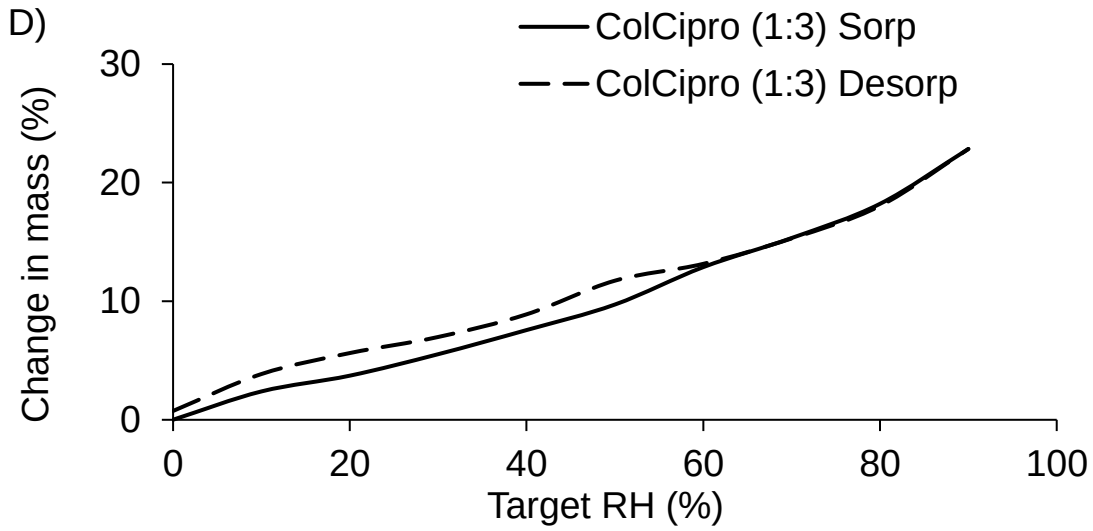
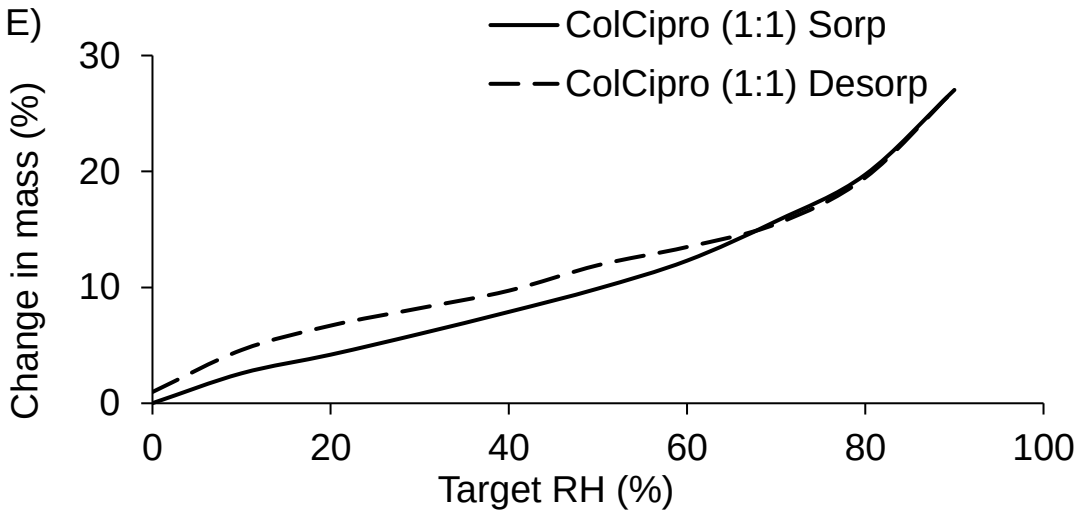


Figure 5-6 continued



5.4.1.9 In-vitro Aerosol Performance

An increase in fine particle fraction (FPF) of Ciprofloxacin was observed by increasing Colistin concentration from 10% to 50% (w/w) (Figure 5-9). The aerosol performance of co-spray-dried Colistin-Ciprofloxacin formulation in the mass ratio (1:9) (45.7 ± 4.2%), (1:3) (55.3 ± 3.1%) and (1:1) (67.1 ± 3.8%) was significantly higher (p <0.0001) compared to the spray-dried Ciprofloxacin alone (28 ± 3.2%). Even for the ColCipro (1:9) and ColCipro (1:3), in which crystallization was observed after 3 days, no significant change was measured in the aerosol performance (Figure 5-7A and 5-7B).

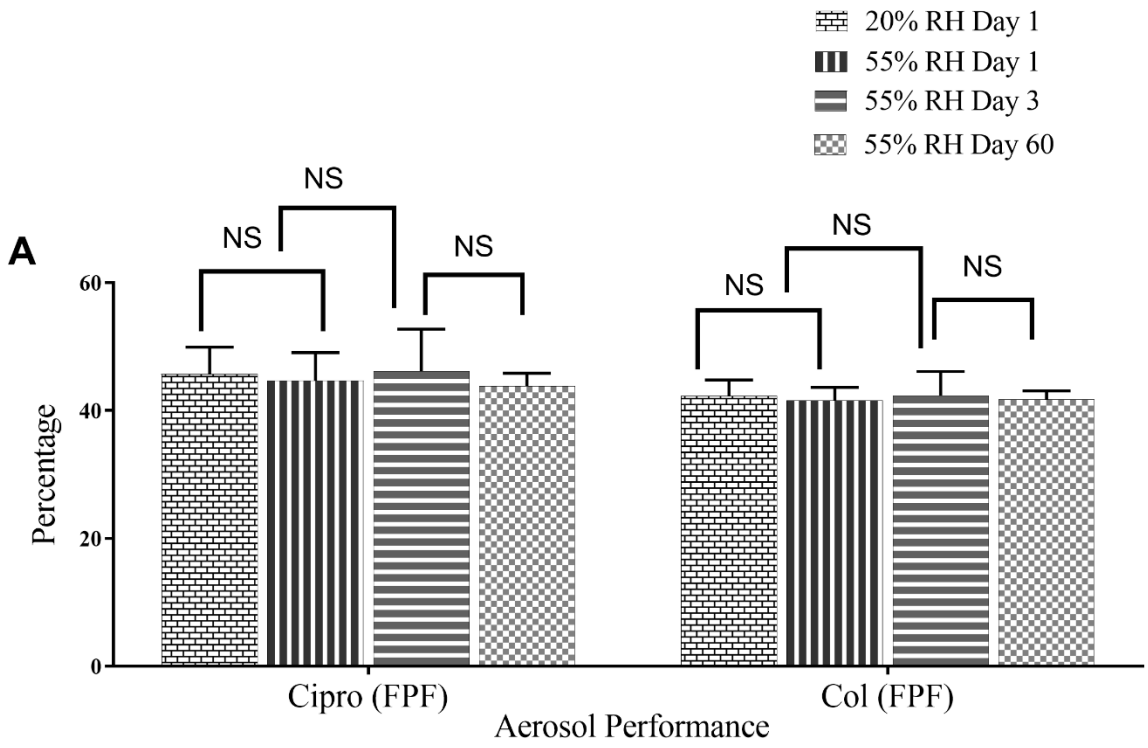
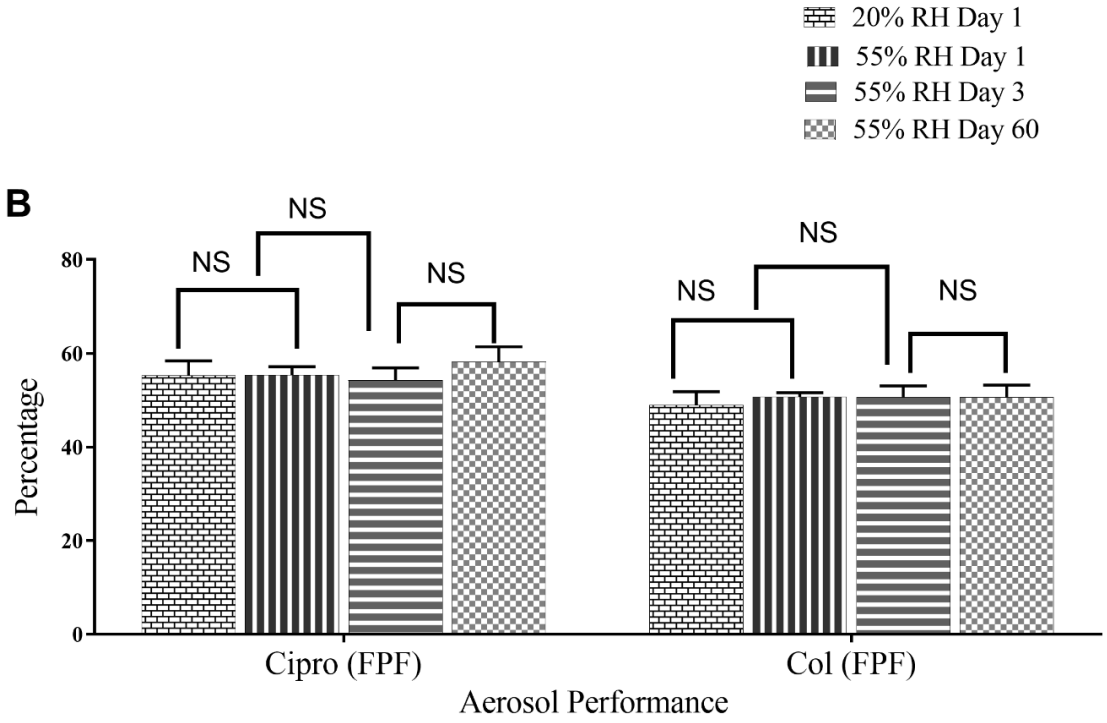
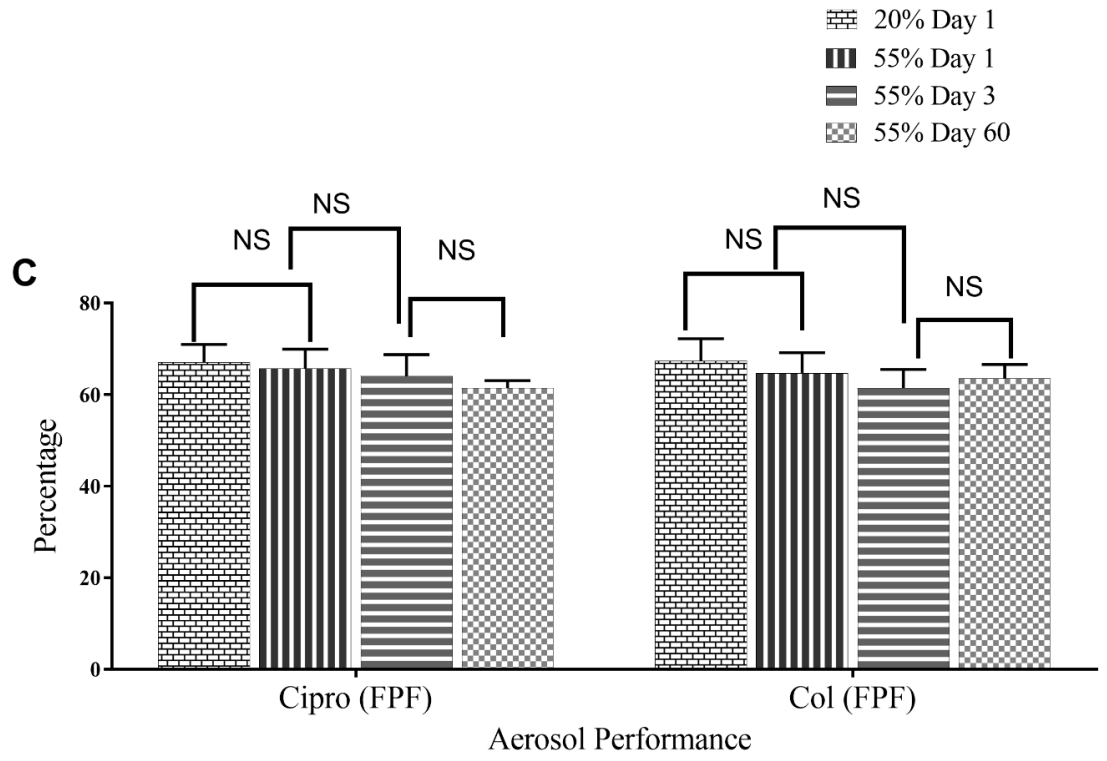


Figure 5-7**:** Fine particle fraction as a percentage of recovered dose of Ciprofloxacin and Colistin in the co-spray-dried formulations at different mass ratios (A) ColCipro (1:9); (B) ColCipro (1:3); and (C) ColCipro (1:1) which were stored at 20% RH and 55% RH (mean ± SD, n=4; *, p <0.05; **, p <0.01; ***, p < 0.001; ****, p<0.0001; NS, no significant difference).

Figure 5-7 continued





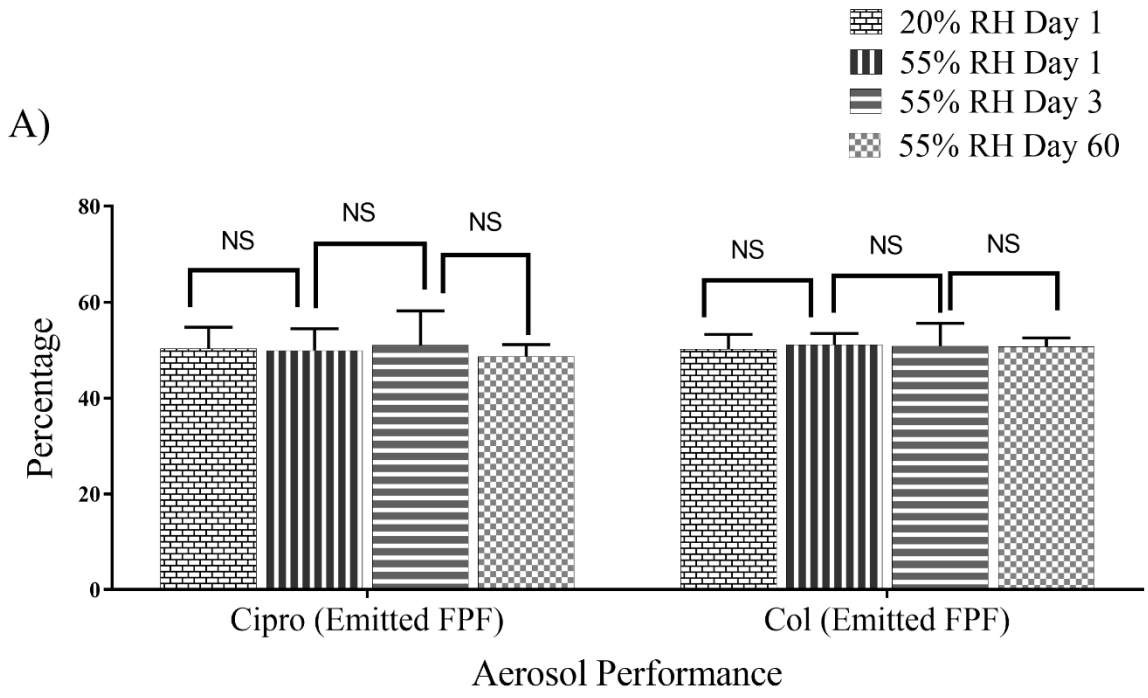
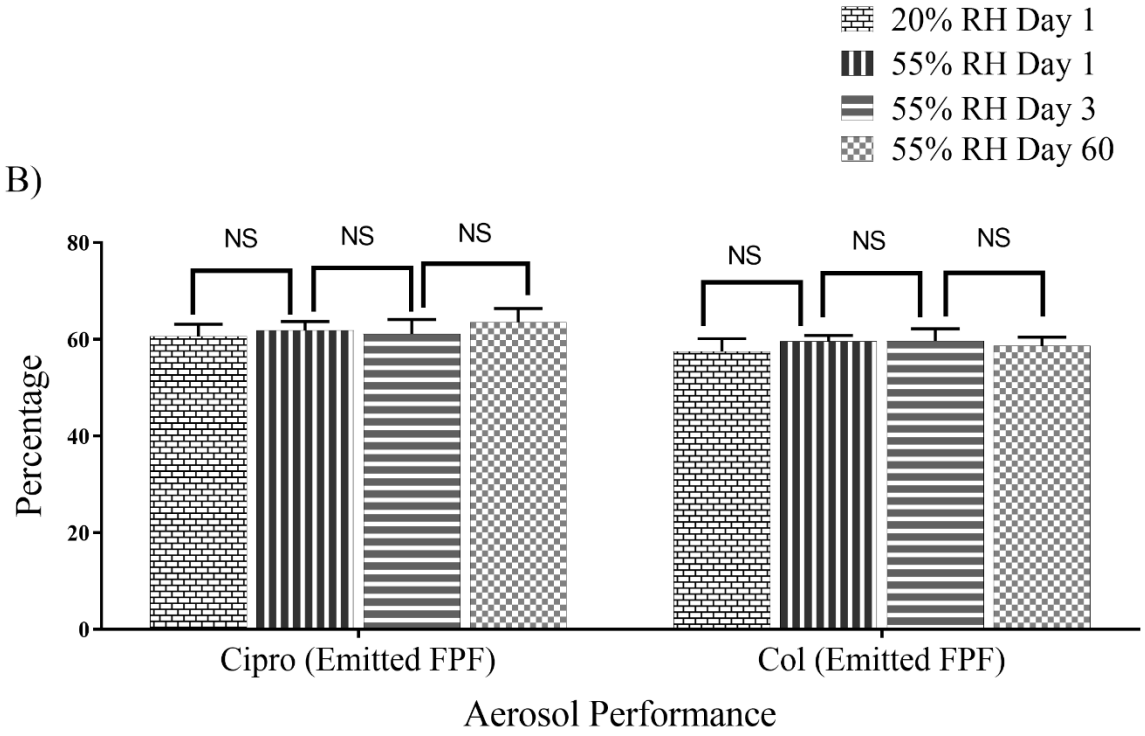
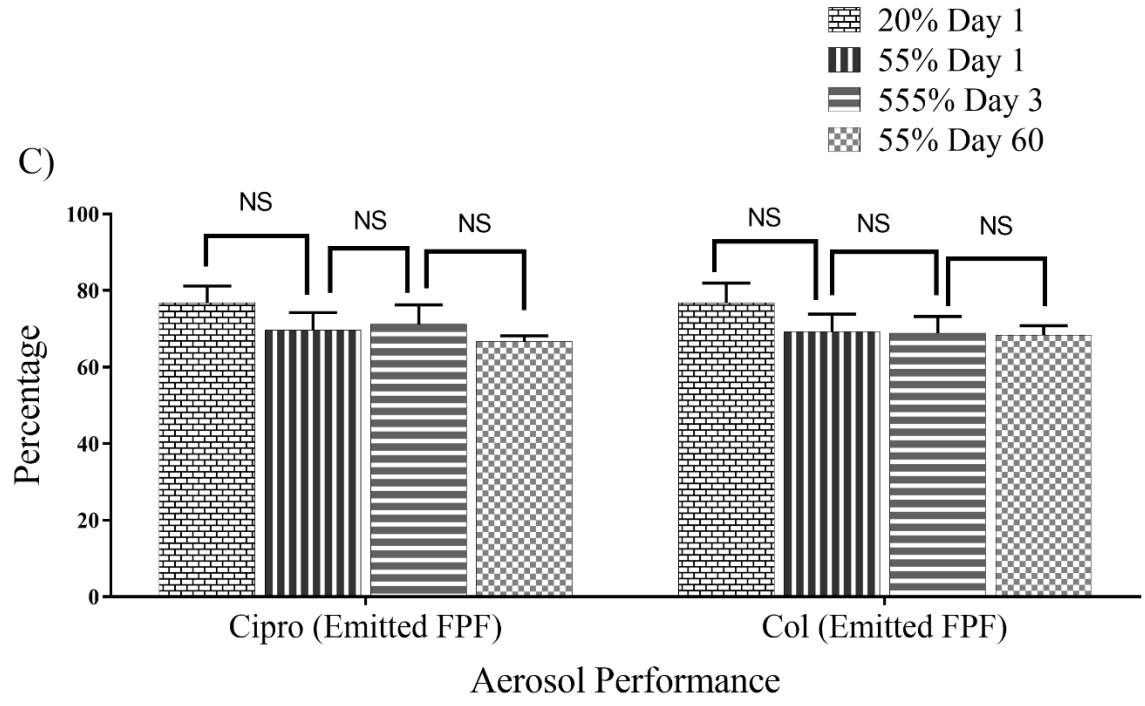


Figure 5-8: Fine particle fraction as a percentage of emitted dose of Ciprofloxacin and Colistin in the co-spray-dried formulations at different mass ratios (A) ColCipro (1:9); (B) ColCipro (1:3); and (C) ColCipro (1:1) which were stored at 20% RH and 55% RH (mean ± SD, n=4; *, p <0.05; **, p <0.01; ***, p < 0.001; ****, p<0.0001; NS, no significant difference).

Figure 5-8 continued





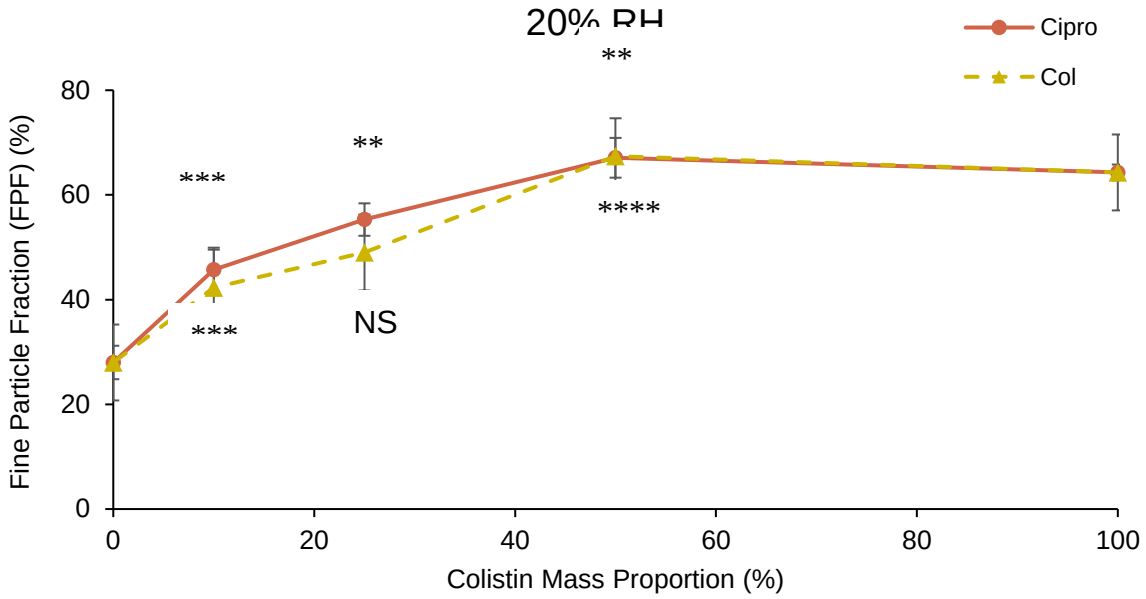


Figure 5-9: Effect of Colistin concentration on aerosol performance of co-spray-dried formulations after storage at 20% RH for 1 day (mean ± SD, n=4; *, p <0.05; **, p <0.01; ***, p < 0.001; ****, p<0.0001; NS, no significant difference).

5.4.1.10 Change in morphology upon storage of 60 days

SEM images of the spray-dried Ciprofloxacin alone, spray-dried Colistin alone, and co-spray-dried powder formulations stored at 20% RH and 55% RH for 60 days are shown in Figure 5-10. The round dimpled shaped spray-dried Ciprofloxacin powder formulation underwent a drastic change to rough particles upon storage at 55% RH after 3 days (Figure 5-3) and retained that rough surface for up to 60 days at 55% RH (Figure 5-10). However, there was no significant change in particle morphology with the co-spray-dried Colistin-Ciprofloxacin formulation in the mass ratio (1:1), (1:3) and (1:9) stored at 55% RH for 60 days as compared to the corresponding formulations stored at 20% RH. Similarly, no change in particle morphology was observed for the spray-dried Colistin alone stored at 55% RH for 60 days as compared to that stored at 20% RH.

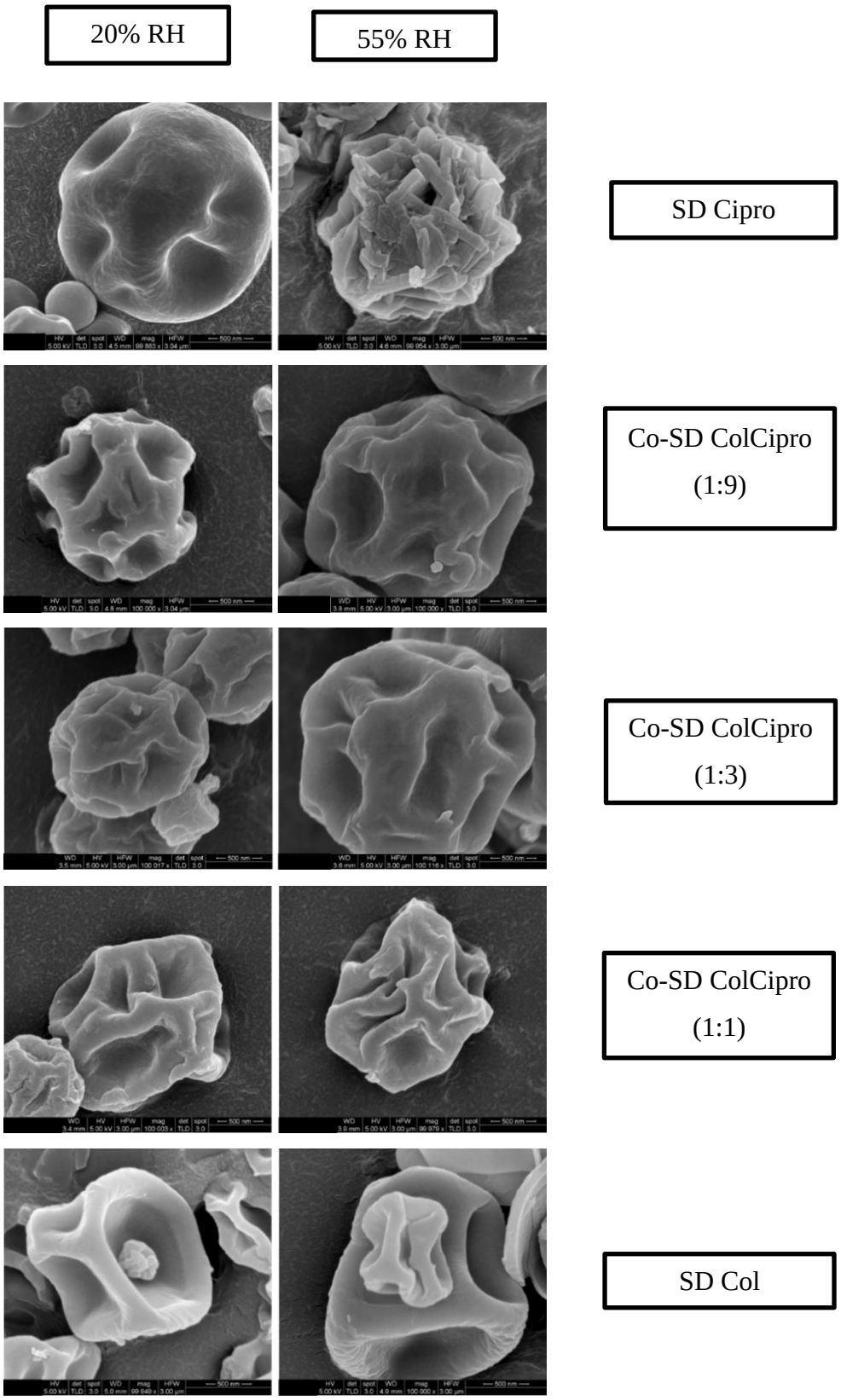


Figure 5-10: SEM images of the spray-dried formulations stored at 20% and 55% RH for 60 days.

5.4.2 Physical and aerosol stability of the co-spray-dried formulation (1:1) at 75% RH

Since the co-spray-dried Colistin-Ciprofloxacin formulation in the mass ratio (1:1) was amorphous at 55% RH for 60 days without significant changes in surface morphology or aerosol performance, we further investigated the performance of this formulation at 75% RH.

5.4.2.1 PXRD

Figure 5-11 depicts the PXRD patterns for the co-spray-dried Ciprofloxacin-Colistin formulation (1:1) upon storage at 75% RH for 7 days. The co-spray-dried Ciprofloxacin-Colistin formulation at 20% RH is amorphous; however, Ciprofloxacin crystallized upon exposure to 75% RH after 1 day. A subsequent increase in degree of crystallization was observed from day 1 to day 7 for the co-spray-dried Ciprofloxacin-Colistin formulation at 75% RH; although such increase was apparent. We further investigated the effects of such crystallization on the surface morphology and aerosol performance of the co-spray-dried formulation.

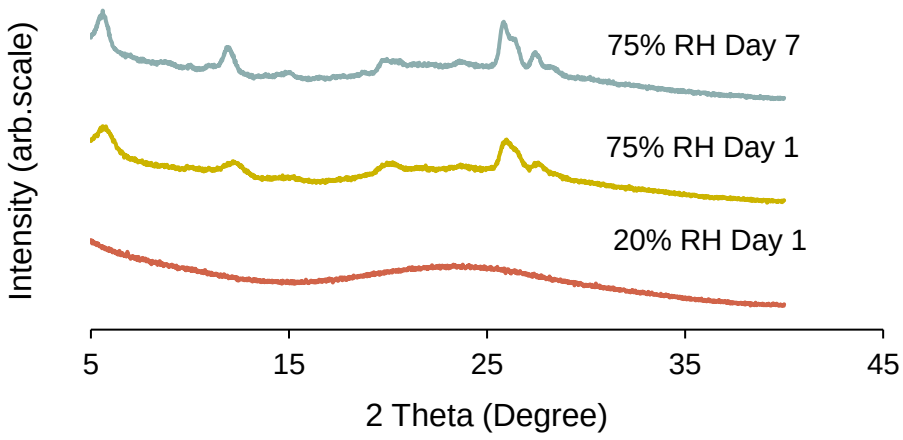


Figure 5-11: PXRD patterns for the co-spray-dried Ciprofloxacin-Colistin formulation in the mass ratio (1:1) stored at 75% RH for up to 7 days.

5.4.2.2 SEM

The co-spray-dried Ciprofloxacin-Colistin formulation had a smooth surface with spherical shape at 20% RH. No change in surface morphpology of the co-spray-dried formulation was observed upon storage at 75% RH for one day. However, upon storage at 75% RH for 7 days, the powders appeared to be fused (Figure 5-12). Based on the XPS data (Table 5-4) we observed that Colistin is enriched at the surface of the co-spray-dried Ciprofloxacin-Colistin formulation in the mass ratio (1:1) and it has been studied previously that Colistin has a greater tendency to absorb moisture [311].

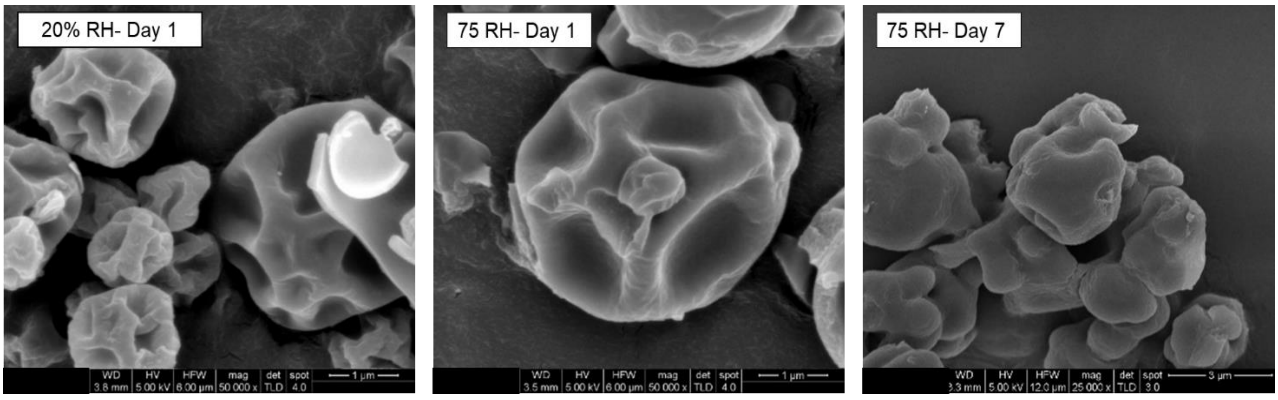


Figure 5-12: SEM micrographs of the co-spray-dried Ciprofloxacin-Colistin formulation in the mass ratio (1:1) stored at 75% RH for up to 7 days.

5.4.2.3 In-vitro Aerosol Performance

Upon storage at 75% RH for one day, no significant change ($p > 0.05$) in FPF for both Colistin (68.6 ± 2.1%) and Ciprofloxacin (71.5 ± 1.7%) was observed as compared to the FPF of both Colistin (67.4 ± 4.9%) and Ciprofloxacin (67.1 ± 3.8%) at 20% RH for 1 day. However, a significant decrease ($p < 0.0001$) in FPF was observed when the co-spray-dried Ciprofloxacin-Colistin formulation was stored at 75% RH for 7 days (Figure 5-13). The FPF for Ciprofloxacin and Colistin decreased to 19.2 ± 1.4% and 19.5 ± 1.3% respecetively when stored at 75% RH for

7 days. As previous studies showed that co-spray-drying with leucine may minimize the negative effects of moisture on aerosolization, we added L-leucine in the formulation to prevent such moisture effects [154].

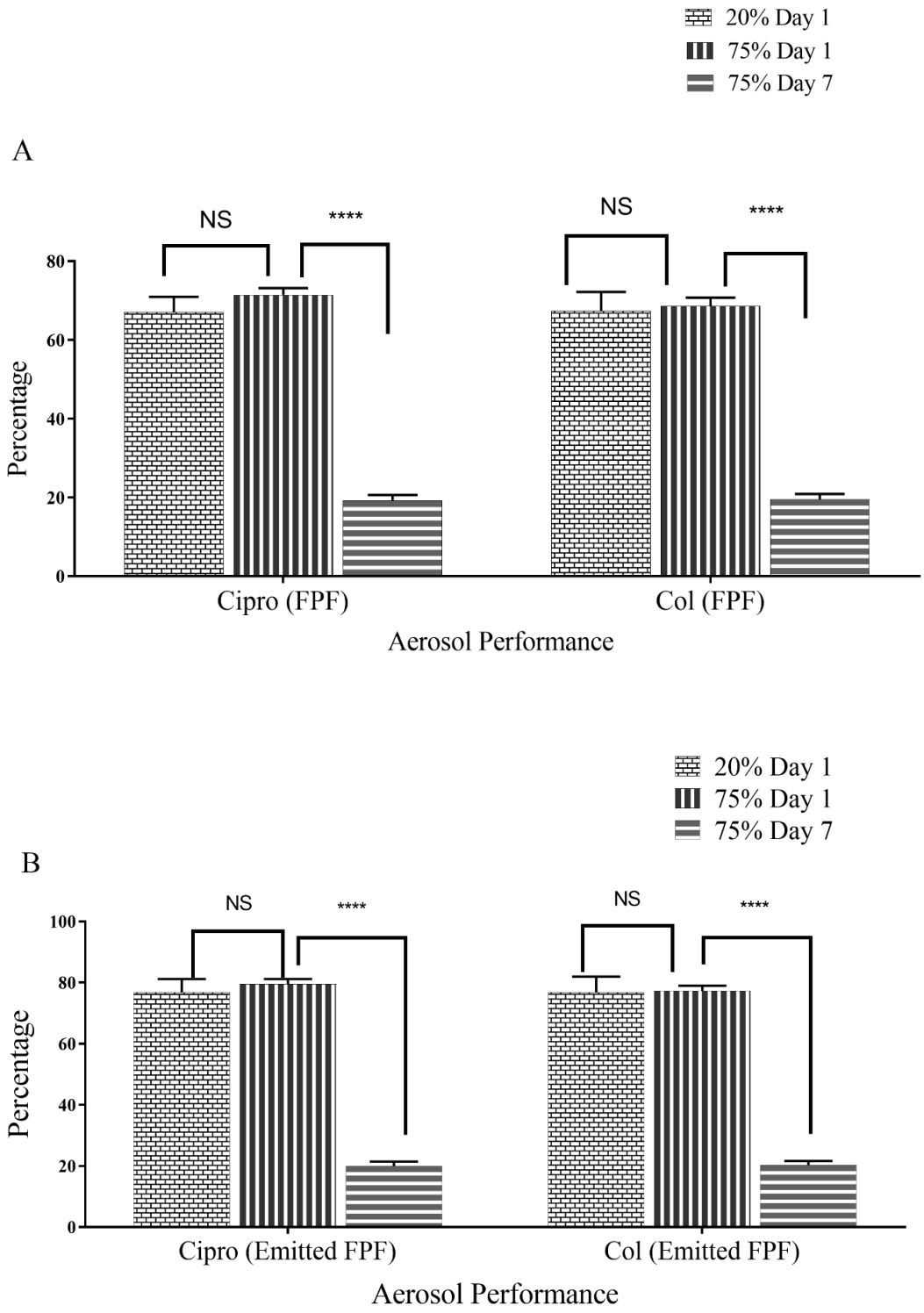


Figure 5-13: (A) Fine particle fraction as a percentage of recovered dose (B) Fine particle fraction as a percentage of emitted dose of Ciprofloxacin and Colistin in the co-spray-dried formulation with mass ratio (1:1) stored at 75% RH for 7 days (mean ± SD, n=4; *, p <0.05; **, p <0.01; ***, p < 0.001; ****, p<0.0001; NS, no significant difference).

5.4.3 Enhancing physical and aerosolization stability by adding L-leucine

5.4.3.1 PXRD

Figure 5-14 depicts the PXRD patterns of the co-spray-dried Colistin-Ciprofloxacin-leucine (ColCipLeu) formulation in the mass ratio (1:1:1) upon storage at 75% RH for 7 days. The co-spray-dried ColCipLeu (1:1:1) formulation at both 20% RH and 75% RH was found to be crystalline at Day 1. Interestingly, no Ciprofloxacin peaks were observed at Day 1 as seen with ColCipro (1:1) co-spray-dried formulations stored at 75% RH for 1 day (Figure 5-11). However, the crystalline peaks corresponded to L-leucine. Upon storage at 75% RH for 7 days, slight crystallization of Ciprofloxacin was observed for the co-spray-dried ColCipLeu (1:1:1) formulation. We have shown previously that 10% ((w/w)) L-leucine alleviates crystallization of Ciprofloxacin at 55% RH. Thus, we hypothesize that at 75% RH in presence of both Colistin and L-leucine the crystallization of Ciprofloxacin is substantially reduced.

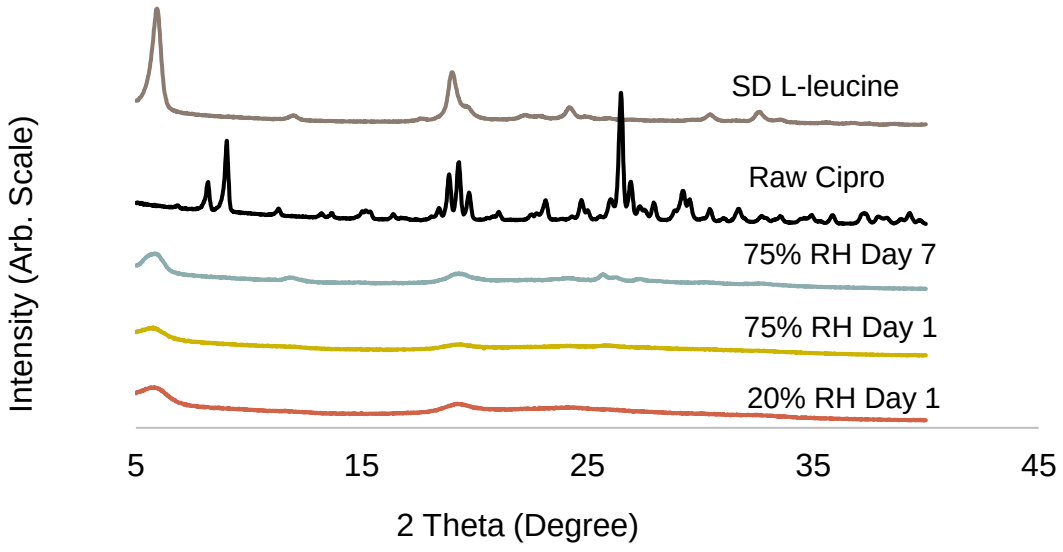


Figure 5-14: PXRD patterns for the co-spray-dried Colistin-Ciprofloxacin-leucine (ColCipLeu) formulation in the mass ratio (1:1:1) stored at 75% RH for up to 7 days, spray-dried L-leucine and raw Ciprofloxacin

5.4.3.2 SEM

The co-spray-dried Colistin-Ciprofloxacin-leucine formulation in the mass ratio (1:1:1) had a dimpled and rough surface at 20% RH. No change in morphpology of the co-spray-dried formulation was observed upon storage at 75% RH for 7 days (Figure 5-15).

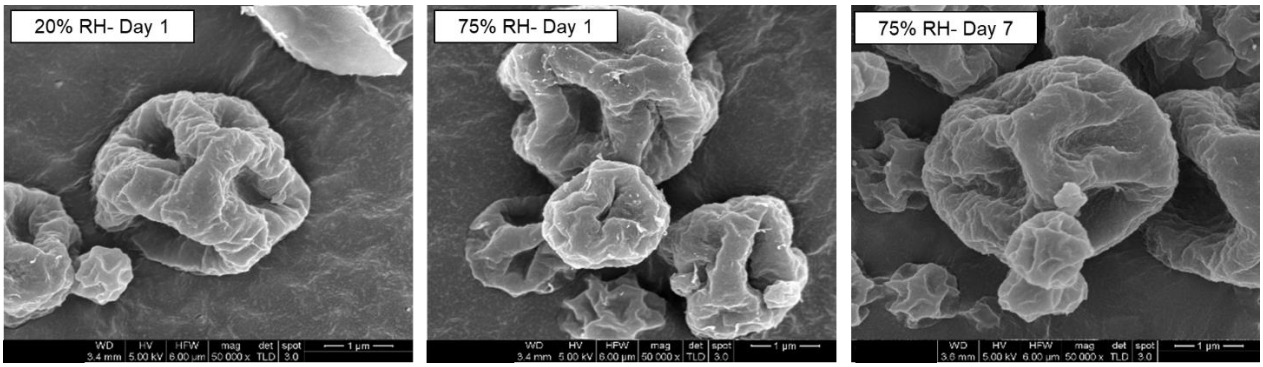


Figure 5-15: SEM micrographs of the co-spray-dried Colistin-Ciprofloxacin-leucine formulation in the mass ratio (1:1:1) stored at 75% RH for up to 7 days.

5.4.3.3 In-vitro Aerosol Performance

Figure 5-16 depicts the in-vitro aerosol performance for co-spray-dried ColCipLeu formulation in the mass ratio (1:1:1) stored at 20% RH for 1 day, 75% RH for 1 day and 7 days respectively. FPF for both Colistin (62.9 ± 6.1%) and Ciprofloxacin (63.1 ± 4.7%) stored at 75% RH for 1 day did not change significantly as compared to the FPF of both Colistin (58.7 ± 2.2%) and Ciprofloxacin (62.1 ± 2.2%) at 75% RH for 7 days. Adding L-leucine prevented the change in surface morphology and aerosol performance upon storage of this formulation at 75% RH for up to 7 days.

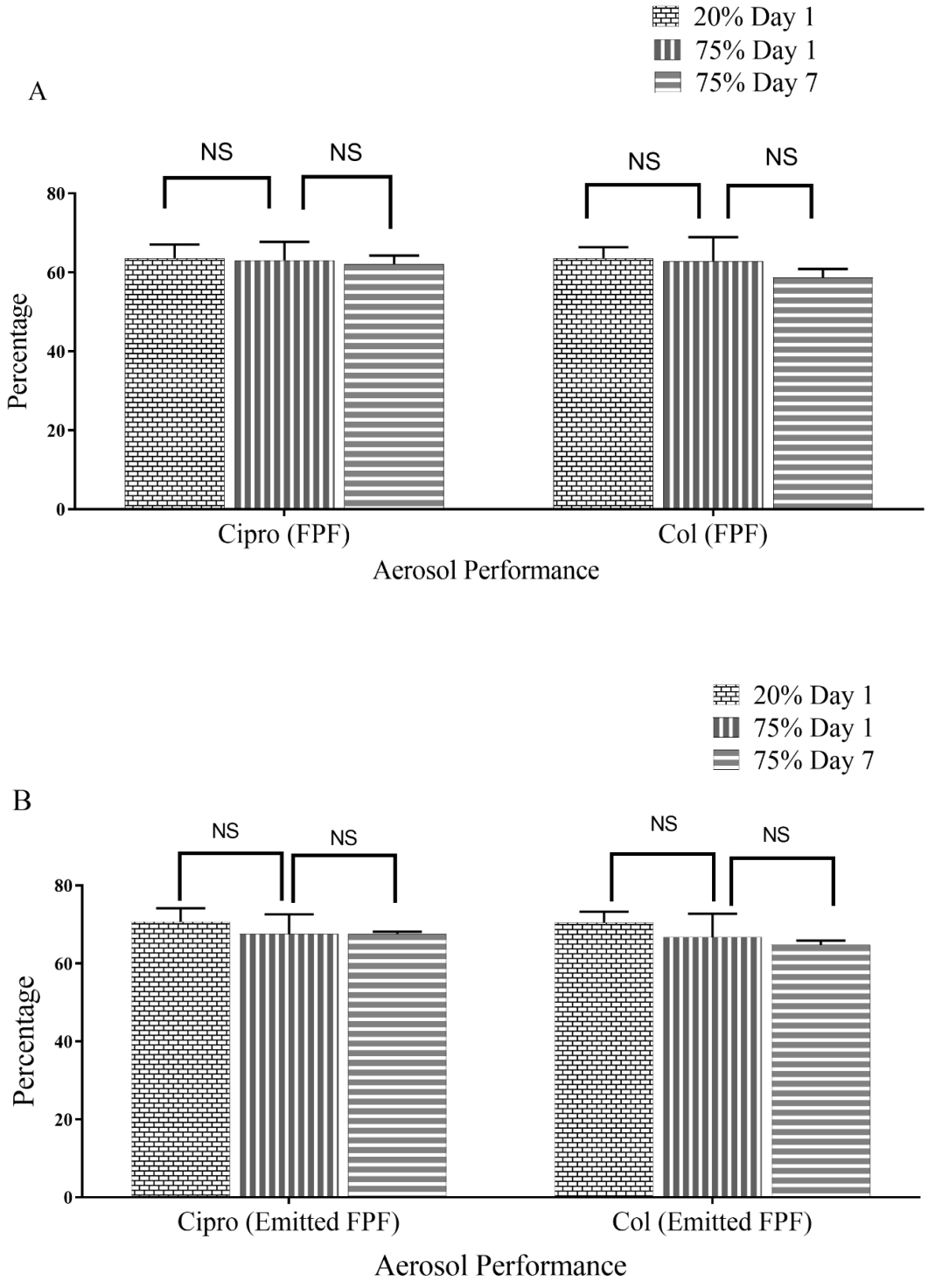


Figure 5-16: (A) Fine particle fraction as a percentage of recovered dose (B) Fine particle fraction as a percentage of emitted dose of Ciprofloxacin and Colistin in the co-spray-dried ColCipLeu formulation with mass ratios (1:1:1) stored at 75% RH for 7 days (mean ± SD, n=4; *, p <0.05; **, p <0.01; ***, p < 0.001; ****, p<0.0001; NS, no significant difference)

5.4.3.4 XPS

The measured L-leucine surface concentration (44%) was higher than the theoretical Leucine concentration (33%) in the ColCipLeu (1:1:1) co-spray-dried powder formulation (Table 5-6). Measured surface Ciprofloxacin (22%) was significantly lower than the theoretical (33%). While measured (34%) and theoretical (33%) surface Colistin concentration was nearly identical. Both Colistin and L-leucine are known to be surface active agents; however, based on the XPS data it indicated that L-leucine was more surface active than Colistin.

Table 5-6: Theoretical and measured (by XPS) surface compositions for the co-spray-dried ColCipLeu formulation in the mass ratio (1:1:1).

Formulation	**% Surface Composition (Theoretical)**			**% Surface Composition (Measured)**		
	Colistin	**L-leucine**	**Ciprofloxacin**	**Colistin**	**L-leucine**	**Ciprofloxacin**
ColCipLeu (1:1:1)	33	33	33	34	44	22

5.5 Discussion

Particle engineering such as spray-drying is being explored to produce particles of optimal morphologies and surface properties that provide optimum aerosol performance. However, many spray-dried small molecules such as Ciprofloxacin are amorphous and tend to crystalize upon storage [75]. Our earlier study showed the amorphous spray-dried Ciprofloxacin crystallized at RH of 55% within one hour, which led to a significant increase in FPF from 35.5 ± 1.7% at Day

1 to 42.3 ± 0.9% at Day 3 ($p < 0.01$) [75]. Unlike Ciprofloxacin, amorphous form of the spray-dried Colistin formulations can be retained on storage at 60% RH and 25°C for up to 3 months with no significant change in aerosol performance indicating the physical stability [67, 303]. In this study, we aimed to examine if incorporation of synergistic Colistin can improve physical stability of amorphous Ciprofloxacin particles. Data showed the co-spray-dried formulation of Ciprofloxacin with Colistin in a mass ratio of 1:1 remained amorphous for up to 60 days when stored at 55% RH (Figure 5-1E). Additionally, FT-IR results suggested that intermolecular interactions like hydrogen bonding between Colistin and Ciprofloxacin inhibited crystallization of amorphous spray-dried Ciprofloxacin, and this inhibition effect was most significant at the weight ratio of 1:1 (Figure 5-2). From the significant changes in IR spectra of co-spray-dried Ciprofloxacin and Colistin, it can be suggested that hydrogen bond can be formed between the carboxyl C=O group of Ciprofloxacin as a hydrogen acceptor and the amide II N-H of Colistin as a hydrogen donor. Hydrogen bond formation of amide II N-H could influence the bond length and strength of its adjacent group which is the amide I C=O causing the IR band of the amide I C=O to be shifted.

This suggestion can be supported by the fact that there are earlier reports which showed that the carboxyl group of fluoroquinolone antibiotics such as ofloxacin and Ciprofloxacin can interact with other chemical containing hydrogen donors such as N-H or O-H group [312-314]. Therefore, all those changes in FT-IR spectrum give a strong evidence for the intermolecular interactions via hydrogen bond between Ciprofloxacin and Colistin. Among all the samples, the peak shift was largest for Colistin-Ciprofloxacin in the mass ratio of 1:1, suggesting that the strongest hydrogen bond could be formed at this ratio. The fact that only Colistin-Ciprofloxacin (1:1) co-spray-dried formulation remained amorphous without crystallization of Ciprofloxacin

during the storage at 55% RH for 60 days supports this assumption. There was no change in the particle morphology and no significant change in aerosol performance ($p > 0.05$), which indicated the physical and aerosolization stability (Figure 5-10 and 5-7C, respectively). Co-spray-dried ColCipro formulation in the mass ratio (1:3) and (1:9) crystallized after 3 days of storage at 55% RH (Figure 5-1D); however, no change in particle morphology and aerosol performance was observed in these formulations at 55% RH for up to 60 days.

We propose the amorphous Colistin with larger molecular weight acts as a polymer-like matrix that minimizes the mobility and interactions between Ciprofloxacin-Ciprofloxacin and Ciprofloxacin-water molecules, therefore inhibits the crystallization tendency for amorphous Ciprofloxacin [315]. Also, Colistin improves aerosol performance of Ciprofloxacin when co-spray-dried (Figure 8). There was an increase in aerosol performance of Ciprofloxacin by increasing Colistin concentrations from 10% to 50% (w/w). The FPF of co-spray-dried Colistin-Ciprofloxacin formulation even in the mass ratio (1:9) was significantly higher ($p < 0.0001$) than that of spray dried Ciprofloxacin alone. We hypothesize that Colistin improved the aerosol performance by enriching on the particle surfaces, which was confirmed by XPS, ToF-SIMS, and EDX. Our earlier studies showed Colistin has self-assembling and surface-active properties [316] with low surface energy [298]; thus, surface enrichment of Colistin resulted in improved aerosolization and intermolecular interactions due to hydrogen bonding inhibited crystallization of Ciprofloxacin.

The co-spray-dried Ciprofloxacin-Colistin formulation (1:1) crystallized upon exposure to 75% RH for a day and subsequent increase in degree of crystallization was observed as stored at 75% RH from Day 1 to Day 7. Colistin acts as a matrix forming intermolecular bonds with Ciprofloxacin; however, at the high humidity, water molecule likely weakens the bonding

causing the drug to crystallize [317, 318]. Upon storage at 75% RH for 7 days the powders appeared to be fused with a significant decrease in FPF of Ciprofloxacin from 71.5 ± 1.7% at Day 1 down to 19.2 ± 1.4% at day 7. It has been observed that at high relative humidity conditions such as 75% RH, Colistin powders absorbed a significant amount of water, which led to substantial deterioration of aerosolization due to enhanced inter-particulate capillary forces [319]. When the hygroscopic powders are stored at such humid environment for some time, water condensed on the particle surfaces may dissolve the surface component (i.e., Colistin) and form liquid bridges between particles. Such liquid bridges likely cause strong bonding between contacted particles and lead to poor aerosolization [303].

In order to overcome such negative effects of elevated humidity, we propose to add an excipient, L-leucine, in the combination formulation. The amino acid, L-leucine, has been widely used to reduce cohesion [320] and improve aerosolization of cohesive fine particles [258-260]. Our studies showed that effects of L-leucine on aerosolization enhancement depend on whether L-leucine is enriched on the particle surfaces [275]. Li et al. reported that enrichment of crystalline L-leucine on particle surface could provide protection against moisture on dispersion of hygroscopic powders [154]. The data showed a slight crystallization of Ciprofloxacin in the co-spray-dried Colistin-Ciprofloxacin-leucine [ColCipLeu (1:1:1)] upon storage at 75% RH for 7 days; but there was no change in surface morphology of the co-spray-dried formulation. XPS data revealed that L-leucine was enriched on the surface of the co-spray-dried ColCipLeu (1:1:1) formulation, which contributed to the unchanged FPF when it was stored at 75% RH for 7 days. Our study also indicated L-leucine is more surface-active than Colistin, leading to more L-leucine on the composite particle surface.

5.6 Conclusions

We developed a combination DPI formulation of Ciprofloxacin and Colistin through co-spray-drying. Colistin in the formulation inhibited the tendency of amorphous Ciprofloxacin to crystallize when stored at 55% RH, resulting in enhanced physical stability. Such inhibitory effect could be due to polymer-like properties of Colistin that acts as a matrix material and reduces the molecular mobility of Ciprofloxacin, as suggested by FT-IR results. Moreover, addition of Colistin improved the aerosolization as compared to the spray-dried Ciprofloxacin alone formulation, which is attributed to enrichment of Colistin on the surface of the co-spray-dried formulation as measured by XPS, EDX, and ToF-SIMS. Further addition of L-leucine even prevented moisture-induced deteroation in aerosolization as stored at 75% RH for 7 days.

Our study demonstrated, for the first time, that co-spray-drying Ciprofloxacin with a synergistic antibiotic Colistin not only enhances the physical stability of amorphous powder formulation through intermolecular interactions, but also improves the aerosolization through surface enrichment of Colistin. The in-vivo synegistic efficacy of such combination formulation are under investigation in a mouse lung infection model

CHAPTER 6. THESIS SUMMARY

This thesis investigated the impact of storage humidity on physical stability and aerosol performance of high-dose dry powder inhaler formulations.

Spray dried Ciprofloxacin powder was amorphous in nature and crystallized on storage at RH > 55%, thereby altering the aerosol performance. Crystallization under humid conditions altered the surface morphology of Ciprofloxacin particles and affected its aerosol performance. Interestingly, there was an improvement in aerosol performance of the spray dried Ciprofloxacin formulation stored at 55% RH. This was due to moisture-induced crystallization that caused increase in surface roughness and consequent decrease in particle-particle interactions. On the other hand, there was a drastic decrease in aerosol performance at 75% RH which was due to particle fusion in presence of excessive moisture.

Such significant changes in aerosol performance of DPIs upon storage may cause critical quality concerns in product stability. Therefore, it is crucial to understand the mechanisms of such impact of crystallization on aerosol performance to ensure the quality and stability of the DPI products. To overcome physical instability associated with spray dried amorphous powders, we have developed several novel composite formulations, through co-spray drying Ciprofloxacin with various excipients (lactose, sucrose, trehalose, mannitol and l-leucine) or synergistic antibiotics such as Colistin.

Among all excipients studied, l-leucine was shown as the best excipient to alleviate crystallization of amorphous Ciprofloxacin. Co-spray dried Ciprofloxacin with 50% (w/w) l-

leucine resulted in no significant change in aerosol performance for powders stored at 20% and 55% RH. It is interesting to note that unlike the spray dried Ciprofloxacin alone powders, the co-spray dried Ciprofloxacine-l-leucine formulation showed no major change in particle morphology and alleviated crystallization of Ciprofloxacin when stored at 55% RH for 45 days. The favorable impact of l-leucine on stability and aerosol performance of the spray dried Ciprofloxacin formulation was evident even at a very low concentration of l-leucine (10%). Co-spray dried formulations of Ciprofloxacin with lactose, sucrose, trehalose and mannitol fused upon storage at 55% RH and thus showed a drastic decrease in aerosol performance upon storage at 55% RH for up to 10 days. Our study demonstrated that such enhanced physical stability with l-leucine is due to its surface-enrichment properties upon spray drying as confirmed by X-ray photoelectron spectroscopy (XPS) data. **This study provides insights in physical and aerosolization stability of spray dried DPI formulations for high-dose medications, which are critical for the product quality.**

To limit the use of excipient in high-dose medication, we selected another synergistic antibiotic, Colistin, to be spray dried together with Ciprofloxacin. Colistin is chosen because the amorphous form of the spray dried Colistin formulations can be retained on storage at 60% RH and 25°C for up to 3 months with no significant change in aerosol performance [149]. Addition of Colistin in the formulation inhibited the tendency of amorphous Ciprofloxacin to crystallize when stored at 55% RH, resulting in enhanced physical stability. Such inhibitory effect could be due to polymer-like properties of Colistin that acts as a matrix material and reduces the molecular mobility of Ciprofloxacin, as suggested by Solid State Fourier Transform Infrared Spectroscopy (FT-IR) results. Moreover, addition of Colistin improved the aerosolization as compared to the

spray dried Ciprofloxacin alone formulation, which is attributed to enrichment of Colistin on the surface of the co-spray dried formulation as measured by X-ray Photoelectron Spectroscopy (XPS) and Time-of-flight Secondary Ion Mass Spectrometry (ToF-SIMS). **Our study demonstrated, for the first time, that co-spray drying Ciprofloxacin with a synergistic antibiotic Colistin not only enhances the physical stability of amorphous powder formulation through intermolecular interactions, but also improves the aerosolization through surface enrichment of Colistin.**

APPENDIX

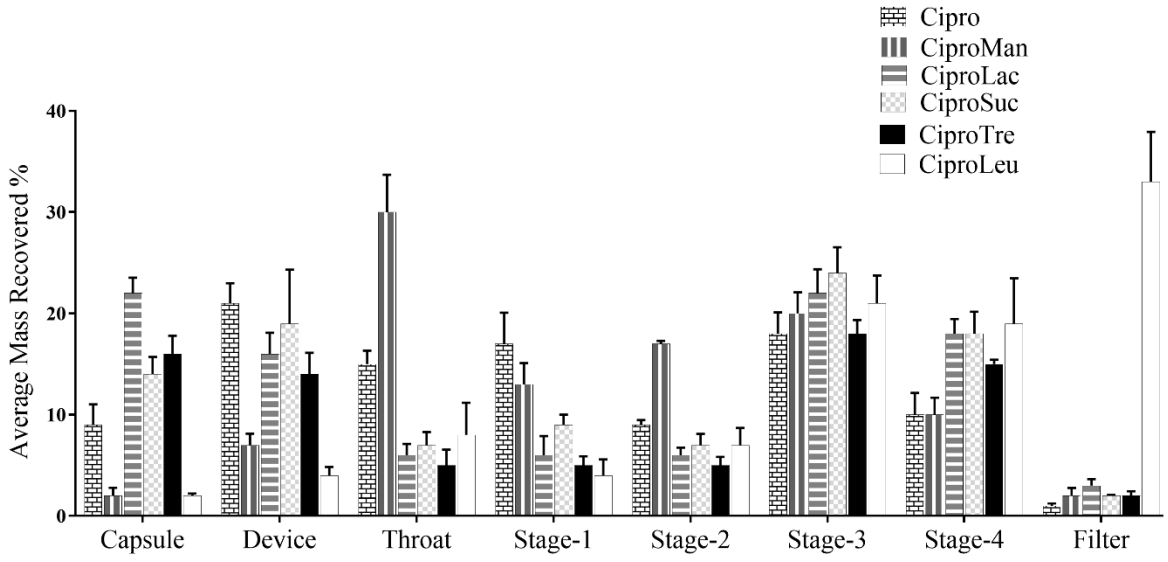


Figure A1: Deposition profiles of the co-spray-dried Ciprofloxacin-excipient stored at 20% RH for 1 day (mean ± SD, n=4)

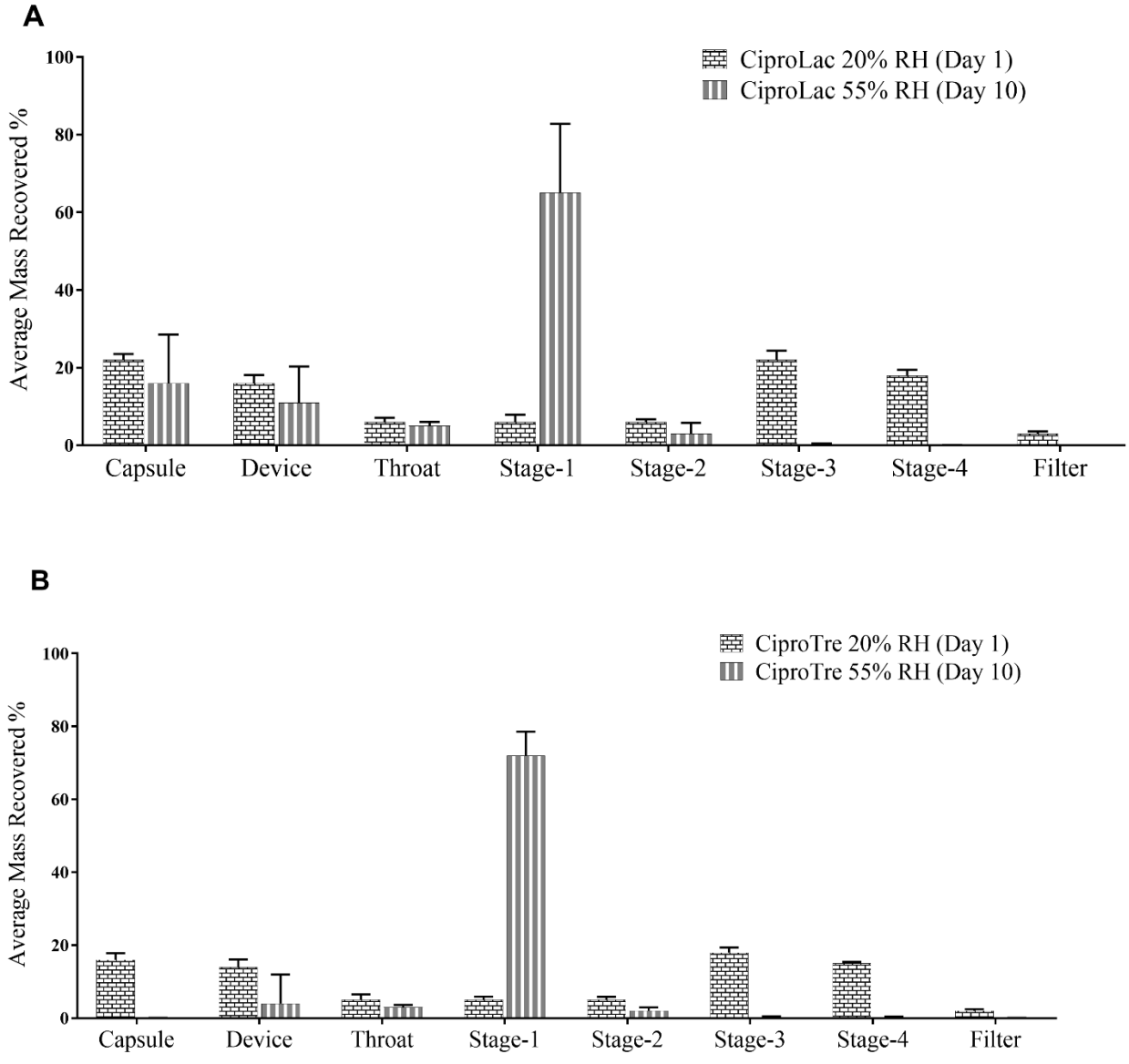
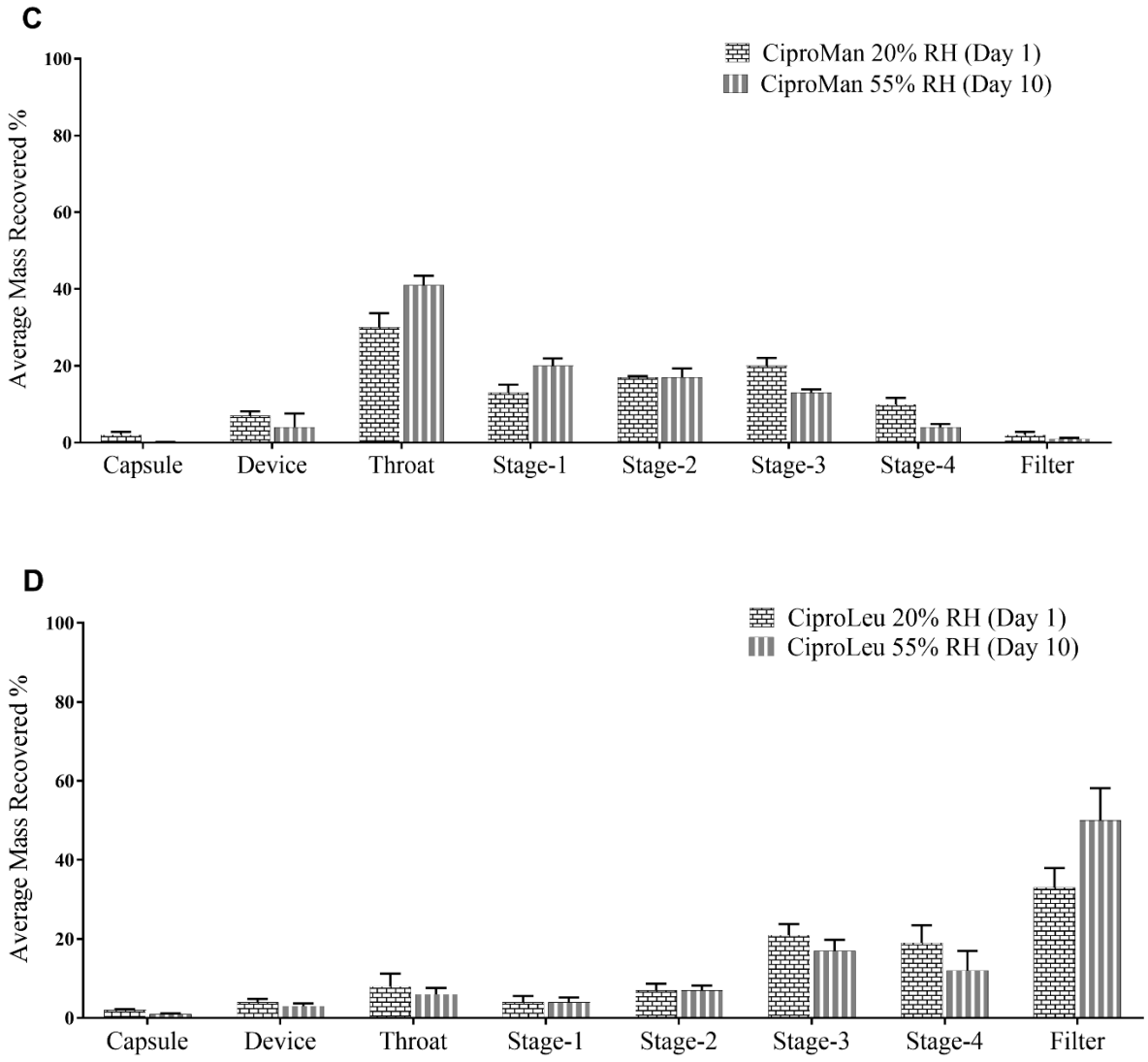


Figure A2: Deposition profiles of the co-spray-dried (A) Ciprofloxacin-lactose (B) Ciprofloxacin-trehalose (C) Ciprofloxacin-mannitol and (D) Ciprofloxacin-L-leucine formulations stored at 20% for 1 day and 55% storage humidity for 10 days (mean ± SD, n=4))

Figure A.2 continued



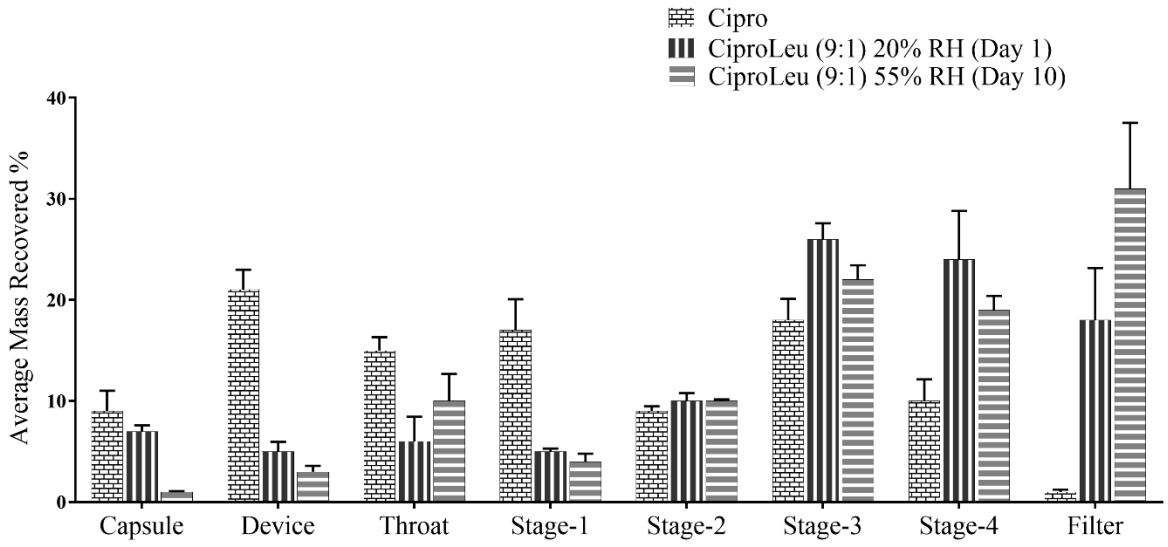


Figure A3: Deposition profiles of the co-spray-dried Ciprofloxacin-L-leucine formulations in the mass ratio (9:1) stored at 20% for 1 day and 55% storage humidity for 10 days (mean ± SD, n=4)

REFERENCES

1. *Chapter 67 - Lower Respiratory Tract Infections A2 - Zaoutis, Lisa B*, in *Comprehensive Pediatric Hospital Medicine*, V.W. Chiang, Editor. 2007, Mosby: Philadelphia. p. 382-393.

2. Abubakar, I., T. Tillmann, and A. Banerjee, *Global, regional, and national age-sex specific all-cause and cause-specific mortality for 240 causes of death, 1990-2013: a systematic analysis for the Global Burden of Disease Study 2013.* Lancet, 2015. **385**(9963): p. 117-171.

3. Troeger, C., et al., *Estimates of the global, regional, and national morbidity, mortality, and aetiologies of lower respiratory tract infections in 195 countries: a systematic analysis for the Global Burden of Disease Study 2015.* The Lancet Infectious Diseases, 2017. **17**(11): p. 1133-1161.

4. Ding, C., et al., *Prevalence of Pseudomonas aeruginosa and antimicrobial-resistant Pseudomonas aeruginosa in patients with pneumonia in mainland China: a systematic review and meta-analysis.* Int J Infect Dis, 2016. **49**: p. 119-28.

5. Zhou, Q.T., et al., *Emerging inhalation aerosol devices and strategies: where are we headed?* Advanced drug delivery reviews, 2014. **75**: p. 3-17.

6. Rau, J.L., *The inhalation of drugs: advantages and problems.* Respir Care, 2005. **50**(3): p. 367-82.

7. Lin, Y.-W., et al., *Powder production and particle engineering for dry powder inhaler formulations.* Current pharmaceutical design, 2015. **21**(27): p. 3902-3916.

8. Ibrahim, M., R. Verma, and L. Garcia-Contreras, *Inhalation drug delivery devices: technology update.* Medical devices (Auckland, NZ), 2015. **8**: p. 131.

9. Fischer, A., et al., *Novel devices for individualized controlled inhalation can optimize aerosol therapy in efficacy, patient care and power of clinical trials.* Eur J Med Res, 2009. **14 Suppl 4**: p. 71-7.

10. Tiddens, H.A., et al., *Inhaled antibiotics: dry or wet?* Eur Respir J, 2014. **44**(5): p. 1308-18.

11. Chiou, D. and T.A.G. Langrish, *Crystallization of Amorphous Components in Spray-Dried Powders.* Drying Technology, 2007. **25**(9): p. 1427-1435.

12. Wu, L., et al., *Studies on the spray dried lactose as carrier for dry powder inhalation.* Asian Journal of Pharmaceutical Sciences, 2014. **9**(6): p. 336-341.

13. Costantino, H.R., et al., *Effect of mannitol crystallization on the stability and aerosol performance of a spray-dried pharmaceutical protein, recombinant humanized anti-IgE monoclonal antibody.* J Pharm Sci, 1998. **87**(11): p. 1406-11.

14. Adi, H., et al., *Cospray dried antibiotics for dry powder lung delivery.* Journal of pharmaceutical sciences, 2008. **97**(8): p. 3356-3366.

15. Adi, H., et al., *Co-spray-dried mannitol–ciprofloxacin dry powder inhaler formulation for cystic fibrosis and chronic obstructive pulmonary disease.* European Journal of Pharmaceutical Sciences, 2010. **40**(3): p. 239-247.

16. Chalkley, L. and H. Koornhof, *Antimicrobial activity of ciprofloxacin against Pseudomonas aeruginosa, Escherichia coli, and Staphylococcus aureus determined by the killing curve method: antibiotic comparisons and synergistic interactions.* Antimicrobial agents and chemotherapy, 1985. **28**(2): p. 331-342.

17. Ball, A.P. and G.S. Tillotson, *Lower respiratory tract infection therapy--the role of ciprofloxacin.* J Int Med Res, 1995. **23**(5): p. 315-27.

18. Stass, H., et al., *Ciprofloxacin Dry Powder for Inhalation in Patients with Non-Cystic Fibrosis Bronchiectasis or Chronic Obstructive Pulmonary Disease, and in Healthy Volunteers.* J Aerosol Med Pulm Drug Deliv, 2017. **30**(1): p. 53-63.

19. Wilson, R., et al., *Ciprofloxacin dry powder for inhalation in non-cystic fibrosis bronchiectasis: a phase II randomised study.* European Respiratory Journal, 2013. **41**(5): p. 1107-1115.

20. Quittner, A., et al., *Effects Of Ciprofloxacin Dry Powder For Inhalation (Ciprofloxacin DPI) On Health-Related Quality Of Life In Patients With Non-Cystic Fibrosis Bronchiectasis (NCFB): Results From The Phase III RESPIRE 1 Study*, in *D94. ADVANCES IN CYSTIC FIBROSIS AND NON-CYSTIC FIBROSIS BRONCHIECTASIS*. 2017, Am Thoracic Soc. p. A7303-A7303.

21. Sanders, M., *Inhalation therapy: an historical review.* Primary care respiratory journal, 2007. **16**(2): p. 71.

22. de Boer, A., et al., *Dry powder inhalation: past, present and future.* Expert opinion on drug delivery, 2017. **14**(4): p. 499-512.

23. Rubin, B.K., *Pediatric aerosol therapy: new devices and new drugs*. 2011, Respiratory Care.

24. Stein, S.W. and C.G. Thiel, *The History of Therapeutic Aerosols: A Chronological Review.* Journal of aerosol medicine and pulmonary drug delivery, 2017. **30**(1): p. 20-41.

25. Nokhodchi, A. and G.P. Martin, *Pulmonary Drug Delivery: Advances and Challenges*. 2015: John Wiley & Sons.

26. Anselmo, A.C., Y. Gokarn, and S. Mitragotri, *Non-invasive delivery strategies for biologics.* Nature Reviews Drug Discovery, 2018. **18**: p. 19.

27. Zhou, Q., et al., *Inhaled formulations and pulmonary drug delivery systems for respiratory infections.* Advanced Drug Delivery Reviews, 2015. **85**: p. 83-99.

28. Ibrahim, M., R. Verma, and L. Garcia-Contreras, *Inhalation drug delivery devices: technology update.* Medical devices (Auckland, N.Z.), 2015. **8**: p. 131-139.

29. Gilbert, I., L.A. Millette, and M. Riebe, *Inhalation device options for the management of chronic obstructive pulmonary disease AU - DePietro, Michael.* Postgraduate Medicine, 2018. **130**(1): p. 83-97.

30. Geller, D.E., *Comparing clinical features of the nebulizer, metered-dose inhaler, and dry powder inhaler.* Respiratory care, 2005. **50**(10): p. 1313-1322.

31. Daniels, T., N. Mills, and P. Whitaker, *Nebuliser systems for drug delivery in cystic fibrosis.* The Cochrane Library, 2013.

32. Muchão, F.P. and L.V.R.F. Silva Filho, *Advances in inhalation therapy in pediatrics.* Jornal de pediatria, 2010. **86**(5): p. 367-376.

33. Stein, S.W., et al., *Advances in metered dose inhaler technology: hardware development.* AAPS PharmSciTech, 2014. **15**(2): p. 326-338.

34. Terzano, C., *Metered dose inhalers and spacer devices.* European review for medical and pharmacological sciences, 1999. **3**: p. 159-170.

35. Myers, T.R., *The science guiding selection of an aerosol delivery device.* Respiratory care, 2013. **58**(11): p. 1963-1973.

36. Melani, A.S., et al., *Inhaler mishandling remains common in real life and is associated with reduced disease control.* Respiratory medicine, 2011. **105**(6): p. 930-938.

37. Zuyderhoudt, K.F.M., *Inhalers and housing caps for inhalers*. 2016, Google Patents.

38. Leach, C.L. and G.L. Colice, *A pilot study to assess lung deposition of HFA-beclomethasone and CFC-beclomethasone from a pressurized metered dose inhaler with and without add-on spacers and using varying breathhold times.* Journal of aerosol medicine and pulmonary drug delivery, 2010. **23**(6): p. 355-361.

39. Mansfield, L.E. and R. Maynes, *A Valved Holding Chamber Does Not Decrease the Bronchodilator Activity of Budesonide–Formoterol Combination Metered Dose Inhaler.* Journal of Asthma, 2013. **50**(1): p. 71-74.

40. Newman, S.P., *Principles of metered-dose inhaler design.* Respiratory care, 2005. **50**(9): p. 1177-1190.

41. Molina, M.J. and F.S. Rowland, *Stratospheric sink for chlorofluoromethanes: chlorine atom-catalysed destruction of ozone.* Nature, 1974. **249**(5460): p. 810-812.

42. Sanders, M., *Inhalation therapy: an historical review.* Prim Care Respir J, 2007. **16**(2): p. 71-81.

43. Jones, M.D., et al., *An investigation into the relationship between carrier-based dry powder inhalation performance and formulation cohesive–adhesive force balances.* European Journal of Pharmaceutics and Biopharmaceutics, 2008. **69**(2): p. 496-507.

44. Khan, I., et al., *Liposome-based carrier systems and devices used for pulmonary drug delivery*, in *Biomaterials and Medical Tribology*. 2012, Elsevier. p. 395-443.

45. Noakes, T., *Medical aerosol propellants.* Journal of fluorine chemistry, 2002. **118**(1-2): p. 35-45.

46. de Boer, A.H., et al., *Dry powder inhalation: past, present and future.* Expert Opinion on Drug Delivery, 2016: p. 1-14.

47. Smith, I.J. and M. Parry-Billings, *The inhalers of the future? A review of dry powder devices on the market today.* Pulmonary Pharmacology & Therapeutics, 2003. **16**(2): p. 79-95.

48. Sumby, B., et al., *Review of dry powder inhalers.* Adv Drug Deliv Rev, 1997. **26**(1): p. 51-58.

49. Coates, M.S., et al., *Effect of design on the performance of a dry powder inhaler using computational fluid dynamics. Part 1: Grid structure and mouthpiece length.* Journal of pharmaceutical sciences, 2004. **93**(11): p. 2863-2876.

50. Voss, A. and W.H. Finlay, *Deagglomeration of dry powder pharmaceutical aerosols.* International journal of pharmaceutics, 2002. **248**(1-2): p. 39-50.

51. Harrison, L.I., et al., *Comparative pulmonary function and pharmacokinetics of fluticasone propionate and salmeterol xinafoate delivered by two dry powder inhalers to patients with asthma.* Journal of aerosol medicine and pulmonary drug delivery, 2011. **24**(5): p. 245-252.

52. Broaddus, V.C., et al., *Murray & Nadel's Textbook of Respiratory Medicine E-Book.* 2015: Elsevier Health Sciences.

53. Carvalho, S.R., et al., *Dry powder inhalation for pulmonary delivery: recent advances and continuing challenges.* Pulmonary Drug Delivery. Advances and Challenges (Advances in Pharmaceutical Technology), John Wiley & Sons, Chichester, UK, 2015: p. 35-62.

54. Lavorini, F., *The Challenge of Delivering Therapeutic Aerosols to Asthma Patients*. Vol. 2013. 2013. 102418.

55. Berkenfeld, K., A. Lamprecht, and J.T. McConville, *Devices for dry powder drug delivery to the lung.* AAPS PharmSciTech, 2015. **16**(3): p. 479-490.

56. Brambilla, G., et al., *Designing a novel dry powder inhaler: the NEXT DPI (part 1).* Proceedings of RDD, 2006: p. 553-6.

57. Sou, T., et al., *New developments in dry powder pulmonary vaccine delivery.* Trends in biotechnology, 2011. **29**(4): p. 191-198.

58. Telko, M.J. and A.J. Hickey, *Dry powder inhaler formulation.* Respir Care, 2005. **50**(9): p. 1209-27.

59. Zhang, X., et al., *Effect of powder properties on the aerosolization performance of nanoporous mannitol particles as dry powder inhalation carriers.* Powder Technology, 2018.

60. Peng, T., et al., *Influence of physical properties of carrier on the performance of dry powder inhalers.* Acta Pharmaceutica Sinica B, 2016. **6**(4): p. 308-318.

61. French, D.L., D.A. Edwards, and R.W. Niven, *The influence of formulation on emission, deaggregation and deposition of dry powders for inhalation.* Journal of Aerosol Science, 1996. **27**(5): p. 769-783.

62. Hickey, A.J., et al., *Physical characterization of component particles included in dry powder inhalers. II. Dynamic characteristics.* Journal of pharmaceutical sciences, 2007. **96**(5): p. 1302-1319.

63. Newman, S. and W. Busse, *Evolution of dry powder inhaler design, formulation, and performance.* Respiratory medicine, 2002. **96**(5): p. 293-304.

64. Srichana, T., G. Martin, and C. Marriott, *On the relationship between drug and carrier deposition from dry powder inhalers in vitro.* International Journal of Pharmaceutics, 1998. **167**(1-2): p. 13-23.

65. Edwards, D.A., et al., *Large porous particles for pulmonary drug delivery.* Science, 1997. **276**(5320): p. 1868-1872.

66. Edwards, D.A., A. Ben-Jebria, and R. Langer, *Recent advances in pulmonary drug delivery using large, porous inhaled particles.* Journal of applied physiology, 1998. **85**(2): p. 379-385.

67. Zhou, Q.T., et al., *Synergistic antibiotic combination powders of colistin and rifampicin provide high aerosolization efficiency and moisture protection.* The AAPS journal, 2014. **16**(1): p. 37-47.

68. Zhou, Q.T., et al., *Effect of surface coating with magnesium stearate via mechanical dry powder coating approach on the aerosol performance of micronized drug powders from dry powder inhalers.* AAPS PharmSciTech, 2013. **14**(1): p. 38-44.

69. Chow, A.H., et al., *Particle engineering for pulmonary drug delivery.* Pharmaceutical research, 2007. **24**(3): p. 411-437.

70. Hickey, A.J., *Pharmaceutical inhalation aerosol technology*. 2003: CRC Press.

71. Chan, J.G., et al., *A novel inhalable form of rifapentine.* Journal of pharmaceutical sciences, 2014. **103**(5): p. 1411-1421.

72. Gonda, I. and A. Abd El Khalik, *On the calculation of aerodynamic diameters of fibers.* Aerosol science and technology, 1985. **4**(2): p. 233-238.

73. Chan, H.-K. and I. Gonda, *Aerodynamic properties of elongated particles of cromoglycic acid.* Journal of aerosol science, 1989. **20**(2): p. 157-168.

74. Son, Y.-J. and J.T. McConville, *A new respirable form of rifampicin.* European Journal of Pharmaceutics and Biopharmaceutics, 2011. **78**(3): p. 366-376.

75. Shetty, N., et al., *Effects of Moisture-Induced Crystallization on the Aerosol Performance of Spray Dried Amorphous Ciprofloxacin Powder Formulations.* Pharm Res, 2018. **35**(1): p. 7.

76. Lechuga-Ballesteros, D., et al., *Trileucine improves aerosol performance and stability of spray-dried powders for inhalation.* Journal of pharmaceutical sciences, 2008. **97**(1): p. 287-302.

77. Shoyele, S.A. and S. Cawthorne, *Particle engineering techniques for inhaled biopharmaceuticals.* Advanced drug delivery reviews, 2006. **58**(9-10): p. 1009-1029.

78. Pilcer, G. and K. Amighi, *Formulation strategy and use of excipients in pulmonary drug delivery.* International Journal of Pharmaceutics, 2010. **392**(1–2): p. 1-19.

79. Nakach, M., et al., *Comparison of various milling technologies for grinding pharmaceutical powders.* International Journal of Mineral Processing, 2004. **74**: p. S173-S181.

80. Malcolmson, R.J. and J.K. Embleton, *Dry powder formulations for pulmonary delivery.* Pharmaceutical Science & Technology Today, 1998. **1**(9): p. 394-398.

81. Ticehurst, M.D., et al., *Characterisation of the influence of micronisation on the crystallinity and physical stability of revatropate hydrobromide.* International journal of pharmaceutics, 2000. **193**(2): p. 247-259.

82. Taylor, K., K. Pancholi, and D. Wong, *In-vitro Evaluation of Dry Powder Inhaler Formulations of Micronized and Milled Nedocromil Sodium.* Pharmacy and pharmacology communications, 1999. **5**(4): p. 255-257.

83. Vehring, R., *Pharmaceutical particle engineering via spray drying.* Pharmaceutical research, 2008. **25**(5): p. 999-1022.

84. Steckel, H., J. Thies, and B. Müller, *Micronizing of steroids for pulmonary delivery by supercritical carbon dioxide.* International Journal of Pharmaceutics, 1997. **152**(1): p. 99-110.

85. Johanson, K., *Selecting the Proper Mill for Your Product.* Chemical Engineering, 2013. **120**(11): p. 47.

86. Ameri, M. and Y.-F. Maa, *Spray drying of biopharmaceuticals: stability and process considerations.* Drying Technology, 2006. **24**(6): p. 763-768.

87. Haque, M.K. and Y. Roos, *Water plasticization and crystallization of lactose in spray-dried lactose/protein mixtures.* Journal of food science, 2004. **69**(1).

88. Sosnik, A. and K.P. Seremeta, *Advantages and challenges of the spray-drying technology for the production of pure drug particles and drug-loaded polymeric carriers.* Advances in Colloid and Interface Science, 2015. **223**: p. 40-54.

89. Traini, D., *Inhalation drug delivery.* Inhalation Drug Delivery: Techniques and Products, 2013: p. 1-14.

90. Moulton, B. and M.J. Zaworotko, *From molecules to crystal engineering: supramolecular isomerism and polymorphism in network solids.* Chemical Reviews, 2001. **101**(6): p. 1629-1658.

91. Vippagunta, S.R., H.G. Brittain, and D.J. Grant, *Crystalline solids.* Advanced drug delivery reviews, 2001. **48**(1): p. 3-26.

92. Storey, R.A. and I. Ymen, *Solid state characterization of pharmaceuticals*. 2011: John Wiley & Sons.

93. Yu, L., *Amorphous pharmaceutical solids: preparation, characterization and stabilization.* Advanced drug delivery reviews, 2001. **48**(1): p. 27-42.

94. Brodka-Pfeiffer, K., et al., *Conditioning following powder micronization: influence on particle growth of salbutamol sulfate.* Drug development and industrial pharmacy, 2003. **29**(10): p. 1077-1084.

95. Cheow, W.S., et al., *Spray-freeze-drying production of thermally sensitive polymeric nanoparticle aggregates for inhaled drug delivery: effect of freeze-drying adjuvants.* International journal of pharmaceutics, 2011. **404**(1-2): p. 289-300.

96. Niwa, T., D. Mizutani, and K. Danjo, *Spray freeze-dried porous microparticles of a poorly water-soluble drug for respiratory delivery.* Chemical and Pharmaceutical Bulletin, 2012. **60**(7): p. 870-876.

97. Yu, Z., et al., *Spray freezing into liquid nitrogen for highly stable protein nanostructured microparticles.* European Journal of Pharmaceutics and Biopharmaceutics, 2004. **58**(3): p. 529-537.

98. Audouy, S.A., et al., *Development of a dried influenza whole inactivated virus vaccine for pulmonary immunization.* Vaccine, 2011. **29**(26): p. 4345-4352.

99. Maa, Y.-F., et al., *Protein inhalation powders: spray drying vs spray freeze drying.* Pharmaceutical research, 1999. **16**(2): p. 249-254.

100. Qian, L. and H. Zhang, *Controlled freezing and freeze drying: a versatile route for porous and micro-/nano-structured materials.* Journal of chemical technology and biotechnology, 2011. **86**(2): p. 172-184.

101. Kang, S.-M., J.-M. Song, and Y.-C. Kim, *Microneedle and mucosal delivery of influenza vaccines.* Expert review of vaccines, 2012. **11**(5): p. 547-560.

102. Tomar, J., et al., *Dry influenza vaccines: towards a stable, effective and convenient alternative to conventional parenteral influenza vaccination.* Expert review of vaccines, 2016. **15**(11): p. 1431-1447.

103. Tsakaloudi, V., G. Kogias, and V. Zaspalis, *Freeze Granulation: A novel technique for low-loss Mn-Zn ferrites.* Small. **3**: p. 2.4108.

104. Pasquali, I., R. Bettini, and F. Giordano, *Solid-state chemistry and particle engineering with supercritical fluids in pharmaceutics.* European journal of pharmaceutical sciences, 2006. **27**(4): p. 299-310.

105. Knez, Z. and E. Weidner, *Particles formation and particle design using supercritical fluids.* Current opinion in solid state and materials science, 2003. **7**(4-5): p. 353-361.

106. Jung, J. and M. Perrut, *Particle design using supercritical fluids: literature and patent survey.* The Journal of Supercritical Fluids, 2001. **20**(3): p. 179-219.

107. Shekunov, B.Y., et al., *Aerosolisation behaviour of micronised and supercritically-processed powders.* Journal of Aerosol Science, 2003. **34**(5): p. 553-568.

108. Rehman, M., et al., *Optimisation of powders for pulmonary delivery using supercritical fluid technology.* European journal of pharmaceutical sciences, 2004. **22**(1): p. 1-17.

109. Kim, Y.H. and K.S. Shing, *Supercritical fluid-micronized ipratropium bromide for pulmonary drug delivery.* Powder Technology, 2008. **182**(1): p. 25-32.

110. Steckel, H., L. Pichert, and B.W. Müller, *Influence of process parameters in the ASES process on particle properties of budesonide for pulmonary delivery.* European journal of pharmaceutics and biopharmaceutics, 2004. **57**(3): p. 507-512.

111. Naik, S. and B. Chaudhuri, *Quantifying Dry Milling in Pharmaceutical Processing: A Review on Experimental and Modeling Approaches.* J Pharm Sci, 2015. **104**(8): p. 2401-13.

112. Lin, Y.W., et al., *Powder production and particle engineering for dry powder inhaler formulations.* Curr Pharm Des, 2015. **21**(27): p. 3902-16.

113. Lau, M., P.M. Young, and D. Traini, *A review of co-milling techniques for the production of high dose dry powder inhaler formulation.* Drug Dev Ind Pharm, 2017. **43**(8): p. 1229-1238.

114. Hoppentocht, M., et al., *Technological and practical challenges of dry powder inhalers and formulations.* Advanced Drug Delivery Reviews, 2014. **75**: p. 18-31.

115. Coelho, M.C. and N. Harnby, *Moisture bonding in powders.* Powder Technology, 1978. **20**(2): p. 201-205.

116. Feeley, J.C., et al., *Determination of surface properties and flow characteristics of salbutamol sulphate, before and after micronisation.* International Journal of Pharmaceutics, 1998. **172**(1): p. 89-96.

117. Young, P.M., et al., *The influence of mechanical processing of dry powder inhaler carriers on drug aerosolization performance.* J Pharm Sci, 2007. **96**(5): p. 1331-41.

118. Ticehurst, M.D., et al., *Characterisation of the influence of micronisation on the crystallinity and physical stability of revatropate hydrobromide.* Int J Pharm, 2000. **193**(2): p. 247-59.

119. Boshhiha, A. and N. Urbanetz, *Influence of micronization on the physical properties of salbutamol sulfate as a model drug used in dry powder inhalation.* Libyan International Medical University Journal, 2018. **3**(1): p. 8-15.

120. Padmadisastra, Y., R.A. Kennedy, and P.J. Stewart, *Influence of carrier moisture adsorption capacity on the degree of adhesion of interactive mixtures.* International journal of pharmaceutics, 1994. **104**(1): p. R1-R4.

121. Young, P.M. and R. Price, *The influence of humidity on the aerosolisation of micronised and SEDS produced salbutamol sulphate.* Eur J Pharm Sci, 2004. **22**(4): p. 235-40.

122. Young, P.M., et al., *Effect of humidity on aerosolization of micronized drugs.* Drug Dev Ind Pharm, 2003. **29**(9): p. 959-66.

123. Brodka-Pfeiffer, K., et al., *Conditioning following powder micronization: influence on particle growth of salbutamol sulfate.* Drug Dev Ind Pharm, 2003. **29**(10): p. 1077-84.

124. Depasquale, R., et al., *The influence of secondary processing on the structural relaxation dynamics of fluticasone propionate.* AAPS PharmSciTech, 2014. **16**(3): p. 589-600.

125. Qu, L., D.A. Morton, and Q.T. Zhou, *Particle engineering via mechanical dry coating in the design of pharmaceutical solid dosage forms.* Current pharmaceutical design, 2015. **21**(40): p. 5802-5814.

126. Zhou, Q.T. and D.A. Morton, *Drug–lactose binding aspects in adhesive mixtures: controlling performance in dry powder inhaler formulations by altering lactose carrier surfaces.* Advanced drug delivery reviews, 2012. **64**(3): p. 275-284.

127. Zhou, Q.T., et al., *Improving aerosolization of drug powders by reducing powder intrinsic cohesion via a mechanical dry coating approach.* International journal of pharmaceutics, 2010. **394**(1-2): p. 50-59.

128. Begat, P., et al., *The Influence of Force Control Agents on the Cohesive-Adhesive Balance in Dry Powder Inhaler Formulations.* KONA Powder and Particle Journal, 2005. **23**: p. 109-121.

129. Begat, P., et al., *The role of force control agents in high-dose dry powder inhaler formulations.* J Pharm Sci, 2009. **98**(8): p. 2770-83.

130. Zhou, Q.T., et al., *Effect of surface coating with magnesium stearate via mechanical dry powder coating approach on the aerosol performance of micronized drug powders from dry powder inhalers.* AAPS PharmSciTech, 2012. **14**(1): p. 38-44.

131. Zhou, Q.T., et al., *Understanding the influence of powder flowability, fluidization and de-agglomeration characteristics on the aerosolization of pharmaceutical model powders.* European Journal of Pharmaceutical Sciences, 2010. **40**(5): p. 412-421.

132. Kumon, M., et al., *Novel approach to DPI carrier lactose with mechanofusion process with additives and evaluation by IGC.* Chem Pharm Bull (Tokyo), 2006. **54**(11): p. 1508-14.

133. Lau, M., P.M. Young, and D. Traini, *Co-milled API-lactose systems for inhalation therapy: impact of magnesium stearate on physico-chemical stability and aerosolization performance.* Drug Dev Ind Pharm, 2017. **43**(6): p. 980-988.

134. Balani, P.N., et al., *Influence of excipients in comilling on mitigating milling-induced amorphization or structural disorder of crystalline pharmaceutical actives.* J Pharm Sci, 2010. **99**(5): p. 2462-74.

135. Balani, P.N., et al., *Influence of polymer content on stabilizing milled amorphous salbutamol sulphate.* Int J Pharm, 2010. **391**(1-2): p. 125-36.

136. Curtin, V., et al., *Reducing mechanical activation-induced amorphisation of salbutamol sulphate by co-processing with selected carboxylic acids.* Int J Pharm, 2013. **456**(2): p. 508-16.

137. El-Gendy, N., et al., *Development of budesonide nanocluster dry powder aerosols: processing.* J Pharm Sci, 2012. **101**(9): p. 3425-33.

138. Chan, H.K. and N.Y. Chew, *Novel alternative methods for the delivery of drugs for the treatment of asthma.* Adv Drug Deliv Rev, 2003. **55**(7): p. 793-805.

139. Saleem, I., K. Petkar, and S. Somavarapu, *Chapter Nineteen - Rationale for Pulmonary Vaccine Delivery: Formulation and Device Considerations*, in *Micro and Nanotechnology in Vaccine Development*, M. Skwarczynski and I. Toth, Editors. 2017, William Andrew Publishing. p. 357-371.

140. Walters, R.H., et al., *Next generation drying technologies for pharmaceutical applications.* Journal of pharmaceutical sciences, 2014. **103**(9): p. 2673-2695.

141. Bhatnagar, B., et al., *Drying technologies for biopharmaceutical applications: Recent developments and future direction AU - Langford, Alex.* Drying Technology, 2018. **36**(6): p. 677-684.

142. Chiou, D. and T. A. G. Langrish, *Crystallization of Amorphous Components in Spray-Dried Powders*. Vol. 25. 2007. 1427-1435.

143. Weers, J.G., D.P. Miller, and T.E. Tarara, *Spray-Dried PulmoSphere Formulations for Inhalation Comprising Crystalline Drug Particles.* AAPS PharmSciTech, 2019. **20**(3): p. 103.

144. Bosquillon, C., et al., *Influence of formulation excipients and physical characteristics of inhalation dry powders on their aerosolization performance.* Journal of Controlled Release, 2001. **70**(3): p. 329-339.

145. Shetty, N., et al., *Influence of excipients on physical and aerosolization stability of spray dried high-dose powder formulations for inhalation.* International Journal of Pharmaceutics, 2018. **544**(1): p. 222-234.

146. Li, L., et al., *Investigation of L-leucine in reducing the moisture-induced deterioration of spray-dried salbutamol sulfate power for inhalation.* International Journal of Pharmaceutics, 2017. **530**(1): p. 30-39.

147. Shetty, N., et al., *Improved Physical Stability and Aerosolization of Inhalable Amorphous Ciprofloxacin Powder Formulations by Incorporating Synergistic Colistin.* Mol Pharm, 2018. **15**(9): p. 4004-4020.

148. Yu, J., et al., *Protective effect of sodium stearate on the moisture-induced deterioration of hygroscopic spray-dried powders.* Int J Pharm, 2018. **541**(1-2): p. 11-18.

149. Zhou, Q.T., et al., *Colistin powders with high aerosolisation efficiency for respiratory infection: preparation and in vitro evaluation.* Journal of pharmaceutical sciences, 2013. **102**(10): p. 3736-3747.

150. Yu, J., et al., *Protection of hydrophobic amino acids against moisture-induced deterioration in the aerosolization performance of highly hygroscopic spray-dried powders.* European Journal of Pharmaceutics and Biopharmaceutics, 2017. **119**: p. 224-234.

151. Visser, J., *Van der Waals and other cohesive forces affecting powder fluidization.* Powder Technology, 1989. **58**(1): p. 1-10.

152. Zhou, Q., et al., *How Much Surface Coating of Hydrophobic Azithromycin Is Sufficient to Prevent Moisture-Induced Decrease in Aerosolisation of Hygroscopic Amorphous Colistin Powder?* The AAPS Journal, 2016. **18**(5): p. 1213-1224.

153. Yu, J., et al., *Protective effect of sodium stearate on the moisture-induced deterioration of hygroscopic spray-dried powders.* International journal of pharmaceutics, 2018. **541**(1-2): p. 11-18.

154. Li, L., et al., *l-Leucine as an excipient against moisture on in vitro aerosolization performances of highly hygroscopic spray-dried powders.* European Journal of Pharmaceutics and Biopharmaceutics, 2016. **102**: p. 132-141.

155. Cui, Y., et al., *Moisture-Resistant Co-Spray-Dried Netilmicin with l-Leucine as Dry Powder Inhalation for the Treatment of Respiratory Infections.* Pharmaceutics, 2018. **10**(4): p. 252.

156. Abiad, M.G., O.H. Campanella, and M.T. Carvajal, *Effect of Spray Drying Conditions on the Physicochemical Properties and Enthalpy Relaxation of α-Lactose.* International Journal of Food Properties, 2014. **17**(6): p. 1303-1316.

157. Karimi-Jafari, M., et al., *Creating cocrystals: a review of pharmaceutical cocrystal preparation routes and applications.* Crystal Growth & Design, 2018. **18**(10): p. 6370-6387.

158. Alhalaweh, A., et al., *Theophylline cocrystals prepared by spray drying: physicochemical properties and aerosolization performance.* AAPS PharmSciTech, 2013. **14**(1): p. 265-76.

159. Karashima, M., et al., *Enhanced pulmonary absorption of poorly soluble itraconazole by micronized cocrystal dry powder formulations.* Eur J Pharm Biopharm, 2017. **115**: p. 65-72.

160. Lee, G., *Spray-drying of proteins*, in *Rational design of stable protein formulations*. 2002, Springer. p. 135-158.

161. Wang, W., *Advanced protein formulations.* Protein Science, 2015. **24**(7): p. 1031-1039.

162. Grasmeijer, N., *Improving protein stabilization by spray drying.* Ridderprint, Groningen, 2015.

163. Maa, Y.-F. and S.J. Prestrelski, *Biopharmaceutical powders particle formation and formulation considerations.* Current pharmaceutical biotechnology, 2000. **1**(3): p. 283-302.

164. Moussa, E.M., et al., *Effects of Drying Process on an IgG1 Monoclonal Antibody Using Solid-State Hydrogen Deuterium Exchange with Mass Spectrometric Analysis (ssHDX-MS).* Pharmaceutical research, 2018. **35**(1): p. 12.

165. Chang, L.L. and M.J. Pikal, *Mechanisms of protein stabilization in the solid state.* Journal of pharmaceutical sciences, 2009. **98**(9): p. 2886-2908.

166. Costantino, H.R., et al., *Effect of mannitol crystallization on the stability and aerosol performance of a spray-dried pharmaceutical protein, recombinant humanized anti-IgE monoclonal antibody.* Journal of pharmaceutical sciences, 1998. **87**(11): p. 1406-1411.

167. Hulse, W.L., et al., *Do co-spray dried excipients offer better lysozyme stabilisation than single excipients?* european journal of pharmaceutical sciences, 2008. **33**(3): p. 294-305.

168. Saluja, V., et al., *A comparison between spray drying and spray freeze drying to produce an influenza subunit vaccine powder for inhalation.* Journal of Controlled Release, 2010. **144**(2): p. 127-133.

169. Chow, M.Y., et al., *Inhaled powder formulation of naked siRNA using spray drying technology with L-leucine as dispersion enhancer.* International journal of pharmaceutics, 2017. **530**(1-2): p. 40-52.

170. Liang, W., et al., *Inhalable dry powder formulations of siRNA and pH-responsive peptides with antiviral activity against H1N1 influenza virus.* Molecular pharmaceutics, 2015. **12**(3): p. 910-921.

171. Chow, M.Y.T., et al., *Inhaled powder formulation of naked siRNA using spray drying technology with l-leucine as dispersion enhancer.* International Journal of Pharmaceutics, 2017. **530**(1): p. 40-52.

172. Sou, T., et al., *The effect of amino acid excipients on morphology and solid-state properties of multi-component spray-dried formulations for pulmonary delivery of biomacromolecules.* Eur J Pharm Biopharm, 2013. **83**(2): p. 234-43.

173. Seville, P.C., et al., *Amino acid-modified spray-dried powders with enhanced aerosolisation properties for pulmonary drug delivery.* Powder Technology, 2007. **178**(1): p. 40-50.

174. Mangal, S., et al., *Relationship between surface concentration of l-leucine and bulk powder properties in spray dried formulations.* European Journal of Pharmaceutics and Biopharmaceutics, 2015. **94**: p. 160-169.

175. Wang, W., *Lyophilization and development of solid protein pharmaceuticals.* International journal of pharmaceutics, 2000. **203**(1-2): p. 1-60.

176. Tang, X.C. and M.J. Pikal, *Design of freeze-drying processes for pharmaceuticals: practical advice.* Pharmaceutical research, 2004. **21**(2): p. 191-200.

177. Yu, Z., et al., *Preparation and characterization of microparticles containing peptide produced by a novel process: spray freezing into liquid.* European journal of pharmaceutics and biopharmaceutics, 2002. **54**(2): p. 221-228.

178. Johnson, K.A., *Preparation of peptide and protein powders for inhalation.* Advanced drug delivery reviews, 1997. **26**(1): p. 3-15.

179. Ferrati, S., et al., *Influence of Formulation Factors on the Aerosol Performance and Stability of Lysozyme Powders: a Systematic Approach.* AAPS PharmSciTech, 2018: p. 1-12.

180. Patel, S.M., T. Doen, and M.J. Pikal, *Determination of End Point of Primary Drying in Freeze-Drying Process Control.* AAPS PharmSciTech, 2010. **11**(1): p. 73-84.

181. Rey, L. and J.C. May, *Freeze-Drying/Lyophilization Of Pharmaceutical & Biological Products, Revised and Expanded*. 2004: CRC Press.

182. Sonner, C., Y.F. Maa, and G. Lee, *Spray-freeze-drying for protein powder preparation: Particle characterization and a case study with trypsinogen stability.* Journal of Pharmaceutical Sciences, 2002. **91**(10): p. 2122-2139.

183. Liang, W., et al., *Spray freeze drying of small nucleic acids as inhaled powder for pulmonary delivery.* Asian Journal of Pharmaceutical Sciences, 2018. **13**(2): p. 163-172.

184. Chen, L., et al., *Amorphous powders for inhalation drug delivery.* Adv Drug Deliv Rev, 2016. **100**: p. 102-15.

185. Webb, S.D., et al., *Surface adsorption of recombinant human interferon-gamma in lyophilized and spray-lyophilized formulations.* J Pharm Sci, 2002. **91**(6): p. 1474-87.

186. Costantino, H.R., et al., *Protein spray-freeze drying. Effect of atomization conditions on particle size and stability.* Pharmaceutical research, 2000. **17**(11): p. 1374-1382.

187. Carpenter, J.F., et al., *Rational design of stable lyophilized protein formulations: some practical advice.* Pharmaceutical research, 1997. **14**(8): p. 969-975.

188. Gradon, L. and T.R. Sosnowski, *Formation of particles for dry powder inhalers.* Advanced Powder Technology, 2014. **25**(1): p. 43-55.

189. Webb, S.D., et al., *Surface adsorption of recombinant human interferon-γ in lyophilized and spray-lyophilized formulations.* Journal of pharmaceutical sciences, 2002. **91**(6): p. 1474-1487.

190. Murugappan, S., et al., *Physical and immunogenic stability of spray freeze-dried influenza vaccine powder for pulmonary delivery: comparison of inulin, dextran, or a mixture of dextran and trehalose as protectants.* European Journal of Pharmaceutics and Biopharmaceutics, 2013. **85**(3): p. 716-725.

191. Mauer, L., D. Smith, and T. Labuza, *Effect of water content, temperature and storage on the glass transition, moisture sorption characteristics and stickiness of β-casein.* International Journal of Food Properties, 2000. **3**(2): p. 233-248.

192. Otake, H., T. Okuda, and H. Okamoto, *Development of Spray-Freeze-Dried Powders for Inhalation with High Inhalation Performance and Antihygroscopic Property.* Chem Pharm Bull (Tokyo), 2016. **64**(3): p. 239-45.

193. Leung, S.S.Y., et al., *Production of Inhalation Phage Powders Using Spray Freeze Drying and Spray Drying Techniques for Treatment of Respiratory Infections.* Pharmaceutical research, 2016. **33**(6): p. 1486-1496.

194. Liang, W., et al., *Using two-fluid nozzle for spray freeze drying to produce porous powder formulation of naked siRNA for inhalation.* International Journal of Pharmaceutics, 2018. **552**(1): p. 67-75.

195. Kaialy, W. and A. Nokhodchi, *Dry powder inhalers: Physicochemical and aerosolization properties of several size-fractions of a promising alterative carrier, freeze-dried mannitol.* European Journal of Pharmaceutical Sciences, 2015. **68**: p. 56-67.

196. Emami, F., et al., *Drying Technologies for the Stability and Bioavailability of Biopharmaceuticals.* Pharmaceutics, 2018. **10**(3): p. 131.

197. Subramaniam, B., R.A. Rajewski, and K. Snavely, *Pharmaceutical processing with supercritical carbon dioxide.* Journal of pharmaceutical sciences, 1997. **86**(8): p. 885-890.

198. Sievers, R., et al., *Supercritical CO2-assisted methods for the production and pulmonary administration of pharmaceutical aerosols.* Journal of Aerosol Science, 1998(29): p. S1271-S1272.

199. Fages, J., et al., *Particle generation for pharmaceutical applications using supercritical fluid technology.* Powder Technology, 2004. **141**(3): p. 219-226.

200. Debenedetti, P.G., et al., *Rapid expansion of supercritical solutions (RESS): fundamentals and applications.* Fluid Phase Equilibria, 1993. **82**: p. 311-321.

201. Matson, D.W., et al., *Rapid expansion of supercritical fluid solutions: solute formation of powders, thin films, and fibers.* Industrial & engineering chemistry research, 1987. **26**(11): p. 2298-2306.

202. Sellers, S.P., et al., *Dry powders of stable protein formulations from aqueous solutions prepared using supercritical CO2-assisted aerosolization.* Journal of Pharmaceutical Sciences, 2001. **90**(6): p. 785-797.

203. Yeo, S.D., et al., *Formation of microparticulate protein powder using a supercritical fluid antisolvent.* Biotechnology and Bioengineering, 1993. **41**(3): p. 341-346.

204. Sievers, R., *Formation of aqueous small droplet aerosols assisted by supercritical carbon dioxide.* Aerosol Science & Technology, 1999. **30**(1): p. 3-15.

205. Rehman, M., et al., *Optimisation of powders for pulmonary delivery using supercritical fluid technology.* Eur J Pharm Sci, 2004. **22**(1): p. 1-17.

206. Jovanović, N., et al., *Stable sugar-based protein formulations by supercritical fluid drying.* International journal of pharmaceutics, 2008. **346**(1-2): p. 102-108.

207. Jovanović, N., et al., *Stabilization of Proteins in Dry Powder Formulations Using Supercritical Fluid Technology.* Pharmaceutical Research, 2004. **21**(11): p. 1955-1969.

208. Weers, J. and T. Tarara, *The PulmoSphere™ platform for pulmonary drug delivery.* Therapeutic delivery, 2014. **5**(3): p. 277-295.

209. Miller, D.P., et al., *Physical Characterization of Tobramycin Inhalation Powder: II. State Diagram of an Amorphous Engineered Particle Formulation.* Mol Pharm, 2017. **14**(6): p. 1950-1960.

210. Miller, D.P., et al., *Physical Characterization of Tobramycin Inhalation Powder: I. Rational Design of a Stable Engineered-Particle Formulation for Delivery to the Lungs.* Mol Pharm, 2015. **12**(8): p. 2582-93.

211. McShane, P.J., et al., *Ciprofloxacin Dry Powder for Inhalation (ciprofloxacin DPI): Technical design and features of an efficient drug–device combination.* Pulmonary pharmacology & therapeutics, 2018.

212. Xu, L.-M., et al., *Engineering drug ultrafine particles of beclomethasone dipropionate for dry powder inhalation.* International Journal of Pharmaceutics, 2012. **436**(1): p. 1-9.

213. Saboti, D., et al., *Novel Budesonide Particles for Dry Powder Inhalation Prepared Using a Microfluidic Reactor Coupled With Ultrasonic Spray Freeze Drying.* J Pharm Sci, 2017. **106**(7): p. 1881-1888.

214. Thiele, J., et al., *Early development drug formulation on a chip: fabrication of nanoparticles using a microfluidic spray dryer.* Lab Chip, 2011. **11**(14): p. 2362-8.

215. Mansur, E.A., et al., *A State-of-the-Art Review of Mixing in Microfluidic Mixers.* Chinese Journal of Chemical Engineering, 2008. **16**(4): p. 503-516.

216. Wilson, E.M., J.C. Luft, and J.M. DeSimone, *Formulation of High-Performance Dry Powder Aerosols for Pulmonary Protein Delivery.* Pharm Res, 2018. **35**(10): p. 195.

217. Xu, J., et al., *Rendering protein-based particles transiently insoluble for therapeutic applications.* J Am Chem Soc, 2012. **134**(21): p. 8774-7.

218. Zhou, Q.T., et al., *Inhaled formulations and pulmonary drug delivery systems for respiratory infections.* Adv Drug Deliv Rev, 2015. **85**: p. 83-99.

219. Williams, R.O., D.R. Taft, and J.T. McConville, *Advanced drug formulation design to optimize therapeutic outcomes*. 2007: CRC Press.

220. Zhou, Q., et al., *Emerging inhalation aerosol devices and strategies: Where are we headed?* Advanced Drug Delivery Reviews, 2014. **75**: p. 3-17.

221. Hassan, M.S. and R.W. Lau, *Effect of particle shape on dry particle inhalation: study of flowability, aerosolization, and deposition properties.* AAPS PharmSciTech, 2009. **10**(4): p. 1252-62.

222. Chavan, V. and R. Dalby, *Effect of rise in simulated inspiratory flow rate and carrier particle size on powder emptying from dry powder inhalers.* AAPS PharmSci, 2000. **2**(2): p. E10.

223. Chew, N.Y., D.F. Bagster, and H.K. Chan, *Effect of particle size, air flow and inhaler device on the aerosolisation of disodium cromoglycate powders.* Int J Pharm, 2000. **206**(1-2): p. 75-83.

224. Zhou, Q.T., et al., *Colistin powders with high aerosolisation efficiency for respiratory infection: preparation and in vitro evaluation.* J Pharm Sci, 2013. **102**(10): p. 3736-47.

225. Zhou, Q. and D.A.V. Morton, *Drug–lactose binding aspects in adhesive mixtures: Controlling performance in dry powder inhaler formulations by altering lactose carrier surfaces.* Advanced Drug Delivery Reviews, 2012. **64**(3): p. 275-284.

226. Chew, N.Y. and H.-K. Chan, *Use of solid corrugated particles to enhance powder aerosol performance.* Pharmaceutical Research, 2001. **18**(11): p. 1570-1577.

227. Bohr, A., C.A. Ruge, and M. Beck-Broichsitter, *Preparation of nanoscale pulmonary drug delivery formulations by spray drying.* Adv Exp Med Biol, 2014. **811**: p. 183-206.

228. Chow, A.H., et al., *Particle engineering for pulmonary drug delivery.* Pharm Res, 2007. **24**(3): p. 411-37.

229. Vehring, R., W.R. Foss, and D. Lechuga-Ballesteros, *Particle formation in spray drying.* Journal of Aerosol Science, 2007. **38**(7): p. 728-746.

230. Anandharamakrishnan, C.a.I., S. P., *Introduction to spray drying, in Spray Drying Techniques for Food Ingredient Encapsulation*. 2015, Chichester, UK: John Wiley & Sons, Ltd.

231. Niazi, M.B.K. and A.A. Broekhuis, *Production of amorphous starch powders by solution spray drying.* Journal of Applied Polymer Science, 2012. **126**(S1): p. E143-E153.

232. Antoniu, S. and D. Azoicai, *Ciprofloxacin DPI in non-cystic fibrosis bronchiectasis: a Phase II randomized study.* Expert Opin Investig Drugs, 2013. **22**(5): p. 671-3.

233. Nie, H., et al., *Impact of metallic stearates on disproportionation of hydrochloride salts of weak bases in solid-state formulations.* Molecular pharmaceutics, 2016. **13**(10): p. 3541-3552.

234. Nečas, D. and P. Klapetek, *Gwyddion: an open-source software for SPM data analysis.* Open Physics, 2012. **10**(1): p. 181-188.

235. Zhou, Q.T., et al., *How Much Surface Coating of Hydrophobic Azithromycin Is Sufficient to Prevent Moisture-Induced Decrease in Aerosolisation of Hygroscopic Amorphous Colistin Powder?* AAPS J, 2016. **18**(5): p. 1213-24.

236. Price, R. and P.M. Young, *Visualization of the crystallization of lactose from the amorphous state.* Journal of Pharmaceutical Sciences, 2004. **93**(1): p. 155-164.

237. Glover, W., et al., *Effect of particle size of dry powder mannitol on the lung deposition in healthy volunteers.* International Journal of Pharmaceutics, 2008. **349**(1–2): p. 314-322.

238. Silva-Júnior, A.A., et al., *Thermal analysis of biodegradable microparticles containing ciprofloxacin hydrochloride obtained by spray drying technique.* Thermochimica Acta, 2008. **467**(1–2): p. 91-98.

239. Pilcer, G., N. Wauthoz, and K. Amighi, *Lactose characteristics and the generation of the aerosol.* Advanced Drug Delivery Reviews, 2012. **64**(3): p. 233-256.

240. Chew, N.Y., et al., *How much particle surface corrugation is sufficient to improve aerosol performance of powders?* Pharm Res, 2005. **22**(1): p. 148-52.

241. Hoppentocht, M., et al., *Technological and practical challenges of dry powder inhalers and formulations.* Advanced Drug Delivery Reviews, 2014. **75**(Supplement C): p. 18-31.

242. Velkov, T., et al., *Inhaled anti-infective chemotherapy for respiratory tract infections: Successes, challenges and the road ahead.* Advanced drug delivery reviews, 2015. **85**: p. 65–82.

243. Muttil, P., C. Wang, and A.J. Hickey, *Inhaled drug delivery for tuberculosis therapy.* Pharmaceutical research, 2009. **26**(11): p. 2401-2416.

244. Chen, L., et al., *Amorphous powders for inhalation drug delivery.* Advanced drug delivery reviews, 2016. **100**: p. 102-115.

245. Chan, H.K., et al., *Physical stability of salmon calcitonin spray-dried powders for inhalation.* Journal of pharmaceutical sciences, 2004. **93**(3): p. 792-804.

246. Young, P.M., et al., *Influence of Humidity on the Electrostatic Charge and Aerosol Performance of Dry Powder Inhaler Carrier based Systems.* Pharmaceutical Research, 2007. **24**(5): p. 963-970.

247. Zhou, Q.T., et al., *Colistin powders with high aerosolisation efficiency for respiratory infection: preparation and in vitro evaluation.* Journal of pharmaceutical sciences, 2013. **102**(10): p. 3736-3747.

248. de Boer, A.H., H.K. Chan, and R. Price, *A critical view on lactose-based drug formulation and device studies for dry powder inhalation: Which are relevant and what interactions to expect?* Advanced Drug Delivery Reviews, 2012. **64**(3): p. 257-274.

249. Kou, X., et al., *Physico-chemical aspects of lactose for inhalation.* Advanced Drug Delivery Reviews, 2012. **64**(3): p. 220-232.

250. Marriott, C. and H.W. Frijlink, *Lactose as a carrier for inhalation products: breathing new life into an old carrier.* Advanced Drug Delivery Reviews, 2012. **64**(3): p. 217-219.

251. Rahimpour, Y., M. Kouhsoltani, and H. Hamishehkar, *Alternative carriers in dry powder inhaler formulations.* Drug Discov Today, 2014. **19**(5): p. 618-26.

252. Adi, H., et al., *Co-spray-dried mannitol-ciprofloxacin dry powder inhaler formulation for cystic fibrosis and chronic obstructive pulmonary disease.* Eur J Pharm Sci, 2010. **40**(3): p. 239-47.

253. Yang, Y., et al., *Mannitol-guided delivery of ciprofloxacin in artificial cystic fibrosis mucus model.* Biotechnology and bioengineering, 2011. **108**(6): p. 1441-1449.

254. Cline, D. and R. Dalby, *Predicting the quality of powders for inhalation from surface energy and area.* Pharmaceutical Research, 2002. **19**(9): p. 1274-1277.

255. Mansour, H.M., Z. Xu, and A.J. Hickey, *Dry powder aerosols generated by standardized entrainment tubes from alternative sugar blends: 3. Trehalose dihydrate and D-mannitol carriers.* Journal of pharmaceutical sciences, 2010. **99**(8): p. 3430-3441.

256. Hamishehkar, H., Y. Rahimpour, and Y. Javadzadeh, *The role of carrier in dry powder inhaler*, in *Recent Advances in Novel Drug Carrier Systems*. 2012, InTech.

257. Jovanović, N., et al., *Distinct effects of sucrose and trehalose on protein stability during supercritical fluid drying and freeze-drying.* European journal of pharmaceutical sciences, 2006. **27**(4): p. 336-345.

258. Arora, S., et al., *Highly respirable dry powder inhalable formulation of voriconazole with enhanced pulmonary bioavailability.* Expert opinion on drug delivery, 2016. **13**(2): p. 183-193.

259. Rabbani, N.R. and P.C. Seville, *The influence of formulation components on the aerosolisation properties of spray-dried powders.* Journal of Controlled Release, 2005. **110**(1): p. 130-140.

260. Chew, N.Y., et al., *Effect of amino acids on the dispersion of disodium cromoglycate powders.* Journal of pharmaceutical sciences, 2005. **94**(10): p. 2289-2300.

261. Li, L., et al., *Investigation of L-leucine in reducing the moisture-induced deterioration of spray-dried salbutamol sulfate power for inhalation.* International Journal of Pharmaceutics, 2017. **530**(1-2): p. 30-39.

262. Boraey, M., et al., *Improvement of the dispersibility of spray-dried budesonide powders using leucine in an ethanol–water cosolvent system*. Vol. 236. 2013. 171–178.

263. Sou, T., et al., *The effect of amino acid excipients on morphology and solid-state properties of multi-component spray-dried formulations for pulmonary delivery of biomacromolecules.* European Journal of Pharmaceutics and Biopharmaceutics, 2013. **83**(2): p. 234-243.

264. Rasband, W.S., *Image J Bathesda, Maryland, U.S. National institute of health*. 2007.

265. Nie, H., et al., *Solid-state spectroscopic investigation of molecular interactions between Clofazimine and Hypromellose phthalate in amorphous solid dispersions.* Molecular pharmaceutics, 2016. **13**(11): p. 3964-3975.

266. Bhandari, B.R., N. Datta, and T. Howes, *Problems associated with spray drying of sugar-rich foods.* Drying technology, 1997. **15**(2): p. 671-684.

267. Yu, X., et al., *Investigating the moisture sorption behavior of amorphous sucrose using a dynamic humidity generating instrument.* Journal of food science, 2008. **73**(1).

268. Makower, B. and W. Dye, *Sugar crystallization, equilibrium moisture content and crystallization of amorphous sucrose and glucose.* Journal of Agricultural and Food Chemistry, 1956. **4**(1): p. 72-77.

269. Tzannis, S.T. and S.J. Prestrelski, *Moisture effects on protein—excipient interactions in spray-dried powders. Nature of destabilizing effects of sucrose.* Journal of pharmaceutical sciences, 1999. **88**(3): p. 360-370.

270. Buckton, G. and P. Darcy, *The use of gravimetric studies to assess the degree of crystallinity of predominantly crystalline powders.* International Journal of Pharmaceutics, 1995. **123**(2): p. 265-271.

271. Muzaffar, K., G.A. Nayik, and P. Kumar, *Stickiness problem associated with spray drying of sugar and acid rich foods: a mini review.* Journal of Nutrition & Food Sciences, 2015(S12): p. 1.

272. Adhikari, S. and T. Kar, *Bulk single crystal growth and characterization of l-leucine–A nonlinear optical material.* Materials Chemistry and Physics, 2012. **133**(2): p. 1055-1059.

273. Rajkumar, B.J. and V. Ramakrishnan, *Infrared and Raman spectra of L-valine nitrate and L-leucine nitrate.* Journal of Raman Spectroscopy, 2000. **31**(12): p. 1107-1112.

274. Joseph, J. and E.D. Jemmis, *Red-, blue-, or no-shift in hydrogen bonds: a unified explanation.* Journal of the American Chemical Society, 2007. **129**(15): p. 4620-4632.

275. Mangal, S., et al., *Physico-Chemical Properties, Aerosolization and Dissolution of Co-Spray Dried Azithromycin Particles with L-Leucine for Inhalation.* Pharmaceutical research, 2018. **35**(2): p. 28.

276. Chan, L.W., L.T. Lim, and P.W. Heng, *Immobilization of fine particles on lactose carrier by precision coating and its effect on the performance of dry powder formulations.* Journal of pharmaceutical sciences, 2003. **92**(5): p. 975-984.

277. Heng, P.W.S., L.W. Chan, and L.T. Lim, *Quantification of the surface morphologies of lactose carriers and their effect on the in vitro deposition of salbutamol sulphate.* Chemical and pharmaceutical bulletin, 2000. **48**(3): p. 393-398.

278. Naini, V., P.R. Byron, and E.M. Phillips, *Physicochemical stability of crystalline sugars and their spray-dried forms: dependence upon relative humidity and suitability for use in powder inhalers.* Drug Development and Industrial Pharmacy, 1998. **24**(10): p. 895-909.

279. Waterer, G. and R. Wunderink, *Respiratory infections: a current and future threat.* Respirology, 2009. **14**(5): p. 651-5.

280. Lister, P.D. and D.J. Wolter, *Resistance Challenges Threatening the Treatment of Pseudomonas aeruginosa Infections with Levofloxacin: The Role of a Levofloxacin-Imipenem Combination for Prevention of Resistance.* 2008.

281. Kaatz, G.W. and S.M. Seo, *Mechanism of ciprofloxacin resistance in Pseudomonas aeruginosa.* Journal of Infectious Diseases, 1988. **158**(3): p. 537-541.

282. Neu, H.C., *Bacterial resistance to fluoroquinolones.* Reviews of infectious diseases, 1988. **10**(Supplement_1): p. S57-S63.

283. Tran, T.B., et al., *Pharmacokinetics/pharmacodynamics of colistin and polymyxin B: are we there yet?* International journal of antimicrobial agents, 2016. **48**(6): p. 592-597.

284. Li, J., et al., *Colistin: the re-emerging antibiotic for multidrug-resistant Gram-negative bacterial infections.* The Lancet infectious diseases, 2006. **6**(9): p. 589-601.

285. Hartzell, J.D., et al., *Nephrotoxicity associated with intravenous colistin (colistimethate sodium) treatment at a tertiary care medical center.* Clinical Infectious Diseases, 2009. **48**(12): p. 1724-1728.

286. Haworth, C.S., et al., *Inhaled colistin in patients with bronchiectasis and chronic Pseudomonas aeruginosa infection.* American journal of respiratory and critical care medicine, 2014. **189**(8): p. 975-982.

287. Lin, Y.-W., et al., *Pharmacokinetics/pharmacodynamics of pulmonary delivery of colistin against Pseudomonas aeruginosa in a mouse lung infection model.* Antimicrobial agents and chemotherapy, 2017. **61**(3): p. e02025-16.

288. Yapa, S.W., et al., *Pulmonary and systemic pharmacokinetics of inhaled and intravenous colistin methanesulfonate in cystic fibrosis patients: targeting advantage of inhalational administration.* Antimicrobial agents and chemotherapy, 2014. **58**(5): p. 2570-2579.

289. Boisson, M., et al., *Comparison of intrapulmonary and systemic pharmacokinetics of colistin methanesulfonate (CMS) and colistin after aerosol delivery and intravenous administration of CMS in critically ill patients.* Antimicrobial agents and chemotherapy, 2014. **58**(12): p. 7331-7339.

290. Prentice, H.G., et al., *Oral ciprofloxacin plus colistin: prophylaxis against bacterial infection in neutropenic patients. A strategy for the prevention of emergence of antimicrobial resistance.* British journal of haematology, 2001. **115**(1): p. 46-52.

291. Buyck, J.M., P.M. Tulkens, and F. Van Bambeke, *Activities of antibiotic combinations against resistant strains of Pseudomonas aeruginosa in a model of infected THP-1 monocytes.* Antimicrob Agents Chemother, 2015. **59**(1): p. 258-68.

292. Furiga, A., et al., *Impairment of Pseudomonas aeruginosa biofilm resistance to antibiotics by combining the drugs with a new quorum-sensing inhibitor.* Antimicrobial agents and chemotherapy, 2016. **60**(3): p. 1676-1686.

293. Koerner-Rettberg, C. and M. Ballmann, *Colistimethate sodium for the treatment of chronic pulmonary infection in cystic fibrosis: an evidence-based review of its place in therapy.* Core evidence, 2014. **9**: p. 99.

294. Pamp, S.J., et al., *Tolerance to the antimicrobial peptide colistin in Pseudomonas aeruginosa biofilms is linked to metabolically active cells, and depends on the pmr and mexAB-oprM genes.* Molecular microbiology, 2008. **68**(1): p. 223-240.

295. Kollef, M.H., et al., *The intensive care medicine research agenda on multidrug-resistant bacteria, antibiotics, and stewardship.* Intensive Care Medicine, 2017: p. 1-11.

296. Lavorini, F., M. Pistolesi, and O.S. Usmani, *Recent advances in capsule-based dry powder inhaler technology.* Multidiscip Respir Med, 2017. **12**: p. 11.

297. Zhou, Q.T., et al., *Inhaled formulations and pulmonary drug delivery systems for respiratory infections.* Advanced drug delivery reviews, 2015. **85**: p. 83-99.

298. Jong, T., et al., *Investigation of the changes in aerosolization behavior between the jet-milled and spray-dried colistin powders through surface energy characterization.* Journal of pharmaceutical sciences, 2016. **105**(3): p. 1156-1163.

299. Park, C.-W., et al., *Advanced spray-dried design, physicochemical characterization, and aerosol dispersion performance of vancomycin and clarithromycin multifunctional*

controlled release particles for targeted respiratory delivery as dry powder inhalation aerosols. International journal of pharmaceutics, 2013. **455**(1-2): p. 374-392.

300. Hickey, A.J., et al., *Physical Characterization of Component Particles Included in Dry Powder Inhalers. II. Dynamic Characteristics.* Journal of Pharmaceutical Sciences. **96**(5): p. 1302-1319.

301. Cun, D., F. Wan, and M. Yang, *Formulation strategies and particle engineering technologies for pulmonary delivery of biopharmaceuticals.* Current pharmaceutical design, 2015. **21**(19): p. 2599-2610.

302. G Thakkar, S., K. Fathe, and H. DC Smyth, *Amorphous or crystalline? A comparison of particle engineering methods and selection.* Current pharmaceutical design, 2015. **21**(40): p. 5789-5801.

303. Zhou, Q., et al., *Colistin Powders with High Aerosolisation Efficiency for Respiratory Infection: Preparation and In Vitro Evaluation.* Journal of Pharmaceutical Sciences, 2013. **102**(10): p. 3736-3747.

304. Choosakoonkriang, S., S. Supaluknari, and P. Puangkaew, *High Performance Liquid Chromatographic Method for Determination of Colistin Sulfate and its Application in Medicated Premixand Animal Feed.* World Academy of Science, Engineering and Technology, International Journal of Chemical, Molecular, Nuclear, Materials and Metallurgical Engineering, 2013. **7**(4): p. 224-228.

305. Zhou, Q.T., et al., *Effect of device design on the aerosolization of a carrier-based dry powder inhaler--a case study on Aerolizer((R)) Foradile ((R)).* Aaps j, 2013. **15**(2): p. 511-22.

306. Asking, L. and B. Olsson, *Calibration at Different Flow Rates of a Multistage Liquid Impinger.* Aerosol Science and Technology, 1997. **27**(1): p. 39-49.

307. Choosakoonkriang, S., S. Supaluknari, and P. Puangkaew. *High performance liquid chromatographic method for determination of colistin sulfate and its application in medicated premix and animal feed*. in *Proceedings of World Academy of Science, Engineering and Technology*. 2013. World Academy of Science, Engineering and Technology (WASET).

308. Freudenthal, O., et al., *Nanoscale investigation of the interaction of colistin with model phospholipid membranes by Langmuir technique, and combined infrared and force spectroscopies.* Biochim Biophys Acta, 2016. **1858**(11): p. 2592-2602.

309. Neugebauer, U., et al., *Vibrational spectroscopic characterization of fluoroquinolones.* Spectrochim Acta A Mol Biomol Spectrosc, 2005. **61**(7): p. 1505-17.

310. Burnett, D.J., F. Thielmann, and J. Booth, *Determining the critical relative humidity for moisture-induced phase transitions.* International Journal of Pharmaceutics, 2004. **287**(1–2): p. 123-133.

311. Zhou, Q.T., et al., *How Much Surface Coating of Hydrophobic Azithromycin Is Sufficient to Prevent Moisture-Induced Decrease in Aerosolisation of Hygroscopic Amorphous Colistin Powder?* The AAPS journal, 2016. **18**(5): p. 1213-1224.

312. Wang, Q., et al., *Controlled release of ciprofloxacin hydrochloride from chitosan/polyethylene glycol blend films.* Carbohydrate Polymers, 2007. **69**(2): p. 336-343.

313. Wang, C.J., et al., *Cation exchange interaction between antibiotic ciprofloxacin and montmorillonite.* J Hazard Mater, 2010. **183**(1-3): p. 309-14.

314. Singh, J. and P.K. Dutta, *Preparation, Antibacterial and Physicochemical Behavior of Chitosan/Ofloxacin Complexes.* International Journal of Polymeric Materials and Polymeric Biomaterials, 2010. **59**(10): p. 793-807.

315. Konno, H. and L.S. Taylor, *Ability of different polymers to inhibit the crystallization of amorphous felodipine in the presence of moisture.* Pharmaceutical research, 2008. **25**(4): p. 969-978.

316. Wallace, S.J., et al., *Self-assembly behavior of colistin and its prodrug colistin methanesulfonate: implications for solution stability and solubilization.* The Journal of Physical Chemistry B, 2010. **114**(14): p. 4836-4840.

317. Rumondor, A.C.F., et al., *Phase Behavior of Poly(vinylpyrrolidone) Containing Amorphous Solid Dispersions in the Presence of Moisture.* Molecular Pharmaceutics, 2009. **6**(5): p. 1492-1505.

318. Rumondor, A.C.F. and L.S. Taylor, *Effect of Polymer Hygroscopicity on the Phase Behavior of Amorphous Solid Dispersions in the Presence of Moisture.* Molecular Pharmaceutics, 2010. **7**(2): p. 477-490.

319. Young, P.M., et al., *The influence of relative humidity on the cohesion properties of micronized drugs used in inhalation therapy.* Journal of pharmaceutical sciences, 2004. **93**(3): p. 753-761.

320. Mangal, S., et al., *Relationship between surface concentration of l-leucine and bulk powder properties in spray dried formulations.* European Journal of Pharmaceutics and Biopharmaceutics, 2015. **94**: p. 160-169.

VITA

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PUBLICATIONS

- **Shetty, N.;** Zeng, L.; Mangal, S.; Nie, H.; Rowles, M. R.; Guo, R.; Han, Y.; Park, J. H.; Zhou, Q. T. Effects of Moisture-Induced Crystallization on the Aerosol Performance of Spray Dried Amorphous Ciprofloxacin Powder Formulations
- **Shetty, N.;** Park, H.; Zemlyanov, D.; Mangal, S.; Bhujbal. S.; Zhou, Q. T. Influence of excipients on physical and aerosolization stability of spray dried high-dose powder formulations for inhalation
- **Shetty, N.;** Ahn, P.; Park, H.; Bhujbal. S.; Zemlyanov, D.; Cavallaro. A.; Mangal, S.; Li, J.; Zhou, Q. Improved physical stability and aerosolization of inhalable amorphous ciprofloxacin powder formulations by incorporating synergistic colistin**.**
- Wang, S., Yu, S., Lin, Y., Zou, P., Chai, G., Yu, H.H., Wickremasinghe, H., **Shetty, N.,** Ling, J., Li, J., Zhou, Q.T.* Co-delivery of ciprofloxacin and colistin in liposomal formulations with enhanced *in vitro* antimicrobial activities against multidrug resistant *Pseudomonas aeruginosa*.
- Mangal,S., Xu, R., Park, H., Zemlyanov, D., **Shetty, N.,** Lin, Y., Morton, D., Chan, H., Li, J., Zhou, Q.* Understanding the impacts of surface compositions on the in-vitro dissolution and aerosolization of co-spray-dried composite powder formulations for inhalation..
- Mangal, S., Huang, J., **Shetty, N.,** Park, H., Lin, Y., Yu,H., Zemlyanov, D., Velkov,T., Li,J., Zhou, Q.* Effects of antibiotic component on in-vitro bacterial killing, physico-chemical properties, aerosolization and dissolution of a ternary-combinational inhalation powder formulation of antibiotics for pan-drug resistant Gram-negative lung infections.

- Mangal, S., Park, H., Nour, R., **Shetty, N.,** Cavallaro. A.; Zemlyanov, D., Thalberg, K., Puri, V., Nicholas, M., Narang, A., Zhou, Q.* Correlations between surface composition and aerosolization of jet-milled dry powder inhaler formulations with pharmaceutical lubricants