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Inhalable combination powder formulations of phage and ciprofloxacin for *P. aeruginosa* respiratory infections

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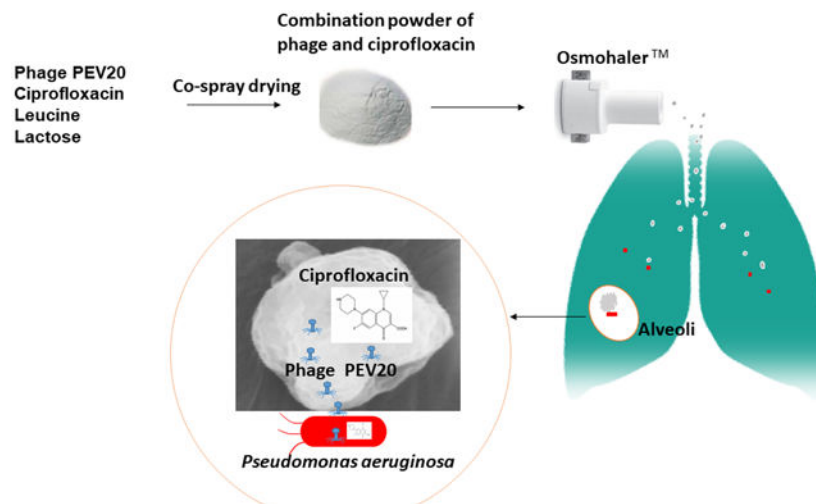
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Abstract

Recently we showed that nebulized ciprofloxacin and phage PEV20 in combination had a synergistic bactericidal effect against antibiotic-resistant *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis. Compared to nebulization, dry powders for inhalation may improve patient handling characteristics and compliance. In the present study, we co-spray dried ciprofloxacin and phage PEV20 using L-leucine with or without lactose as excipients. Two formulations were identified for testing in this study. The mass ratios were set at 1:1:1 for ciprofloxacin, lactose and L-leucine (Formulation A) or 2:1 for ciprofloxacin and L-leucine without lactose (Formulation B). Concentrations of PEV20 were set at 10^8 and 10^9 PFU/mL for two clinical *P. aeruginosa* strains FADD1-PA001 and JIP865, respectively. Formulations A and B were characterized as partially crystalline and the powders recrystallized at >40% relative humidity (RH). Both formulations exhibited strong synergistic antimicrobial killing effect on the two strains. Formulations A and B maintained bactericidal synergy after dispersion using both low and high resistance Osmohaler™. Powder aerosol performance was examined by next generation impactor (NGI) in low resistance inhaler at 100 L/min and by multi-stage liquid impinger (MSLI) in high resistance inhaler at 60 L/min. Fine particle fractions (FPF) obtained by NGI were $64.3 \pm 2.9\%$ and $59.7 \pm 2.1\%$ for A and B, respectively. FPF obtained by MSLI were $71.0 \pm 3.4\%$ and $73.3 \pm 5.0\%$, respectively. In conclusion, it is feasible to prepare stable and inhalable combination powder formulations of phage PEV20 and ciprofloxacin for potential treatment of respiratory infections caused by multi-drug resistant (MDR) *P. aeruginosa*.

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Graphical Abstract



Keywords

Ciprofloxacin; bacteriophages; *Pseudomonas aeruginosa*; Respiratory infection; Spray drying; Cystic fibrosis; Inhalation; L-leucine; Powder formulation

Introduction

Pseudomonas aeruginosa was ranked on the World Health Organization priority list as one of the critical antibiotic-resistant pathogens [1]. This pathogen is a major contributor to morbidity and mortality in patients with cystic fibrosis (CF), chronic obstructive pulmonary disease, non-CF bronchiectasis and sepsis [2–4]. Respiratory infections are difficult to treat because microbes may reside deep in the airways. Repositioning antibiotics for inhalation with higher local drug levels in the lungs could treat refractory pulmonary infections more effectively than oral or parenteral administrations [3].

Ciprofloxacin is a concentration-dependent fluoroquinolone antibiotic, which has been formulated into inhalable liposome [5], metal-complex [6], nanoplex [7] and other dry powders [8, 9] for treating *P. aeruginosa* respiratory infections. However, *P. aeruginosa* rapidly acquires antibiotic-resistance to fluoroquinolones and these “superbugs” can readily survive under monotherapy [10, 11]. To prevent emergence of drug resistance, some concomitant formulation of inhaled antimicrobials have been studied [12–15]. Ciprofloxacin was also previously co-spray dried with doxycycline hydrochloride, but no synergy was observed against *P. aeruginosa* [14].

As an alternative or adjunct to conventional antibiotics, bacteriophage (or phage) therapy has been clinically used to treat multi-drug resistant (MDR) respiratory infections [16–19]. In a recent study, we demonstrated synergistic antimicrobial effect of ciprofloxacin and phage PEV20 combination formulation against MDR *P. aeruginosa* CF isolates [20]. The synergy was retained after aerosolization using commercial nebulizers. Dry powder formulations provide easy storage, transport, long shelf-life and potentially better patient compliance over

liquid formulations [21]. Previously, ciprofloxacin and phage PEV20 were individually formulated into dry powders by spray drying [9, 21]. Spray drying technology is widely used for producing fine particle powders suitable for inhalation delivery using dry powder inhalers (DPIs) [22]. This one-step continuous process is especially suitable for biological and heat-sensitive materials such as phages [23]. In addition, aerosol performance, physico-chemical stability and drug release profiles can be modified using this technology [17].

L-leucine is a dispersion enhancer that can increase the fine particle fraction (FPF) of spray dried ciprofloxacin [24]. Furthermore, enrichment of L-leucine on the particle surface can prevent water absorption, thereby protecting the particles against moisture-induced deterioration of *in vitro* aerosolization performance [25]. Water is known to act as a plasticizer and can cause recrystallization of amorphous powders [24]. Powder recrystallization could potentially increase phage mobility, causing conformational changes and deactivation [21]. Sugars, including lactose, mannitol, and trehalose, have been studied as phage stabilizers against heat and shear stresses during spray drying [26, 27]. For phage PEV20, lactose in combination with L-leucine showed superior protection over trehalose [21]. Hence, lactose and L-leucine were chosen as excipients for producing powder formulations of ciprofloxacin and PEV20.

To the best of our knowledge, inhaled formulations of phage and antibiotic combination in the form of dry powder has not been explored. The aim of this study was to produce synergistic and inhalable dry powder formulations containing phage PEV20 and ciprofloxacin.

Materials and methods

Bacterial strains

Clinical *P. aeruginosa* strains (FADD1-PA001 and JIP865) were freshly subcultured from -80°C stock prior to the experiment. Strain FADD1-PA001 was received from Monash University, Australia and JIP865 from Westmead Hospital, Australia. The two strains were selected based on the antibiotic and phage efficacy screening conducted previously [20].

Phage PEV20 and ciprofloxacin

Phage PEV20 was provided by AmpliPhi Biosciences AU at a titre of 10^{10} plaque forming unit (PFU)/mL in phosphate buffered saline (PBS, 0.01 M phosphate buffer, 0.0027 M KCl and 0.137 M NaCl) [28]. This phage was formerly isolated from the sewage treatment plant in Olympia (WA, USA) by Kutter lab (Evergreen phage lab). Ciprofloxacin hydrochloride was purchased from Sigma-Aldrich Inc.

Spray drying

A Buchi spray dryer (B-290, Buchi Labortechnik AG, Flawil, Switzerland) was employed to prepare powder formulations of ciprofloxacin and phage PEV20 in combination. Liquid feed for the formulations was made up of 18 mL of ciprofloxacin with excipients in ultra-pure water and 2 mL of PEV20 suspension (10^9 PFU/mL for strain FADD1-PA001 or 10^{10} PFU/mL for strain JIP865) in PBS. The solution (18 mL, without phage) of formulation A

contained 8 mg/mL of ciprofloxacin, 8 mg/mL of lactose and 8 mg/mL of L-leucine, and formulation B contained 16 mg/mL of ciprofloxacin and 8 mg/mL of L-leucine. Two additional formulations C and D were spray dried to study the crystallinity of combination powder formulations using X-ray diffraction (XRD). Feed solutions (without phage) of formulation C contained 12 mg/mL of ciprofloxacin and 12 mg/mL of L-leucine, and formulation D contained 8 mg/mL of ciprofloxacin and 16 mg/mL of L-leucine (Table 1). A conventional two fluid nozzle with diameter 0.7mm was used for atomization. The suspension was fed at a constant feed rate of 1.8 mL/min and an atomizing airflow of 742 L/h with an aspiration rate of 35 m³/h. The drying inlet air was heated to 60 °C and the outlet temperature range was between 41 and 42 °C. Dried powders were collected in a vial after passing through the cyclone. Powders were stored in a desiccator with silica beads at room temperature.

Aerosol performance and unit dose dispersion

Formulation A (24mg) or formulation B (12mg) loaded in size 3 hydroxypropyl methylcellulose capsules were dispersed in low and high resistance Osmohaler™ to evaluate aerosol performance. Next Generation Impactor (NGI; Copley, Nottingham UK) with a mouthpiece adapter and a NGI induction port (Copley, Nottingham, UK) was operated for low resistance Osmohaler™ at a flow rate of 100 L/min for 2.4 s, while Multi-stage liquid impinger (MSLI; Copley, Nottingham UK) was operated for high resistance Osmohaler™ at a flow rate of 60 L/min for 4 s. Pressure drop across both devices was 4 kPa. The cut-off diameters of the NGI stages 1–7 at 100 L/min were 6.12, 3.42, 2.18, 1.31, 0.72, 0.40 and 0.24 µm, respectively. In stage 8, a Micro-Orifice Collector was used. Silicon grease was used to coat the impactor stages. Particles deposited on the capsule, inhaler, adapter, induction port, stages 1–7 and MOC were dissolved using 10 mL Milli-Q water. The cut-off diameters of the MSLI stages 1–4 at 60 L/min were 13, 6.8, 3.1 and 1.7 µm, respectively. For each stage, 20 mL Milli-Q water was used to dissolve particles. Concentrations of ciprofloxacin were determined using a high performance liquid chromatography (HPLC) system (Model LC-20, Shimadzu, Kyoto, Japan). A Phenosphere-Next C-18 column (5 mm, 4.6×150 mm, Phenomenex, USA) was used at 35 °C. Mobile phase was a mixture of 0.5% triethylamine in water (pH 3.0) and 100% methanol (78: 22 v/v) and isocratic elution was performed at a flow rate of 0.9 mL/min. Ciprofloxacin was assayed at a wavelength of 277 nm. The NGI or MSLI test was conducted in triplicate for each formulation. The cumulative mass of ciprofloxacin at each cut off diameter was calculated and the fine particle dose (FPD) was determined as the cumulative mass of particles < 5 µm. FPF was the percentile of FPD of the total recovered dose. The same dispersion parameters for low and high resistance inhalers were used for unit dose test. Dispersed powders were captured by a 47 mm diameter sample collection tube equipped with a Suregard® filter. The dry powder impacted on the filter paper was collected for bacterial killing effect study. Study was carried out in triplicate.

Time-kill curve assay

Antibacterial activity of the spray dried powder formulations was assessed using the time-kill curve method [29]. A single bacterial colony was inoculated in 20 mL of nutrient broth (NB) for 18 h at 37°C with continuous shaking (150 revolutions per minute, rpm). Overnight culture (10 mL) was mixed with 20 mL of fresh NB and further incubated for 2 h until early-

log phase was reached. Concentration of the early-log culture was adjusted to 10^6 colony-forming units (CFU)/mL. Formulations A and B were reconstituted in PBS to give a final ciprofloxacin concentration of 80 $\mu\text{g}/\text{mL}$ against strains FADD1-PA001 and 40 $\mu\text{g}/\text{mL}$ against JIP865. Bacterial culture (200 μL) was added to a 96-well plate and then treated with either ciprofloxacin (80 $\mu\text{g}/\text{mL}$ or 40 $\mu\text{g}/\text{mL}$) or PEV20 (10^6 or 10^9 PFU/mL) alone, or with the reconstituted powder formulations, followed by incubation at 37 °C with continuous shaking. Optical density at 600 nm (OD600) was measured at 24 h using a microplate reader (FLUOstar Optima, BMG Labtechnologies, Offenburg, Germany). The assay was repeated five times.

Particle size distribution

Particle size distributions of the spray dried formulations were analyzed by laser diffraction (Malvern Mastersizer 2000, Malvern Instruments Ltd., UK). Powder samples were dispersed via a Scirocco 2000 dry powder module (Malvern Instruments, UK) with air pressure at 4.0 bar. Refractive indices for formulations A and B were 1.54 and 1.46, respectively. Volumetric diameter (D_{10} , D_{50} , and D_{90}) and span (the difference between D_{10} and D_{90} divided by D_{50}) values were reported based on triplicate measurements.

Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM; NeoScope Benchtop JCM-6000, JEOL USA, Inc.) was employed to examine particle morphology of the spray dried powders. Samples were spread on a carbon sticky tape and then mounted on an SEM stub, followed by sputter coating with gold (30 nm thick) using a K550 sputter coater (Quorum Emitech, Kent, UK). The images were captured at 15 kV.

X-ray powder diffraction (XRD)

Powder crystallinity was evaluated by X-ray powder diffraction XRD (Shimadzu XRD-6000, Shimadzu Corporation, Kyoto, Japan). Cu K α radiation was used at a voltage of 40 kV and a current of 30 mA. Data were recorded from 5° to 45° 2 θ using a scan speed of 2° per min and a step size of 0.02°.

Differential scanning calorimetry (DSC)

DSC studies were carried out using a Mettler Toledo DSC 1 model with the STAR^e System (Zurich, Switzerland). Powder formulations A and B (10 mg) were assayed using aluminium pans with a pinhole lid under nitrogen atmosphere (100 mL/min). DSC thermograms were recorded over a temperature range of 25 – 400 °C at a constant rate of 10 °C/min.

Thermogravimetric analysis (TGA)

TGA studies were performed using a Mettler Toledo TGA/DSC 1 model with the STAR^e System (Zurich, Switzerland). Powder samples (5 – 8 mg) were assayed in a ceramic crucible under nitrogen atmosphere (100 mL/min). Sample weight loss was recorded over a temperature range of 22 – 150 °C at a constant rate of 10 °C/min.

Dynamic vapour sorption (DVS)

Moisture sorption isotherms of powder samples (10 – 20 mg) were assayed using DVS instrument (Surface Management Systems, London, United Kingdom). The chamber was kept at 25 °C with continuous nitrogen flow. Each sample was exposed to one absorption and desorption cycle of 0 – 90% - 0 relative humidity (RH), with 10% RH increments and decrements. The criterion for reaching equilibrium moisture content at each RH step was set at a weight change of 0.002% per minute.

Light microscopy

Polarized light microscopy was performed on an Olympus CH30/CH40 biological microscope in transmission mode to confirm recrystallization of powder formulation B after the DVS run. Samples were checked before and after conditioning in DVS up to 60% RH. Sprinkled powders immersed in oil were prepared on glass slides. Images were obtained with a digital camera.

Transmission electron microscopy (TEM)

TEM was performed to study the morphology of PEV20 before and after spray drying. Around 150 mg of combination dry powder formulation was dissolved in 150 mL of PBS using a vortex. A Nanosep centrifugal device with a 10 kDa filter membrane [30] was used for phage enrichment. A final volume of 0.5 mL was obtained. Samples were prepared on carbon copper grids 200 mesh (GSCu200C-50) supplied by ProSciTech Pty Ltd. A drop of 0.1% poly-L-lysine solution was placed on the grid for 1 min and then blotted with a filter paper. A drop of phage sample was loaded onto the grid for 30 min and any excess sample was blotted. The samples were then negatively stained with 1% phosphotungstic acid (pH adjusted to 7.4 with 0.1 M sodium hydroxide) [31] for 20 seconds. Excess stain was blotted and the grids were left to air-dry. A JEOL-JEM 1400 microscope (JEOL, Japan) was used to collect images at $\times 40$ k magnification.

Statistical analysis

Bacterial survival rate at 24 h was calculated by dividing the OD₆₀₀ value of the treated group by that of the negative control group. The additive survival rate was calculated by multiplying the bacterial survival rates of the phage-treated group with that of antibiotic-treated group. Phage-antibiotic synergy was confirmed if the observed bacterial survival rate of phage-antibiotic combinations was statistically lower than the calculated additive survival rate [32]. Student's *t*-test was used to compare the calculated with observed bacterial survival rate for the combination powder. P-value of < 0.05 was considered statistically significant.

Results and Discussion

Taking into consideration the possible thermal stress to phage PEV20 during spray drying, the powder formulations were produced at a relatively low inlet temperature of 60 °C in the present study. The production yields of formulations A and B were 66.9% and 73.8%, respectively, indicating the process efficiency at the chosen inlet temperature. Both formulations had a recovery of over 90% (Table 2) from the NGI and MSLI. The FPF values were $59.7 \pm 2.1\%$ and $64.3 \pm 2.9\%$ for formulations A and B, respectively, obtained from the

different shear forces generated with different fluid dynamic design. Formulation of high-dose antimicrobials for DPI delivery is challenging due to bulk volume of dose, the filled weight in the capsules, and the number of capsules and inhalations required by the patients. In this study, 8 mg of ciprofloxacin was contained in 24 mg and 12 mg of formulations A and B per capsule, respectively. Our formulation design was based on considerations from clinical trials of inhaled ciprofloxacin. Bronchiectasis patients were given 32.5 mg of inhaled ciprofloxacin twice daily for treatment of respiratory infections [9, 37]. The Phase I study predicted that 7.2 mg ciprofloxacin will be deposited in the trachea/bronchi and 5.6 mg in the alveolar space when 32.5 mg was administered. In the Phase II study, a trend for improvement was demonstrated with this dose, but this was not statistically significant compared with the placebo. In our previous study [20], phage PEV20 in combination with ciprofloxacin at 1/4 MIC and 1/2 MIC showed synergy on strain FADD1-PA001 and JIP865, respectively. Therefore, a single dose of inhaled ciprofloxacin in the present study was proportionally reduced to roughly 8 and 16 mg (i.e., 25 or 50% of the 32.5 mg 2 doses) in our powder formulations.

Formulations A and B were both partially crystalline, exhibiting diffraction peaks at similar positions around 6°, 19°, 24°, 26°, 31° and 33° (Fig. 5). To identify the origin of these peaks, we prepared two additional formulations (C and D) which contained less ciprofloxacin and more leucine. The first three peaks and those at 31° and 33° are readily attributed to L-leucine as the peak intensity scaled with the leucine concentration (Fig. 5). These peaks are also consistent with those reported for co-spray dried formulations of leucine and ciprofloxacin, in which ciprofloxacin was identified as amorphous [24]. In contrast, the peak at 26° was ascribed to ciprofloxacin in formulation B as the peak became sharper with increasing ciprofloxacin concentration. This discrepancy was likely due to use of different inlet temperature (120 °C) with different mass ratio of ciprofloxacin and leucine (9:1) and/or total solute concentration (16 mg/mL) of feed solution than the literature. Higher drying temperature and lower feed concentration may affect the time to supersaturation and supersaturation level, nucleation and subsequent crystal growth and packing, which could lead to the change of polymorphic form of ciprofloxacin.

Both formulations A and B showed a sorption profile with the characteristic of a recrystallization event. Powders initially absorbed moisture with increase in mass. Then between 40 – 80% RH, formulation A recrystallized, which caused decrease in mass as water was released from the crystal lattice (Fig. 6). Formulation B recrystallized at 50, 60 and 70% RH. Moisture-induced recrystallization was confirmed by polarized light microscopy showing increased transmitted light (optical birefringence) (Fig. 7). Our data are consistent with previous studies reporting recrystallization of spray dried ciprofloxacin alone [39] or with leucine [24] at 55% RH. Hence, spray dried formulations A and B, being partially amorphous, should be handled and stored at low RH [40].

Besides humidity, storage temperature could also cause recrystallization if the temperature is higher than the glass transition temperature (T_g) of the powder formulation [41]. Formulation A showed a T_g at 54°C (Fig. 8), which was comparable to the value of 63°C for a PEV20 powder formulation containing 70% lactose and 30% leucine [42]. The difference was possibly caused by the decreased lactose content (33%) in formulation A. No T_g was

observed for formulation B. The exothermic peak at 198 °C represents crystallization of amorphous ciprofloxacin, and this is in agreement with a previous study [39]. The moisture contents measured by TGA were $2.0 \pm 0.4\%$ and $2.7 \pm 1.0\%$ for formulations A and B, respectively.

Previous results showed that the effect of temperature and humidity on phage stability in powders are both phage- and formulation-dependent. Trehalose and L-leucine were able to maintain the viability of phage PEV2 with no significant titre reduction when stored at 4°C under vacuum for a year. However, at 20°C the formulation containing 40% leucine reduced $0.9 \log_{10}$ pfu/mL after 1 year storage [43]. Lactose provide better phage PEV20 protection over trehalose in the spray drying process [21]. Spray dried PEV20 powder containing lactose and L-leucine remained stable for 1 year at 20°C when stored inside an aluminium pouch – heat-sealed at <15% RH [21]. Lactose with leucine also provided biological and physical stability for spray dried PEV phage powders at ambient temperature [42]. Although lactose is a reducing sugar and potentially react with proteins, our data to date showed lactose does not impact the biological activity of the phage. These findings may also be applicable to our current phage PEV20 and ciprofloxacin formulations. Hence, stability studies of the powder formulations at different storage conditions are warranted.

Conclusion

Two inhalable, co-spray dried phage PEV20 and ciprofloxacin combination dry powder formulations showed strong synergistic antimicrobial effect on two clinical MDR *P. aeruginosa* strains. Dispersion of the two formulations using a low-resistance Osmohaler™ (connected to an NGI operating at 100 L/min) resulted in an FPF of 60–64%, while a high-resistance Osmohaler™ produced FPF of 71–73% (MSLI at 60 L/min). The antimicrobial synergy was maintained after powder dispersion for both formulations using the two inhalers. Both formulations were characterized as partially crystalline and the powders recrystallized at >40% RH. Our work confirm the feasibility of preparing inhalable and biochemically stable combination formulations of phage PEV20 and ciprofloxacin for treatment of respiratory infection caused by MDR *P. aeruginosa*. The best combination powder formulations for different phages and antibiotics necessitate further investigations.

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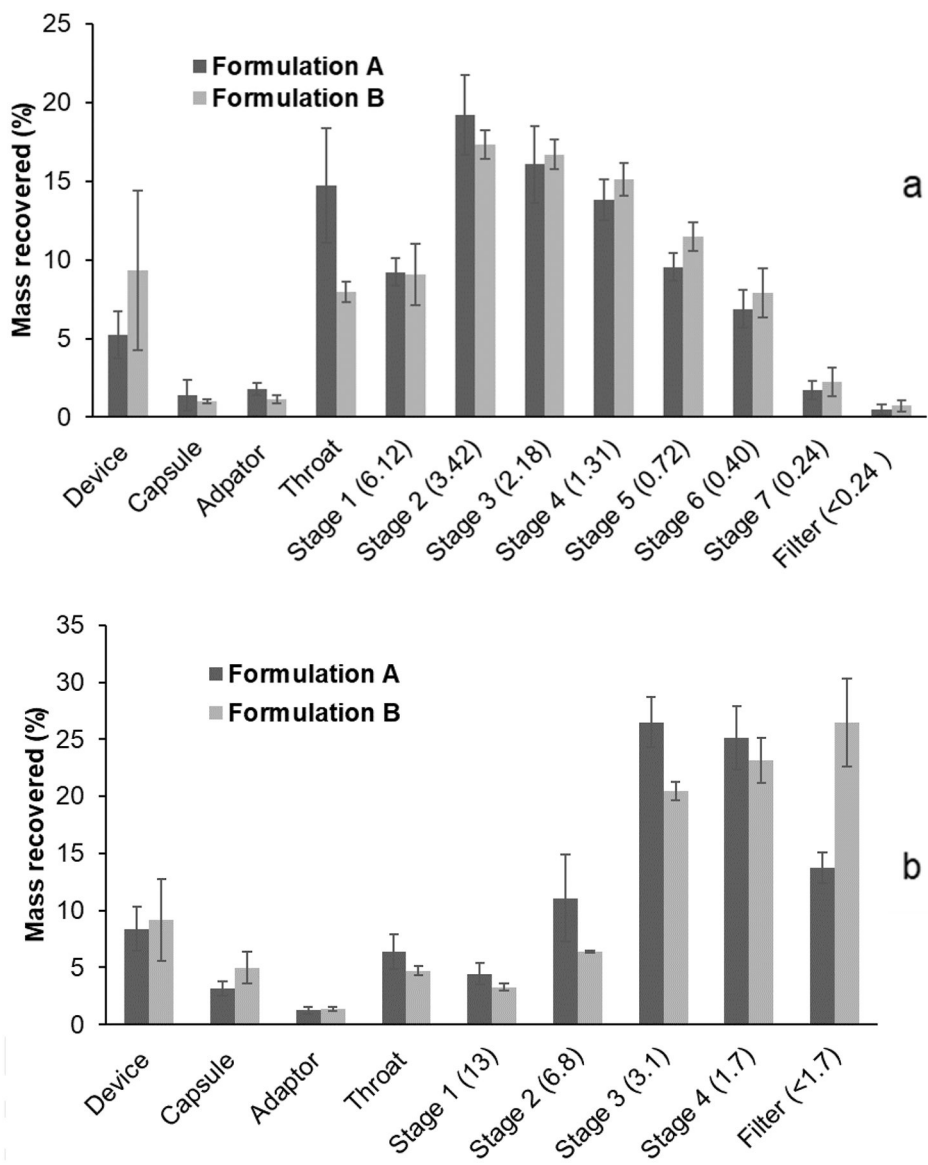


Fig. 1. The NGI (a) and MSLI (b) deposition profiles of formulations A and B (n=3). The aerodynamic cut-off diameter (μm) of each stage is quoted in parentheses. Powders were dispersed in NGI at 100 L/min for 2.4 s using low resistance Osmohaler™ and in MSLI at 60 L/min for 4 s using high resistance Osmohaler™.

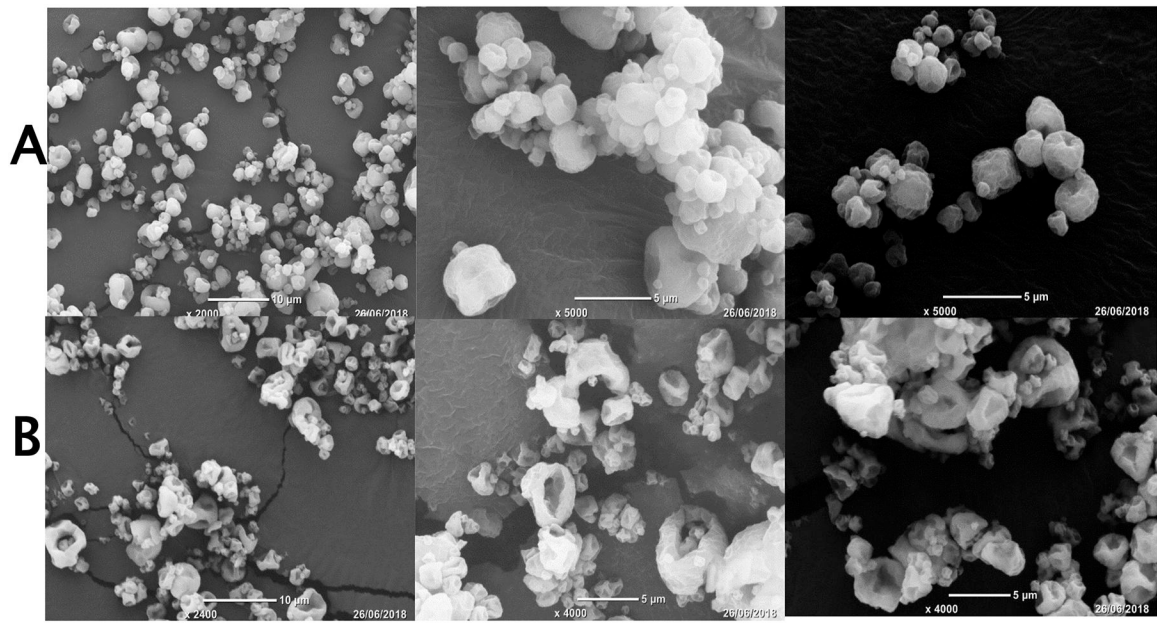


Fig. 2.
SEM images of formulations A and B.

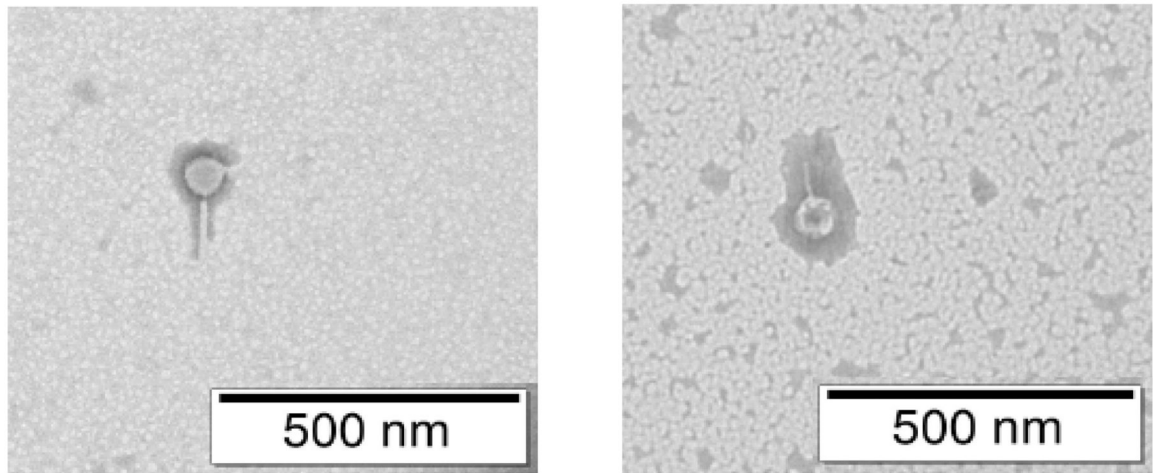
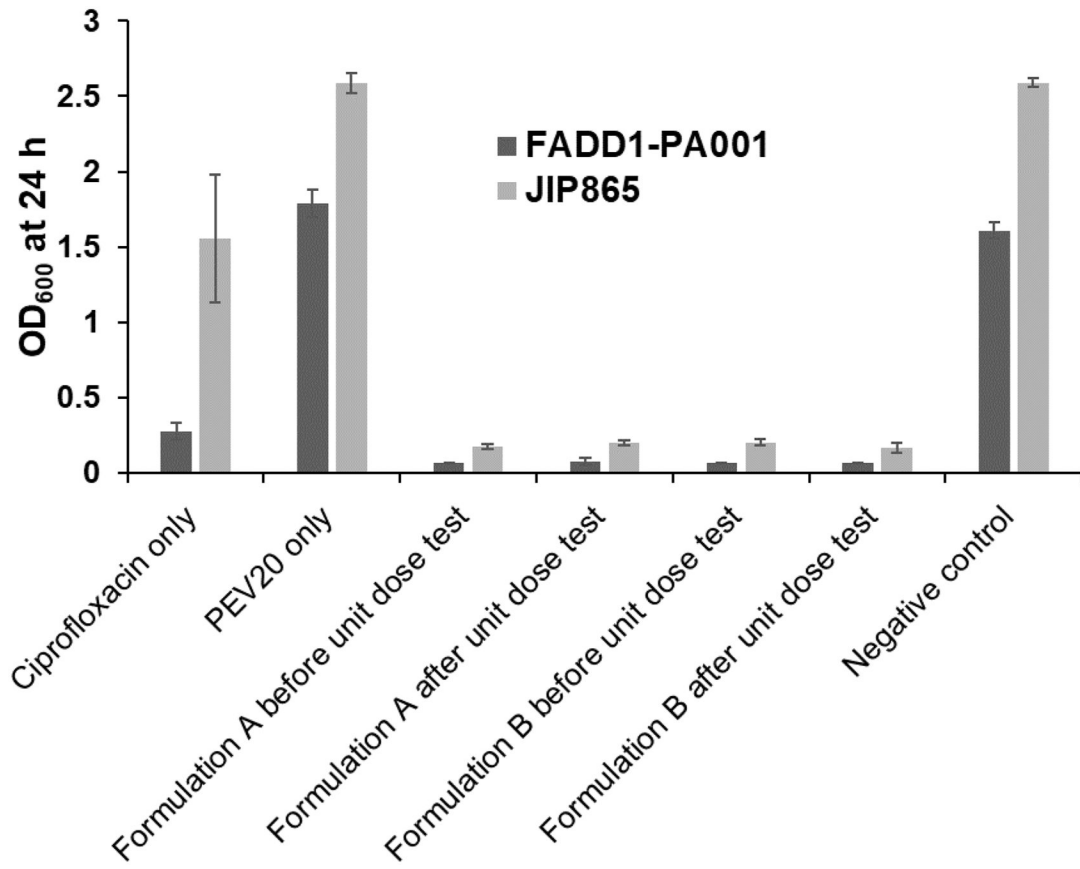


Fig. 3.
TEM images of phage PEV20 before and after spray drying.



a

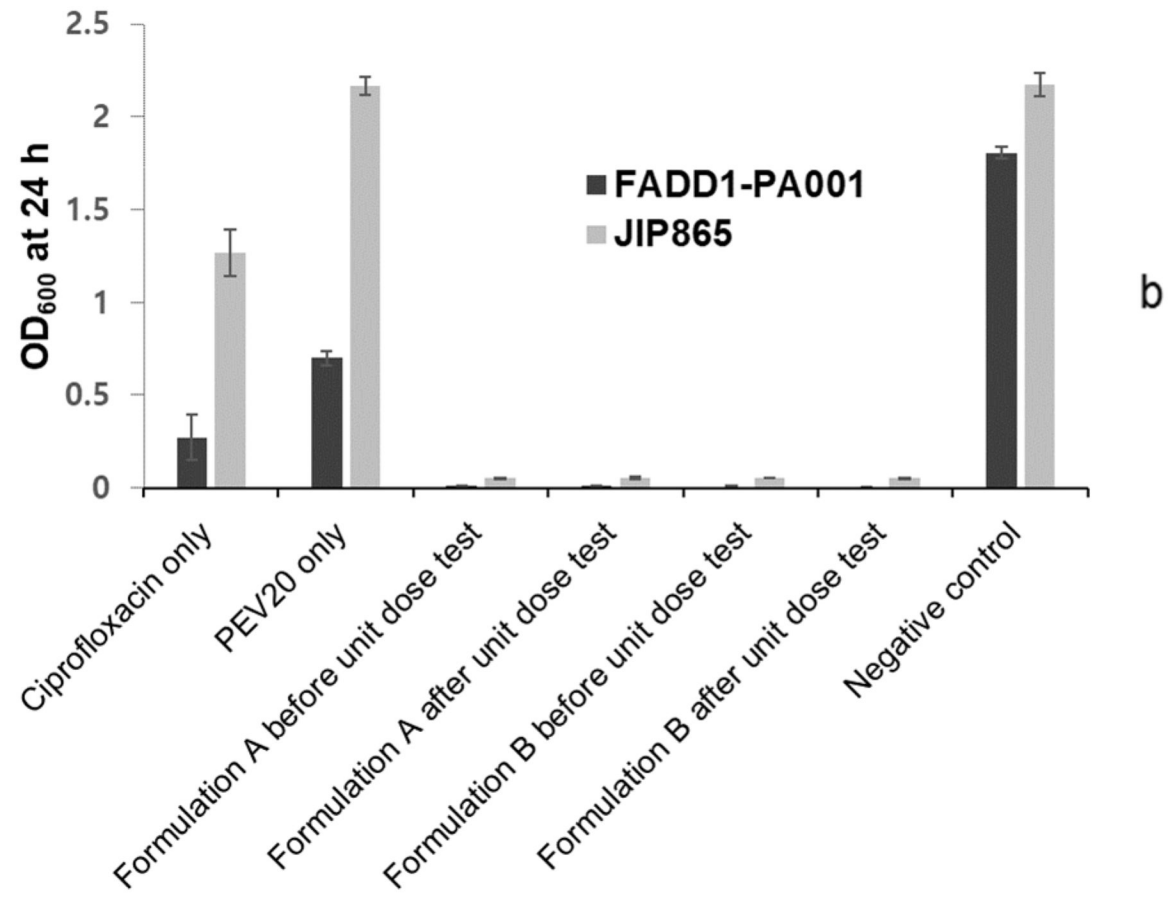


Fig. 4.

Antibacterial activities of ciprofloxacin (80 $\mu\text{g}/\text{mL}$ for strain FADD1-PA001 and 40 $\mu\text{g}/\text{mL}$ for JIP865), PEV20 (10^5 PFU/mL for strain FADD1-PA001 and 10^6 PFU/mL for JIP865), and spray dried combinations before and after unit dose test using low (a) and high (b) resistance Osmohaler™ (n=6).

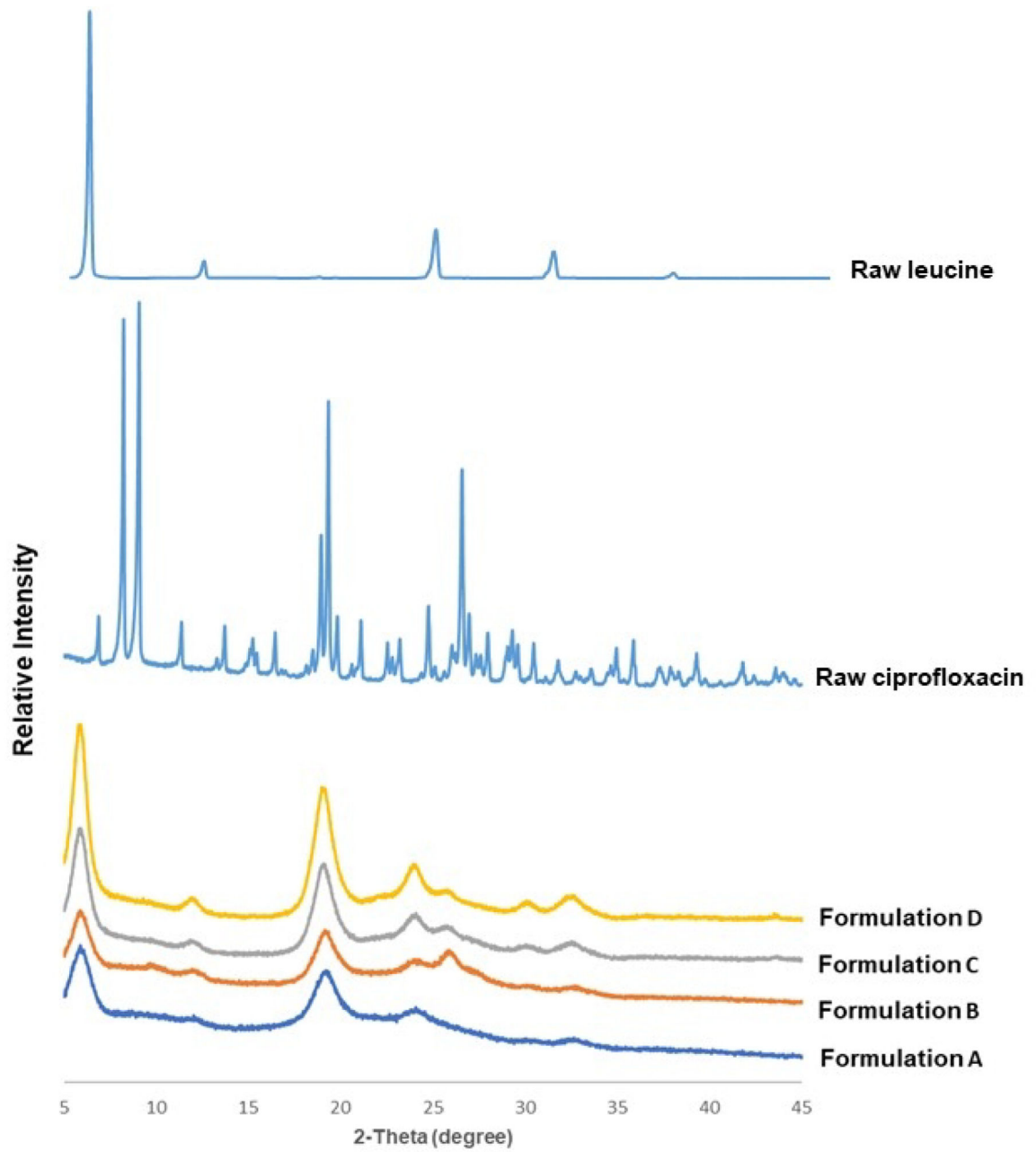


Fig. 5.
X-ray powder diffraction patterns of formulations A, B, C, D, raw leucine and ciprofloxacin

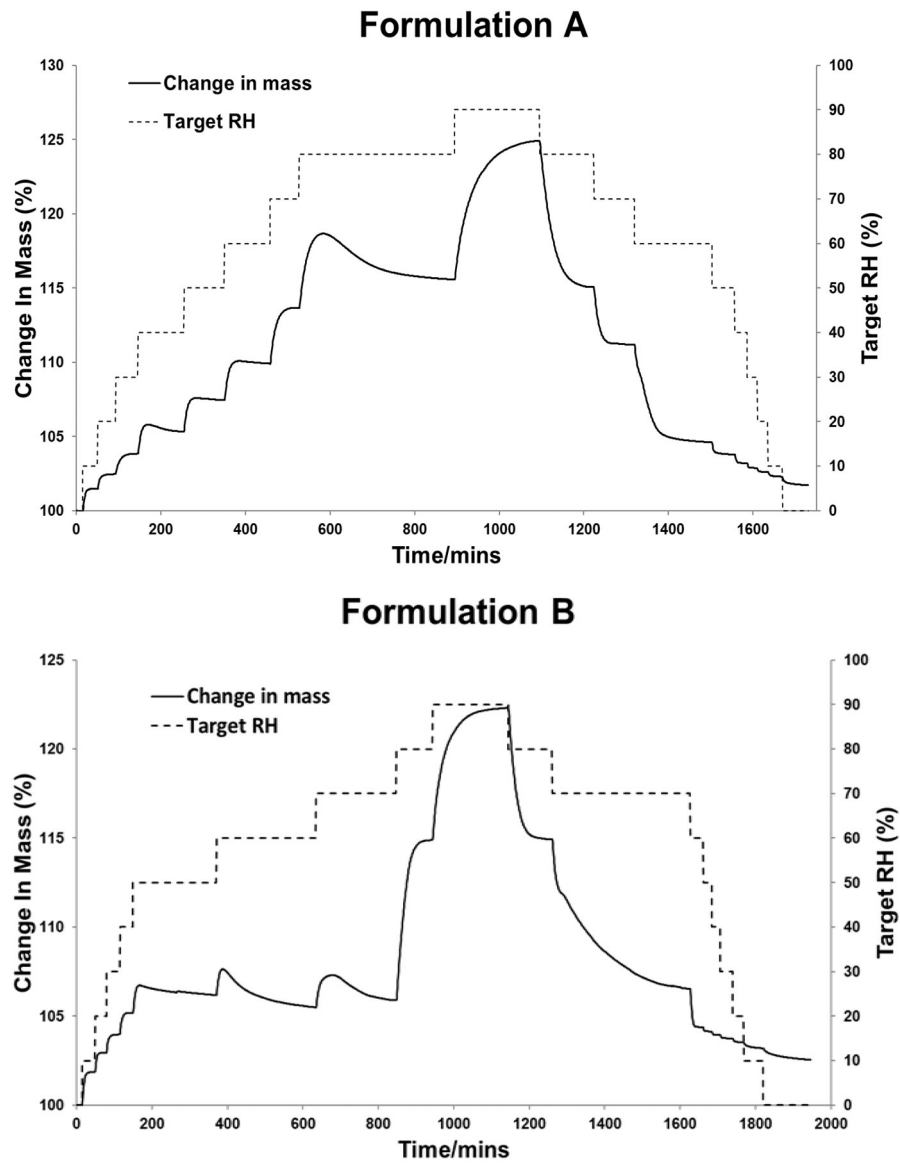


Fig. 6. Moisture sorption kinetic profiles of formulations A and B.

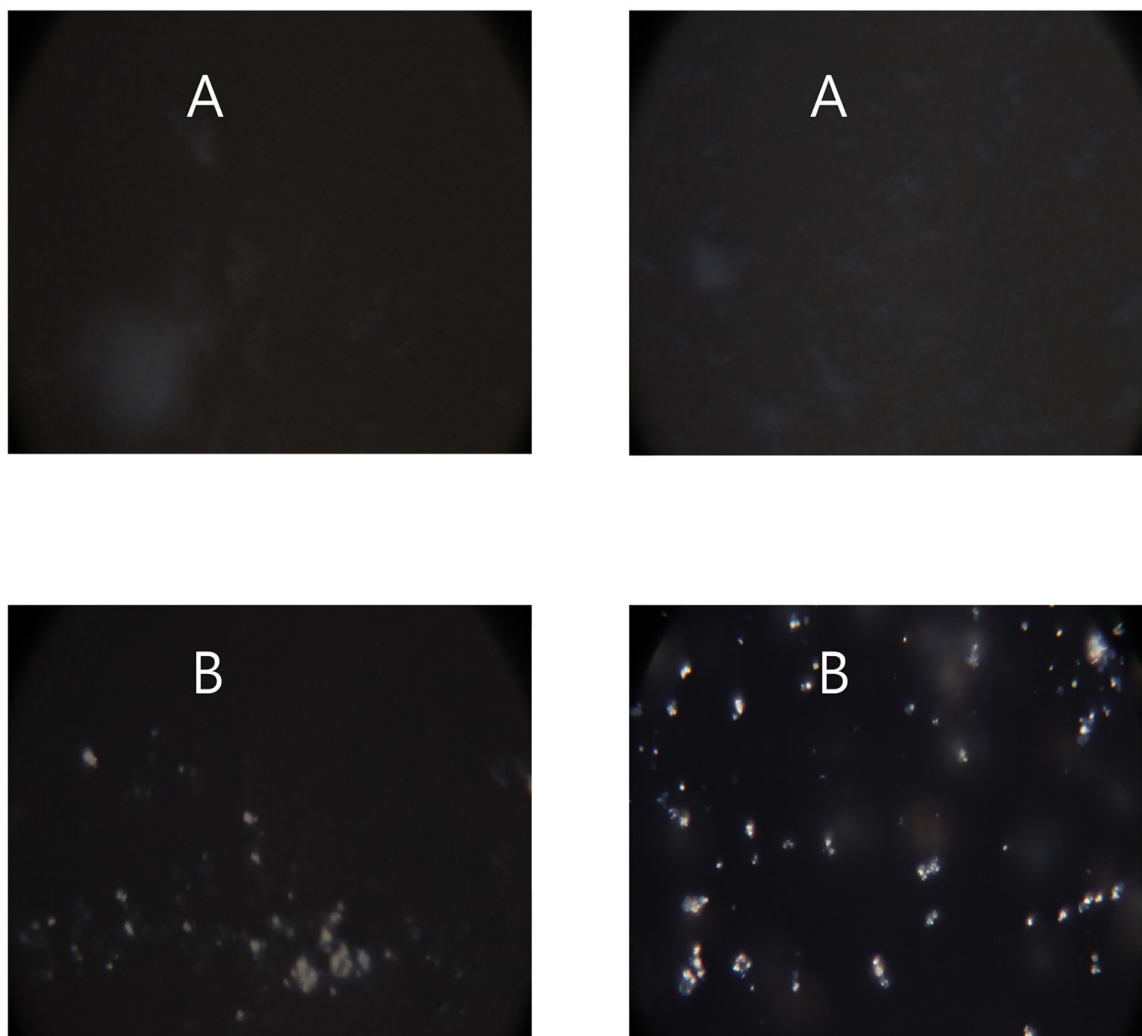


Fig. 7. Polarized light microscope images of formulation A and B before and after exposure to moisture from 0–60% RH

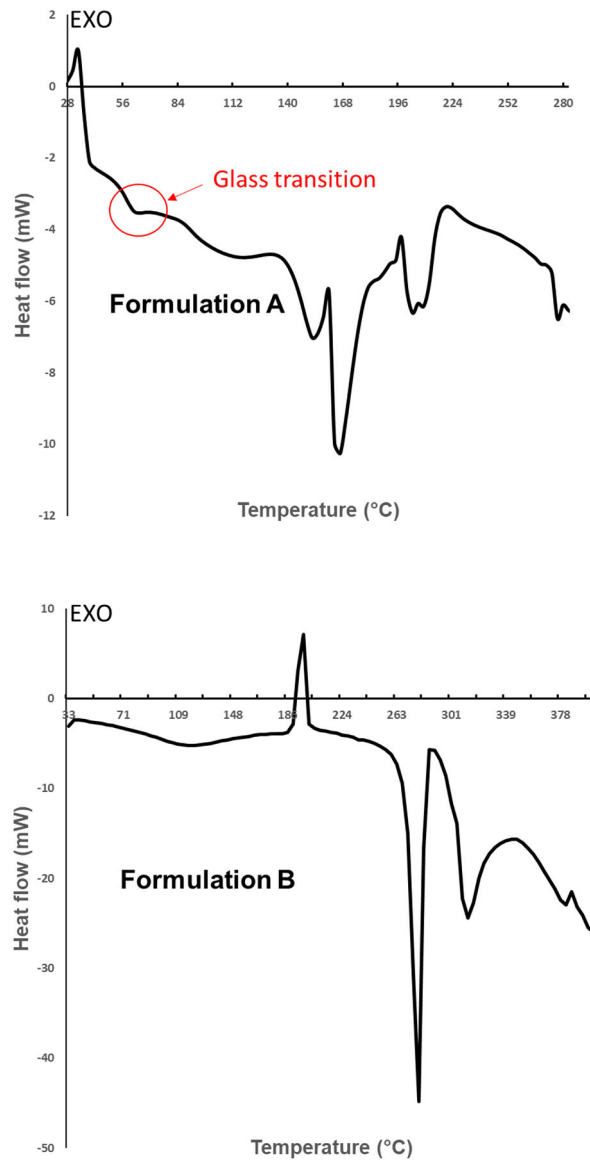


Fig. 8.
DSC thermograms of formulations A and B.

Table 1.

Mass concentration of each excipient in 18 mL water

Formulation	Concentration (mg/mL)		
	Ciprofloxacin	Lactose	L-leucine
A	8	8	8
B	16		8
C	12		12
D	8		16

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Table 2.

FPF of formulations A and B dispersed in NGI at 100 L/min for 2.4 s or MSLI at 60 L/min for 4 s, respectively using the low or high resistance Osmohaler™. Data presented as mean ± standard deviation (n=3).

	NGI		MSLI	
	A	B	A	B
Nominal ^a (mg)	8.1 ± 0.5	10.0 ± 0.3	7.7 ± 1.2	8.8 ± 0.8
Recovered ^b (mg)	7.6 ± 0.1	9.0 ± 0.6	7.5 ± 1.0	8.3 ± 0.4
Recovery %	94.5 ± 4.4	90.2 ± 7.0	97.4 ± 4.1	94.4 ± 4.4
FPD (mg)	4.5 ± 0.2	5.8 ± 0.4	5.3 ± 0.5	6.1 ± 0.6
FPF% (Nominal)	56.5 ± 4.5	58.0 ± 3.7	69.3 ± 5.5	68.3 ± 3.8
FPF% (Recovered)	59.7 ± 2.1	64.3 ± 2.9	71.0 ± 3.4	73.3 ± 5.0
Emitted ^c (mg)	7.1 ± 0.2	8.1 ± 0.6	6.6 ± 0.9	7.1 ± 0.6
FPF% (Emitted)	64.0 ± 3.2	71.5 ± 1.4	80.3 ± 4.3	85.4 ± 1.1

^aNominal: Ciprofloxacin mass content in powder loaded in the capsule

^bRecovered: Ciprofloxacin mass recovered from capsule, inhaler, adaptor and NGI or MSLI

^cEmitted: Ciprofloxacin mass exited the capsule and inhaler

Table 3.

Primary particle size distribution of formulations A and B. Data presented as mean \pm standard deviation (n=3).

Formulation	D ₁₀ (μm)	D ₅₀ (μm)	D ₉₀ (μm)	Span
A	1.0 \pm 0.1	2.5 \pm 0.2	5.1 \pm 0.3	1.6 \pm 0.0
B	1.2 \pm 0.1	2.4 \pm 0.0	5.1 \pm 0.6	1.6 \pm 0.3

Table 4.

Calculated and observed bacterial survival rate of *P. aeruginosa* strains FADD1-PA001 or JIP 865 of co-spray dried PEV20-ciprofloxacin formulation A and B before and after unit dose test using low and high resistance Osmohaler™ (n=6).

Strains	Formulations	Unit dose test	Low resistance		High resistance	
			Calculated	Observed	Calculated	Observed
FADD1-PA001	A	Before	0.2±0.06	0.04±0.001 *	0.06±0.03	0.005±0.0008 *
		After		0.05±0.002 *		0.006±0.0003 *
	B	Before	0.6±0.2	0.04±0.002 *	0.6±0.08	0.004±0.0022 *
		After		0.04±0.002 *		0.0004±0.0002 *
JIP 865	A	Before	0.6±0.2	0.07±0.006 *	0.6±0.08	0.02±0.002 *
		After		0.08±0.007 *		0.02±0.005 *
	B	Before	0.6±0.2	0.08±0.009 *	0.6±0.08	0.02±0.001 *
		After		0.07±0.01 *		0.02±0.002 *

* Statistically significant (p<0.05) according to Student's *t*-test.