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Improved physical stability and aerosolization of inhalable amorphous ciprofloxacin powder formulations by incorporating synergistic colistin

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Abstract

Objective: 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.

Methods: 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.

Results: 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

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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.

Conclusions: 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.

GRAPHICAL ABSTRACT

Keywords

Dry powder inhaler; co-spray drying; storage humidity; multi-drug resistance; aerosol performance

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 [1]. This is mainly due to the emergence of drug resistance against the first-line antimicrobials [2] 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 [3]. 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 [4].

Combination antibiotic therapy is a promising 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 [5]. Colistin, despite being an old antibiotic, has retained its excellent antibacterial activity against Gram-negative bacteria including *Pseudomonas* species [6]. However, use of high doses of intravenous colistin to treat lung infections may cause serious adverse events such as nephrotoxicity [6, 7]. In contrast, inhaled colistin has shown to be safe and well tolerated in animals and patients with *P. aeruginosa* infections [8–11]. PK/PD data from animal and clinical studies of inhaled colistin have shown in-vivo advantages over systemic administration [8–11].

A combination of oral ciprofloxacin with colistin could successfully prevent emergence of resistance even when used continuously for 10 years as a prophylactic regimen [12]. 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 Gram-negative bacteria [13]. Combination of ciprofloxacin with colistin was also found to be effective against ‘difficult to tackle’ *P. aeruginosa* biofilms [14]. 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 [15, 16].

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 [6]. 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 [17]. On the contrary, dry powder inhalers (DPI) are gaining popularity as they are easy to carry, more chemically stable and suitable for high-dose antibiotics [18, 19]. Inhalation products such as TOBI® Podhaler and Colobreathe are available commercially to treat lower respiratory tract infections [20].

Inhalable powders produced via jet milling are typically cohesive in nature due to high surface energy and consequently exhibit poor aerosolization performance [21, 22]. Physical properties such as particle size, shape, morphology, etc. play a critical role in the clinical efficacy of the DPIs [23–26]. These properties can be altered using specialized particle engineering techniques such as spray drying [22, 27–29]. 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 [30, 31]. 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 [31]. 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 [32], there is a need to minimize the use of excipients for high-dose DPIs [19].

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 [33]. Here we aimed to examine

the effects of colistin on the physical stability and aerosolization of ciprofloxacin in co-spray dried DPI formulations.

MATERIAL AND METHODS

Chemicals

Colistin sulfate (abbreviated as colistin or Col) and ciprofloxacin hydrochloride monohydrate (abbreviated as ciprofloxacin or Cipro) were purchased from BetaPharma® 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).

Spray Drying

Spray-dried formulations as outlined in Table I 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 with a standard two-fluid nozzle (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 °C, aspirator at 35 m³/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 $20 \pm 2\%$ RH at 20 ± 2 °C; (2) a humidity chamber containing saturated magnesium nitrate solution to maintain $55 \pm 2\%$ RH at 20 ± 2 °C.

Additionally, the co-spray dried ColCipro (1:1) powder formulation was stored at $75\% \pm 2\%$ 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.

X-ray Powder Diffraction (PXRD)

Crystallinity of formulations was evaluated using a Rigaku Smartlab™ diffractometer (Rigaku Americas, Texas, USA). Cu-K α radiation source and a highly sensitive D/tex ultra-detector at a voltage of 40kV and current of 44 mA were used [34]. Settings were as follows: 5 to 40° 2 θ at a step size of 0.02° with a scan rate of 4°/min.

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.

Particle Size

Particle size distribution of the powder formulation was measured using an Image J software based on SEM images [35]. The diameter at 10% (d_{10}), 50% (d_{50}) and 90% (d_{90}) undersize was calculated for approximately 100 particles.

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 °C; and in the desorption cycle, the mass change was measured at RH ranging from 90–0% with 10% interval. An equilibrium criterion of $\text{dm}/\text{dt} \leq 0.002\%$ per minute was specified for the system to achieve at each RH step.

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 K α 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 4f $_{7/2}$ at 84.0 eV and Cu 2p $_{3/2}$ 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).

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.

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” AuI instrumental settings, while spatial resolution was optimized by “unbunched” AuI instrumental settings for the Collection of images. ToF-SIMS data were collected randomly from 5 different areas ($75 \times 75 \mu\text{m}$ 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 $[\text{C}_5\text{H}_{12}\text{N}^+]$, ciprofloxacin was at m/z 101 amu corresponding to $[\text{C}_4\text{H}_9\text{N}_2\text{O}^+]$ and l-leucine was at m/z 132 amu corresponding to $[\text{C}_6\text{H}_{14}\text{NO}_2^+]$. The spectra were integrated, and high-resolution surface composition overlays were constructed using WincadenceN software (Physical Electronics Inc., Chanhassen, MN, USA).

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 $400 - 4000 \text{ cm}^{-1}$ 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 [36].

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 [37]. The elution time for ciprofloxacin was $\sim 2 \text{ min}$ 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 [38]. 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, $5 \mu\text{m}$ C18 $150 \times 4.60 \text{ mm}$ 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 .

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 $40\% \text{ RH}$. Each formulation ($10 \pm 1 \text{ mg}$) 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, Plastiaple S.p.A., Osnago, Italy) [39]. The capsules were actuated at an

airflow of 100 L/min for 2.4 s, allowing 4 L of air and a pressure drop of approximately 4 kPa [40]. 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 [33]. 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 high-performance liquid chromatography (HPLC) method described above [41]. The emitted dose was determined as the drug released from the capsule and device; whereas the fine particle fraction (FPF) was defined as particles with an aerodynamic size below 4.9 μm (cut-off diameter of Stage 2) relative to the total recovered drug. The FPF emitted was defined as particles with an aerodynamic size below 4.9 μm (cut-off diameter of Stage 2) relative to the emitted dose.

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.

RESULTS

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

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 1A) and did not show any crystallization up to 60 days when stored at 20 % RH (Figure 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 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 1D). But, the co-spray dried ColCipro (1:1) was amorphous for up to 60 days upon storage at 55% RH (Figure 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.

FTIR—Chemical structures of both molecules used for this study is shown in Figure 2. The FT-IR result are shown in Figure 3 and Table II. 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 [42]. 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 [43]. 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.

Particle Size—Tables III and IV 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 D₅₀ < 1.4 µm and D₉₀ < 3 µm.

Surface Morphology—SEM images are shown in Figure 4. 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 particles were rougher, which is in good agreement to our previous finding [31], 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 4). No change was observed in morphology of the spray-dried colistin sample stored at two RHs (Figure 4). 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.

Time-of-flight secondary ion mass spectrometry (ToF-SIMS)—Figure 5 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.

Energy Dispersive X-ray Spectroscopy (EDX)—Figure 6 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. In addition, there is a possibility of salt disproportionation of Sulphur and Chlorine from the bases [44]. Therefore, more surface sensitive characterization techniques of XPS and ToF-SIMS were employed.

X-ray photoelectron spectroscopy (XPS)—Table V 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.

Dynamic Vapor Sorption—The spray-dried ciprofloxacin alone presented a sorption characteristic of water absorption and crystallization (via water expulsion). Specifically, moisture sorption increased with respect to an 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 (caused by 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 [45]. 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 [46]. In our study we propose that amorphous spray-dried ciprofloxacin crystallized to form a monohydrate. The molecular weight of ciprofloxacin HCl monohydrate is 386, of which 4.6% is the water of crystallization. So, if it crystallized 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 up to 32% moisture upon exposure to 90% RH. No crystallization corresponding to decrease in mass was observed for the spray-dried colistin which is in accordance with PXRD data [47]. 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 7B, 7D & 7E). 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 measurement of 0% RH (Figure 6C). This is in accordance with the PXRD results where ColCipro (1:9) crystallized at Day 3 upon storage at 55% RH (Figure 1D).

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 8). The aerosol performance of co-spray dried colistin-ciprofloxacin formulation in the mass ratio (1:9) ($45.7 \pm 4.2\%$), (1:3) ($55.3 \pm 3.1\%$) and (1:1) ($67.1 \pm 3.8\%$) was significantly higher ($p < 0.0001$) compared to the spray-dried ciprofloxacin alone ($28 \pm 3.2\%$). Additionally, the ColCipro (1:9) and ColCipro (1:3), in which crystallization was

observed after 3 days (Figure 1), no significant change was measured in the FPF expressed as percentage of recovered dose or emitted dose (Figure 9 and 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 11. 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 4) and retained that rough surface for up to 60 days at 55% RH (Figure 11). 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.

Physical stability and aerosol performance 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.

PXRD—Figure 12 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. We further investigated the effects of such crystallization on the surface morphology and aerosol performance of the co-spray dried formulation.

SEM—The co-spray dried ciprofloxacin-colistin formulation had a smooth surface with spherical shape at 20% RH. No change in surface morphology 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 with smoother surfaces (Figure 13). Based on the XPS data (Table 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 [47].

In-vitro Aerosol Performance—Upon storage at 75% RH for one day, no significant change ($p > 0.05$) in FPF for both colistin ($68.6 \pm 2.1\%$) and ciprofloxacin ($71.5 \pm 1.7\%$) was observed as compared to the FPF of both colistin ($67.4 \pm 4.9\%$) and ciprofloxacin ($67.1 \pm 3.8\%$) 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 14). The FPF for ciprofloxacin and colistin decreased to $19.2 \pm 1.4\%$ and $19.5 \pm 1.3\%$ respectively 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 [48].

Enhancing physical and aerosolization stability by adding L-leucine

PXRD—Figure 15 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 12). 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 [49].

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 morphology of the co-spray dried formulation was observed upon storage at 75% RH for 7 days (Figure 16).

In-vitro Aerosol Performance—Figure 17 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 ($62.9 \pm 6.1\%$) and ciprofloxacin ($63.1 \pm 4.7\%$) stored at 75% RH for 1 day did not change significantly as compared to the FPF of both colistin ($58.7 \pm 2.2\%$) and ciprofloxacin ($62.1 \pm 2.2\%$) 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.

XPS and ToF-SIMS—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 VI). 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-enriched than colistin, as confirmed by the ToF-SIMS images (Figure 18).

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 crystallize upon storage [31]. 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 $35.5 \pm 1.7\%$ at Day 1 to $42.3 \pm 0.9\%$ at Day 3 ($p < 0.01$) [31]. 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 [33, 50]. 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 1E). We propose the amorphous colistin with larger molecular weight acts as a polymer-like matrix that minimizes the mobility of ciprofloxacin molecules, therefore inhibits the crystallization tendency for amorphous ciprofloxacin [51]. We have performed DSC measurements on all samples but unfortunately T_g cannot be determined for either colistin or ciprofloxacin [52]. Nevertheless, 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 3). 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 [53–55]. 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 (Figure 11) and no significant change in aerosol performance ($p > 0.05$) (Figure 9), which indicated the physical and aerosolization stability. Co-spray dried ColCipro formulation in the mass ratio (1:3) and (1:9) crystallized after 3 days of storage at 55% RH (Figure 1D); however, no change in particle morphology and aerosol performance was observed in these formulations at 55% RH for up to 60 days (Figure 11).

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. Apparently colistin has a higher Peclet number than ciprofloxacin [56, 57]; our earlier studies showed colistin has self-assembling and surface-active properties [58] with low surface energy [21] and colistin has a much larger molecular weight than ciprofloxacin. 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 [59, 60]. Upon storage at 75% RH for 7 days the powders appeared to be fused with a significant decrease in FPF of ciprofloxacin from $71.5 \pm 1.7\%$ at Day 1 down to $19.2 \pm 1.4\%$ 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 [61]. 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 [33].

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 [62] and improve aerosolization of cohesive fine particles [63–65]. Our studies showed that effects of L-leucine on aerosolization enhancement depend on whether L-leucine is enriched on the particle surfaces [66]. Li et al. reported that enrichment of crystalline L-leucine on particle surface could provide protection against moisture on dispersion of hygroscopic powders [48]. 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.

CONCLUSIONS

We developed a combinational 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 deterioration 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 synergistic efficacy of such combination formulation are under investigation in a mouse lung infection model.

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Figure 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.

Colistin

Ciprofloxacin

Figure 2:
Chemical structures of ciprofloxacin and colistin in their base form.

Figure 3:

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

Figure 4: 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.

Figure 5:

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).

Figure 6:
Surface concentrations based on elemental analysis of chlorine (in ciprofloxacin hydrochloride) and sulfur (in colistin sulfate) measured by EDX.

Figure 8:
Effect of colistin concentration on FPF of co-spray dried formulations after storage at 20% RH for 1 day (mean $\pm$ SD, n=4).

Figure 9:

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 $\pm$ SD, $n=4$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; NS, no significant difference).

Figure 10:

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 $\pm$ SD, $n=4$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; NS, no significant difference).

Figure 11:
SEM images of the spray-dried formulations stored at 20% and 55% RH for 60 days.

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Figure 12:
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.

Figure 13:
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.

Figure 14:

(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 $\pm$ SD, n=4; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; NS, no significant difference).

Figure 15: 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.

Figure 16:
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.

Figure 17:
(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 $\pm$ SD, $n=4$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; NS, no significant difference)

Figure 18: Surface composition distributions of colistin (blue), L-leucine (red) and ciprofloxacin (green) on the surfaces of particles obtained by ToF-SIMS. A- Raw Ciprofloxacin; B- Raw Colistin; C- Raw L-leucine; D- ColCipLeu (1:1:1) (scale bar represents 10 μm).

Table I:

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

Table II:

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

Table III:

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 IV:

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

Table V:

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

Table VI:

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