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Research paper

Ciprofloxacin nanocrystals liposomal powders for controlled drug release via inhalation

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Abstract

This study was conducted to evaluate the feasibility of developing inhalable dry powders of liposomal encapsulated ciprofloxacin nanocrystals (LECN) for controlled drug release. Dry powders of LECN were produced by freeze-thaw followed by spray drying. The formulations contained sucrose as a lyoprotectant in different weight ratios (0.75:1, 1:1 and 2:1 sucrose to lipids), along with 2 % magnesium stearate and 5 % isoleucine as aerosolization enhancers. The powder physical properties (particle size, morphology, crystallinity, moisture content), *in vitro* aerosolization performance, drug encapsulation efficiency and *in vitro* drug release were investigated. The spray dried powders were comprised of spherical particles with a median diameter of ~ 1 µm, partially crystalline, with a low water content (~2 % mass) and did not undergo recrystallization at high relative humidity. When dispersed by an Osmohaler® inhaler at 100 L/min, the powders showed a high aerosol performance with a fine particle fraction (% wt. < 5 µm) of 66-70 %. After reconstitution of the powders in saline, ciprofloxacin nanocrystals were confirmed by cryo-electron microscopy. The drug encapsulation efficiency of the reconstituted liposomes was 71-79 % compared with the stock liquid formulation. Of the three formulations, the one containing a sucrose to lipids wt. ratio of 2:1 demonstrated a prolonged release of ciprofloxacin from the liposomes. In conclusion, ciprofloxacin nanocrystal liposomal powders were prepared that were suitable for inhalation aerosol delivery and controlled drug release.

Keywords

Nanocrystals; Spray drying; ciprofloxacin; liposomes; controlled drug release

Abbreviations

CF, cystic fibrosis; CF BE, cystic fibrosis bronchiectasis; Cryo-TEM, cryogenic transmission electron microscopy; DLS, dynamic light scattering; DPI, dry powder inhaler; DVS, dynamic vapor sorption; ED, emitted dose; EE, encapsulation efficiency; FPF, fine particle fraction; FT, freeze-thawed; FTSD, freeze-thawed then -spray- dried; HBS, HEPES buffered saline; HPMC, hydroxypropyl methylcellulose; LC, loading capacity; LECN, liposomal encapsulated ciprofloxacin nanocrystals; MMAD, mass median aerodynamic diameter; NGI, next generation impactor; NTM, non-tuberculous mycobacteria; PXRD, X-ray powder diffraction; RH, relative humidity; SD, standard deviation; SEM, scanning electron microscopy; TEA, triethylamine

1. Introduction

Respiratory antibiotics delivery to treat lung infections has gained wide popularity in recent years, as it can provide high local concentrations at the site of infection with reduced systemic exposure to minimize side effects (Cipolla and Chan, 2013; Shetty et al., 2018). Approved inhaled antibiotics (e.g. tobramycin, aztreonam and polymyxin) were effective in reducing the decline in lung function and the rate of hospital admission of cystic fibrosis (CF) patients. However, there continues to be an unmet need in CF patients for more effective antibiotic therapy (Justo et al., 2013; Weers, 2015). To that end, ciprofloxacin has strong bactericidal effects against the biofilm forming *P. aeruginosa* but oral and IV adminsitration lead to low concentrations in the lung and concern over systemic side effects. Thus, there is an interest in developing inhaled formulations of ciprofloxacin (Antonela Antoniu, 2012; Cipolla et al., 2016a). Inhaled ciprofloxacin powder of 32.5 mg was adminstered twice-daily over 14 or 28 days to treat patients with non-CF bronchiectasis (BE) or chronic obstructive pulmonary disease (Stass et al., 2017; Wilson et al., 2011). Liposomal ciprofloxacin solutions of 100, 150 or 210 mg for inhalation with once-daily dosing exist as either a sustained-release formulation (100 or 150 mg) or a dual-release formulation (210 mg) combining both an immediate and sustained-release ciprofloxacin component. This combination formulation of free and liposomal ciprofloxacin was effective in reducing the colonization of chronic *P. aeruginosa* infections in non-CF BE patients (Bilton et al., 2011; Serisier et al., 2011). Liposomal formulations can be designed to provide a controlled or sustained release of the encapsulated drug and reduce the rate of systemic absorption thus prolonging the drug's residence time in the lung. This release profile will maintain high local antibiotic

concentrations in the lung (above the minimum inhibitory concentration), thus allowing for less frequent administrations (Cipolla et al., 2014a). In addition, antibiotic-loaded liposomes can be phagocytosed by macrophages, which enables treatment of intracellular infections such as those caused by non-tuberculous mycobacteria (NTM) (Blanchard et al., 2018).

Recently, using a simple freeze-thaw method, we prepared a new liquid formulation of liposomes containing ciprofloxacin nanocrystals which further modulate the release of the drug *in vitro* (Cipolla et al., 2016c). The solid state properties of these nanocrystals were further characterized using cryo-TEM, small angle X-ray scattering (SAXS) and cross-polarized light microscopy (CPLM) (Li et al., 2018). While the liposomes in this formulation retained substantial integrity on storage and following nebulization, liquid formulations of liposomes can undergo chemical degradation (e.g. oxidation and hydrolysis) as well as physical degradation (e.g. fusion, aggregation, drug leakage and conversion to micelles) during storage (Ingvarsson et al., 2011) and during nebulization liposomes are exposed to shear and air-liquid interface, so their integrity could be affected leading to drug leakage and increased vesicle size (Cipolla et al., 2013; Niven and Schreier, 1990; Taylor et al., 1990). In contrast, a powder formulation may improve the product shelf life and eliminate the need for 'cold-chain' storage/distribution (and the associated costs). Furthermore, the powder formulation, if designed properly, can be delivered using a dry powder inhaler, many of which are small in size and portable, and a shorter administration time would likely enhance patient adherence to treatment in comparison with nebulization delivery (Cipolla et al., 2013; Willis et al., 2012). However, the conversion of a liquid liposomal formulation into a stable dry powder formulation which retains its integrity upon reconstitution is a challenge; the loss of water by either evaporation (e.g., spray drying) or sublimation (e.g., lyophilization) can destabilize the liposomes (Ingvarsson et al., 2011).

Dry powders of liposomes can be prepared by spray drying technology which converts a liquid dispersion or suspension of liposomes into dry powder form by atomizing the liquid into a hot air stream, followed by collection of the dry, micron-sized particles in a cyclone (Cipolla et al., 2013). Stabilizing liposomes during spray drying is a major hurdle to overcome, as the process imposes physical stresses on the liposomes including heat, shear force, and dehydration. Although liposomes have been spray dried successfully (Goldbach et al., 1993; Ingvarsson et al., 2011; Kim, 2001; Misra et al., 2009; Patel et al., 2009; Skalko-Basnet et al., 2000), these were not liposomes containing nanocrystals. Transforming a nanosuspension into nano-composite powders suitable for inhalation delivery and controlled release of the drug requires maintenance of the integrity of both the nanocrystals and

liposomes during the process. In the present study, we aim to study the feasibility of converting ciprofloxacin nanocrystals-containing liposomes into a stable dry powder by spray drying.

2. Materials and methods

2.1. Materials

Aqueous formulations of liposomes encapsulating 50 mg mL^{-1} ciprofloxacin HCl and empty liposomes, both in a pH 6.0 histidine buffer, were produced by Exelead (Indianapolis, IN, USA and Northern Lipids Incorporated (Burnaby, BC, Canada) and used as received from Aradigm Corporation (Hayward, CA, USA). Sucrose, sodium chloride, triethylamine (TEA), magnesium stearate, isoleucine, and adult donor bovine serum were all from Sigma-Aldrich (Castle Hill, New South Wales, Australia). All the chemicals were analytical grade except methanol which was HPLC grade. Deionized water was acquired from Modulab Type II Deionization System (Continental Water System, Sydney, Australia). Other materials included Nanosep Omega centrifugal filtration devices, 10k molecular weight (Pall Australia Pty Ltd, Victoria, Australia) and HEPES, free acid (Dojindo, China). An Osmohaler® inhaler was obtained from Pharmaxis Ltd. (Pharmaxis Ltd, Frenches Forest, Australia) and hydroxypropyl methylcellulose (HPMC) capsules of size 3 were from Capsugel (Capsugel, West Ryde, Australia).

2.2. *Preparation of liposomal ciprofloxacin nanocrystals powder formulations*

2.2.1. Preparation of liposomal ciprofloxacin formulations

The lipid composition and the preparation method of ciprofloxacin encapsulated within liposomes were reported previously (Ong et al., 2014; Webb et al., 1998). Briefly, hydrogenated soy phosphatidylcholine and cholesterol in a 7:3 ratio (by weight) were used to produce multilamellar liposomes, which were extruded through a membranes to get unilamellar liposomes followed by solvent removal via diafiltration. The empty liposomes were then mixed with ciprofloxacin for active loading of the drug inside liposomes. Excess ciprofloxacin was removed by diafiltration and > 99% was encapsulated at a concentration of 50 mg mL^{-1}.

2.2.2. *Freeze-thaw of aqueous formulation of liposomes to create nanocrystals*

Ciprofloxacin nanocrystals inside liposomes were produced following the freeze-thaw method reported previously (Cipolla et al., 2016c). Sucrose was used as both cryoprotectant during freeze-thaw and lyoprotectant in the subsequent spray drying step (Chougule et al., 2008; Lo et al., 2004). Ciprofloxacin liposomes at 50 mg mL^{-1} were diluted four-fold with 2 mL of 37.5, 50 or 100 mg mL^{-1} sucrose solution and 1 mL of deionized water. These suspensions were frozen in a 20 mL glass container dipped inside liquid nitrogen for 2 min, and then allowed to thaw in a water bath at room temperature and vortexed to assure homogeneity prior to subsequent spray drying.

2.2.3. Spray drying of aqueous liposomal formulation encapsulating nanocrystals

After the production of ciprofloxacin nanocrystals inside liposomes by a freeze-thaw step, the resulting aqueous suspensions were spray dried using magnesium (Mg) stearate and isoleucine as moisture protectants and dispersibility enhancers (Chan and Chew, 2003; Yu et al., 2017). A spray drying feed suspension (3 mg/mL total solutes) containing one of the three mass ratios of sucrose to lipids (0.75:1, 1:1, and 2:1), 2 % w/w Mg stearate and 5 % isoleucine was pumped with continuous stirring into the spray dryer (B-290 mini spray-dryer, Büchi Falwil, Switzerland) at a feed rate of 1.4 mL/min. The spray dryer was operated under the following conditions: inlet air temperature 50 °C and outlet air temperature 33-35 °C, atomizer setting 742 L/h, aspirator 35 m^3/h. The spray dried powders were collected under controlled humidity (<15 %) at ambient temperature (~ 23°C), then stored inside a dry container over phosphorous pentoxide and protected from light until further characterization.

2.3. Cryogenic transmission electron microscopy (cryo-TEM)

Liposomal ciprofloxacin formulations before and after freeze-thaw were visualized using cryo-TEM to confirm the presence of drug nanocrystals. The same technique was used to observe if the nanocrystals were preserved after spray drying. A JEOL 2100 instrument (JEOL, Japan) operated at 200 kV or a Talos Arctica transmission electron microscope (Thermo Fisher Scientific, USA) operated at 200 kV was used. The control sample (stock, unprocessed liposomal ciprofloxacin) and the freeze-thawed samples were diluted 10 times with water, while the dry powder formulations were reconstituted in water to make the concentration of ciprofloxacin around 1 mg mL^{-1}. Each sample was then applied in a 3 µL aliquot to a glow discharged Lacey formvar/carbon grid (Proschitech, Australia) in a chamber

controlled at 22 °C and 85 % RH. Grid blotting was performed once using filter paper at a blot force of -1 for 1 s, then the grids were plunged into liquid ethane using a Vitrobot (Thermo Fisher Scientific, USA) or a Leica EM GP device (Lecia Microsystem, Germany) to vitrify the samples. The vitrified samples were kept in liquid nitrogen until used for cryo-TEM analysis.

2.4. Dynamic light scattering (DLS)

Liposomes of either the aqueous or dry powder formulations were diluted or reconstituted with saline to yield a ciprofloxacin concentration of ~ 0.1 mg mL^{-1} and analyzed for particle size distribution using a Malvern Zetasizer Nano ZS (Malvern, UK) with the following instrument parameter settings: temperature 23 °C; viscosity 0.887 cP; refractive index 1.34; intensity set point 300 kHz; channel width 10 µs; scattering angle 90°; run time 5 min; mode vesicle and Gaussian distribution. The mean and SD of the liposome size distribution were recorded for three observations of three different batches.

2.5. Assessment of drug encapsulation

As reported previously, the free drug can be separated from the encapsulated drug by using Nanosep Omega centrifugation devices (Pall Australia Pty Ltd, Victoria, Australia) with modified polyethersulfone membrane filters of 10,000 molecular weight cut-offs (Cipolla et al., 2014b). Dry powder formulations were reconstituted with a volume of saline so that the ciprofloxacin concentration would be near 1 mg mL^{-1} (10 mg powder was dissolved in 5 mL saline) and 400 µL was then transferred to the centrifugation device and centrifuged for 18 min at 10,000 rpm (8100 g). The free ciprofloxacin was quantified using HPLC after diluting the filtrate 20 times with distilled water. To measure the total amount of ciprofloxacin, including both the encapsulated and free drug, 1 mL of the reconstituted powder was diluted with 9 mL of 80% methanol to solubilize the liposomes, and then centrifuged for 15 min at 13,400 rpm. The filtrate was diluted 4-fold with deionized water and analyzed by HPLC for ciprofloxacin content. By comparing the free drug content to the total amount of drug, the percent encapsulation was established.

2.6. Particle size and distribution

The volume mean diameter was measured using a Malvern Mastersizer 2000® laser diffractometer combined with a dry sampling accessory (Scirocco 2000, Malvern Instruments, Malvern UK). The real and imaginary refractive indices (RI) for the spray dried

powder were set at 1.40 and 0.10, respectively. These values of RI were viewed to be sufficient as indicated by the low residual values (<1.50%) for all measurements. A low residual value implies that the optical model using the chosen RI values fits well to the measurement data when calculating the particle size distribution. To achieve optimal de-agglomeration of a powder into its primary particles without causing fragmentation of the particles, pressure titration was performed. As the pressure increased from 0.5 to 3.5 bar, D_{50} decreased and then plateaued off until 4 bar. Hence, 4 bar was subsequently used for all the size measurements. A 100 % vibration feed rate and 12 sec measurement time were chosen to give sufficient signals above background. D_{10}, D_{50}, and D_{90} (i.e. the diameter at 10%, 50% and 90% undersize, respectively) and span (i.e. the difference between D_{10} and D_{90} divided by D_{50}) were obtained from the size distribution results. The mean and SD of the size distribution (D_{10}, D_{50} and D_{90}) and the span were reported for three observations of different batches.

2.7. Moisture content

The residual moisture content of the spray dried powders was determined by thermogravimetric analysis (TG/SDTA 851e, Mettler-Toledo, Germany). About 5-10 mg of each dry powder formulation was loaded into an alumina pan and heated from 30 to 150 °C at a heating rate of 10 °C/min under a nitrogen atmosphere, and the percent drop in mass observed between 30 to 100 °C was used to represent water content. The data were reported as mean and SD (n=3).

2.8. Particle morphology

Particle morphology information of the powder formulations were obtained by scanning electron microscopy (Carl Zeiss SMT AG, Oberkochen, Germany). Each powder was distributed on an SEM stub and sputter coated with gold (15 nm thick) using a K550X sputter coater (Quorum Emitech, Kent, UK). Images were captured using 5 kV on the SEM.

2.9. Powder crystallinity

Powder X-ray diffraction (PXRD) (Shimadzu XRD-6000, Shimadzu Corporation, Kyoto, Japan) was applied to measure powder crystallinity after spray drying, with the following instrument parameters used: 40 kV Cu-Ka radiation, current of 30 mA, 2θ range of 5–50° and scan speed of 2°/min. The dry powders examined in the PXRD were prepared by spray drying aqueous suspensions of ciprofloxacin nanocrystals encapsulating liposomes or

empty liposomes, as well as aqueous solutions of drug alone or of each individual excipient (sucrose or isoleucine).

2.10. Dynamic water vapor sorption (DVS)

The moisture sorption behaviour of the spray dried powders was examined using a dynamic vapor sorption system (DVS-1, Surface Measurement Systems Ltd., London, UK). Approximately 10-20 mg of powder was loaded in the sample pan and placed in the equipment chamber under continuous nitrogen gas flow at 25 °C. Relative humidity (RH) inside the chamber was controlled from 0 to 90 % with 10 % increment or decrement steps for both the sorption and desorption cycles. A sample weight change per time (dm/dt) below 0.002% per minute was set as the criterion for moisture equilibrium of the sample.

2.11. In vitro aerosol performance

A next generation impactor (NGI, Copley, Nottingham, UK) coupled to a mouthpiece adapter and a USP throat (dry USP induction port) was applied to evaluate the aerosol performance of the powder formulations. A powder sample of 30 ± 1 mg was loaded in a size 3 HPMC capsule and dispersed through an Osmohaler® inhaler device under ambient conditions. Four liters of air were drawn through the inhaler at 100 L min^{-1} for 2.4 s, with a pressure drop of about 4 kPa across the device (Tiddens et al., 2006). The Osmohaler is essentially the same as the Aerolizer®. It has been reported that an air flow of 105 L/min can be generated by a patient with compromised lung functions using a comfortable inspiratory effort of 40 cm H_2O (i.e., 4 kPa) (Chew and Chan, 2001). After dispersion, powders deposited on the capsule, inhaler, adapter, throat, and stages 1–8 of the NGI were carefully washed with 80% methanol, and the solution samples were collected for HPLC analysis. The total recovered mass is the total mass of the powder deposited on the capsule, inhaler, adapter, throat, and stages 1–8 of the NGI. The emitted dose (ED) was calculated as the percent mass of powder exiting the capsule and device relative to the total recovered mass. The percent mass of powder deposited on each stage of the impactor relative to the total recovered mass was determined. The fine particle fraction (FPF) was calculated as the percent of the total recovered powder mass with a particle size ≤ 5 μm. The fine particle dose (FPD) was the mass of ciprofloxacin collected from the stages with a particle size ≤ 5 μm. Mass median aerodynamic diameter (MMAD) is the particle size below which 50% of the particle population lies on the basis of mass. FPD and MMAD were obtained from plots of the cumulative fraction or mass of ciprofloxacin versus the cut-off diameter of NGI stages,

according to the British Pharmacopeia 2018 (Appendix XII C. Consistency of Formulated Preparations).

2.12. In vitro assay of ciprofloxacin release from liposomes

The measurement of the ciprofloxacin release from the liposomes was undertaken according to a previously validated and reported method (Cipolla et al., 2014b). For comparison, control and freeze-thawed formulations were tested as well as the reconstituted dry powder of spray-dried liposomal formulations. All the aqueous suspensions were diluted with HEPES buffered saline (HBS: 20 mM HEPES, 145 mM NaCl, pH 7.4) to make the ciprofloxacin concentration around 50 µg mL^{-1}. Afterward, the specimens were mixed with an equal volume of chilled (2–8°C) adult donor bovine serum (Sigma-Aldrich, Castle Hill, New South Wales, Australia). Samples which represent time point zero were withdrawn prior to placement of the specimens in a shaking water bath (Labec J-SWB60, Marrickville, Australia) at 37°C and 150 rpm. After incubation for 30, 60, 120, 240, 480 and 720 min, samples were removed at each time point and placed in the ice-water bath; then a one-to-one dilution with chilled (2–8°C) HBS was performed to prevent any further release of ciprofloxacin from the liposomes. A Nanosep centrifugal device was used to separate the released ciprofloxacin from the encapsulated drug; the samples were transferred in 400 µL aliquots to the centrifuge and centrifuged at 10,000 rpm for 18 min. Using HPLC, the released ciprofloxacin in the filtrate was quantified. The resulting values were normalized using 0.93 as a correction factor to compensate for the free drug loss in the filter due to the presence of serum. The ciprofloxacin content was quantified using the method described in Section 2.5. By comparing the released amount of drug to the total drug content, the percent release was calculated. Some of the formulations contained free drug at T0min, so a normalized percentage released was calculated by measuring the release, T30min-T0min, divided by the total possible release, 100-T0min, and converting to a percentage: 100* (T30-T0)/ (100-T0) (Cipolla et al., 2016b). This normalized percentage was used to calculate the similarity factor (f2) and difference factor (f1) for comparative reasons as described in Section 2.14.

2.13. High-performance liquid chromatography (HPLC) for ciprofloxacin assay

The amount of ciprofloxacin was quantified using an HPLC method as reported previously (Cipolla et al., 2014c). In general, HPLC analysis was performed using a Phenosphere-Next C-18 column (5 mm, 4.6 × 150 mm, Phenomenex, USA) at 35 °C. The

mobile phase was a mixture of 0.5 % TEA 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^{-1}. Ciprofloxacin was detected and quantified at a wavelength of 277 nm.

2.14. Statistical analysis

Results are given as means ± SD (n=3). One-way analysis of variance (ANOVA) and Tukey's multiple comparisons test were applied for testing statistical differences. If the probability values are less than 0.05, the statistical differences are considered significant. Difference factor (f1) and similarity factor (f2) analyses were used to compare the *in vitro* release profiles. They are recommended by the FDA for modified release solid oral dosage forms (FDA, 1997) with f2 values greater than 50 indicating similarity and f1 values less than 15 indicating no difference (Riley et al., 2012). As long as the similarity factor analysis was in complete agreement with the difference factor analysis, the similarity factor values were the only values that were reported. Thus, if the f2 values indicated similarity ($f2 > 50$), then the f1 values indicated no difference ($f1 < 15$).

3. Results

3.1. Liposomal morphology by cryo-TEM

The control sample contained uniform spherical liposomes of approximately 90 nm in size (Fig. 1. A). After freeze-thaw, the liposomes contained elongated rod-shaped structures within the vesicles, due to the formation of ciprofloxacin nanocrystals (Fig. 1. B and C), in agreement to previous reports (Cipolla et al., 2016c). After spray drying of the freeze-thawed samples, the same structures were observed in the cryo-TEM micrographs of all formulations (Fig. 1. D, E and F). Most of the ciprofloxacin nanocrystals were between 100 and 200 nm in length. All nanocrystals resided inside intact liposomes. Those samples (Figure 1, D) containing a lower amount of lyoprotectant (0.75:1 sucrose: lipids) showed a few larger liposomes compared to the sample (Figure 1, F) with a higher amount of lyoprotectant (2:1 sucrose: lipids), which implies the importance of sucrose as a protectant during both the freeze-thaw step and spray drying process.

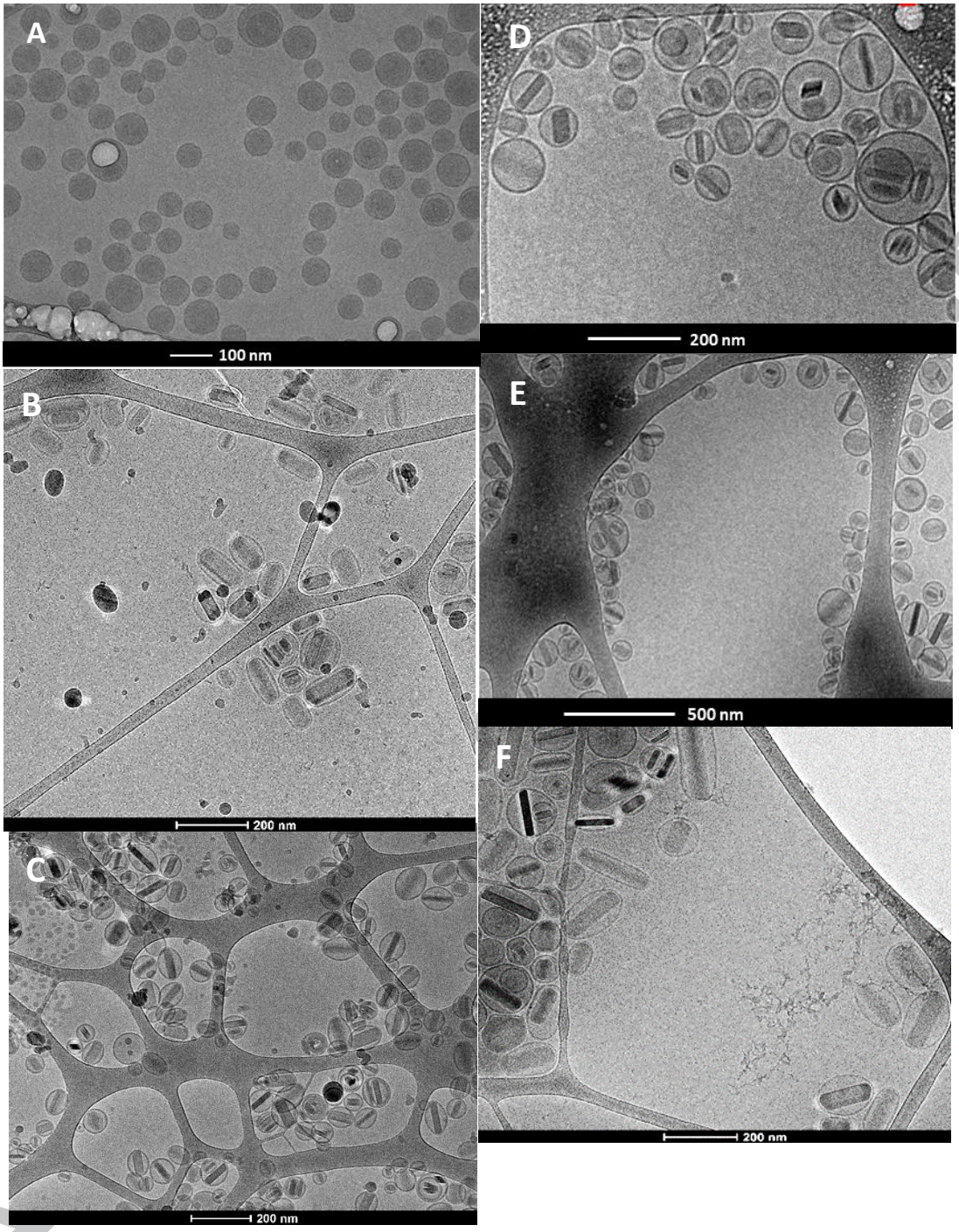

Fig. 1. Cryo-TEM images of liposomes containing ciprofloxacin. (A) Control; (B and C) after freeze-thaw of formulations containing different mass ratios of sucrose to lipids: 1:1 and 2:1, respectively; (D, E and F) after freeze-thaw then spray drying of formulations containing different mass ratios of sucrose to lipids: 0.75:1, 1:1 and 2:1, respectively. The scale bar in the bottom of the micrographs (A) is 100 nm, (B, C, D and F) is 200 nm and (E) is 500 nm.

3.2. Liposomal particle size by DLS

Liposomes containing ciprofloxacin before freeze-thaw had a mean particle diameter of around 100 nm (Table 1). The size increased after freeze-thaw and a further increment was observed after spray drying. In general, the increases in both particle size and polydispersity of the liposomes were dependant on the amount of sucrose. The greatest increase in size (Δ=106.8 nm) was evident for the liposomes containing the lowest sucrose concentration, while the formulation with the highest sucrose concentration showed the highest stability with the mean size of the liposomes increasing by only 26.7 nm.

3.3. Assessment of drug encapsulation

The encapsulation efficiency (EE) for spray dried liposomes was 71-79 % (Table 2). The highest drop of 9 % occurred in the sample with the lowest sucrose content. The other samples containing greater sucrose content retained 78 and 79 % of the encapsulated drug.

Table 1
Size distribution of liposomes by dynamic light scattering for original, freeze-thawed (FT) and freeze-thawed then spray dried samples (FTSD). Mean [SD], n=3.

Sample type (sucrose: lipid) (w/w)	Particle size (nm)	Polydispersity index
Control (0:1)	104.6 [3.4]	0.1 [0.04]
FT (0.75:1)	122.3 [3.3]	0.2 [0.04]
(1:1)	111.3 [4.5]	0.2 [0.02]
FTSD (0.75:1)	211.4 [3.1]	0.5 [0.02]
(1:1)	146.4 [7.6]	0.2 [0.04]
(2:1)	131.3 [7.3]	0.2 [0.06]

3.4. Moisture content

The residual moisture content of the spray dried powder formulations was around 2 % wt. (Table 2). Although the three formulations contain different levels of sucrose, no significant difference was observed in their residual moisture content.

Table 2
Encapsulation efficiency (EE) and moisture content of ciprofloxacin nanocrystals liposomal dry powders. Mean [SD], n=3.

Sucrose: lipid (w/w)	EE (%)	Moisture Content (wt. %)
0.75:1	70.9 [3.7]	1.6 [0.1]
1:1	79.4 [1.0]	2.1 [0.6]
2:1	78.2 [1.2]	2.2 [0.5]

3.5. Particle size distribution

All the formulations contained respirable particles with a narrow size distribution (Table 3), with the D90 being less than 3 µm, which was similar to the previously reported spray-dried liposomal formulations intended for inhalation (Lo et al., 2004; Tang et al., 2015).

Table 3
Particle size distribution of ciprofloxacin nanocrystals liposomal dry powders. Mean [SD], n=3.

Sucrose: lipid (w/w)	D_{10} (µm)	D_{50} (µm)	D_{90} (µm)	Span
0.75:1	0.54 [0.09]	1.14 [0.08]	2.34 [0.22]	1.58 [0.27]
1:1	0.569 [0.04]	1.27 [0.06]	2.66 [0.36]	1.64 [0.24]
2:1	0.40 [0.01]	0.87 [0.02]	1.92 [0.04]	1.74 [0.03]

3.6. Particle morphology

Most of the particles in all the formulations were generally less than 3 µm in size, with none above 6 µm (Fig. 2). However, the shape of the particles changed from corrugated spheres with dimples (Fig. 2. A and B) to spheres with a smooth surface for the formulation containing the highest amount of sucrose (Fig. 2. C).

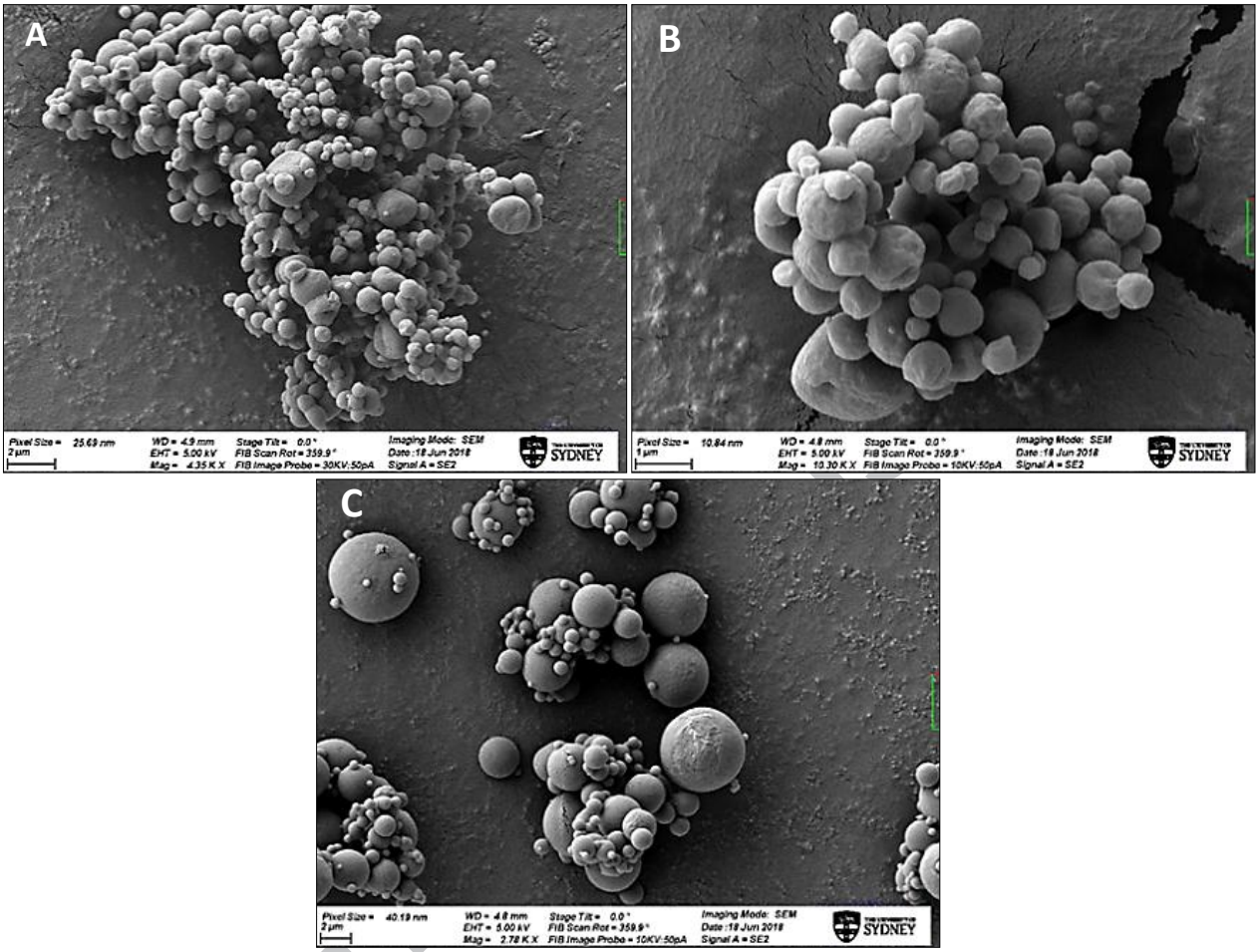

Fig. 2. SEM micrographs of ciprofloxacin nanocrystals liposomal dry powders containing different mass ratios of sucrose to lipids: (A) 0.75:1; (B) 1:1; (C) 2:1.

3.7. Powder crystallinity

The X-ray diffraction patterns of the liposomal powder formulations indicated a partially crystalline state (Fig. 3). The peak at 2θ around 21° matches the overlapping peaks of both Mg stearate and spray dried empty liposomes; since the mass percent of Mg stearate in the powder is only 2 %, the main contributor will more likely be the liposomes. The diffraction peak at around 26° is readily attributed to the spray dried ciprofloxacin. Powders with sucrose to lipid mass ratios 0.75:1 and 1:1 showed more peaks and peaks with higher intensities than for the powder with a 2:1 ratio, which is due to the lower contribution of the amorphous sucrose in those dry powders.

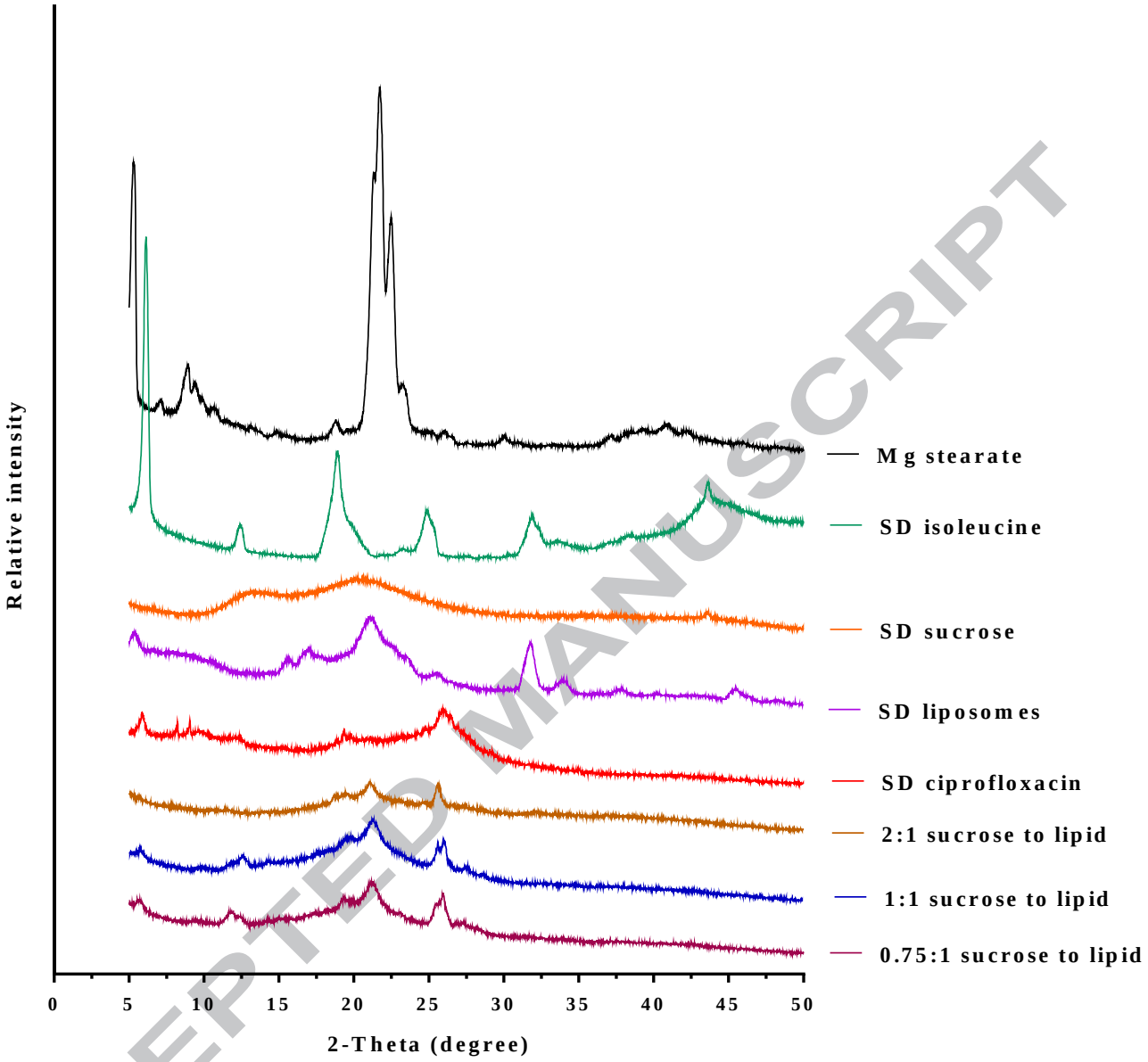

Fig. 3. X-ray powder diffraction patterns of ciprofloxacin nanocrystals liposomal dry powders containing different sucrose to lipids mass ratios (0.75:1, 1:1, 2:1); spray dried raw materials (isoleucine, sucrose, empty liposomes, ciprofloxacin) and Mg stearate included for comparison.

3.8. Dynamic water sorption (DVS)

The dynamic water sorption behaviour showed a gradual increase in mass with increasing relative humidity in all formulations (Fig. 4). The maximum mass increase was 48 % at 90 % RH, which was observed with the powder containing the highest sucrose amount (Fig. 4). All formulations exhibited a similar reversible moisture sorption trend, and most importantly, with no moisture-induced recrystallization.

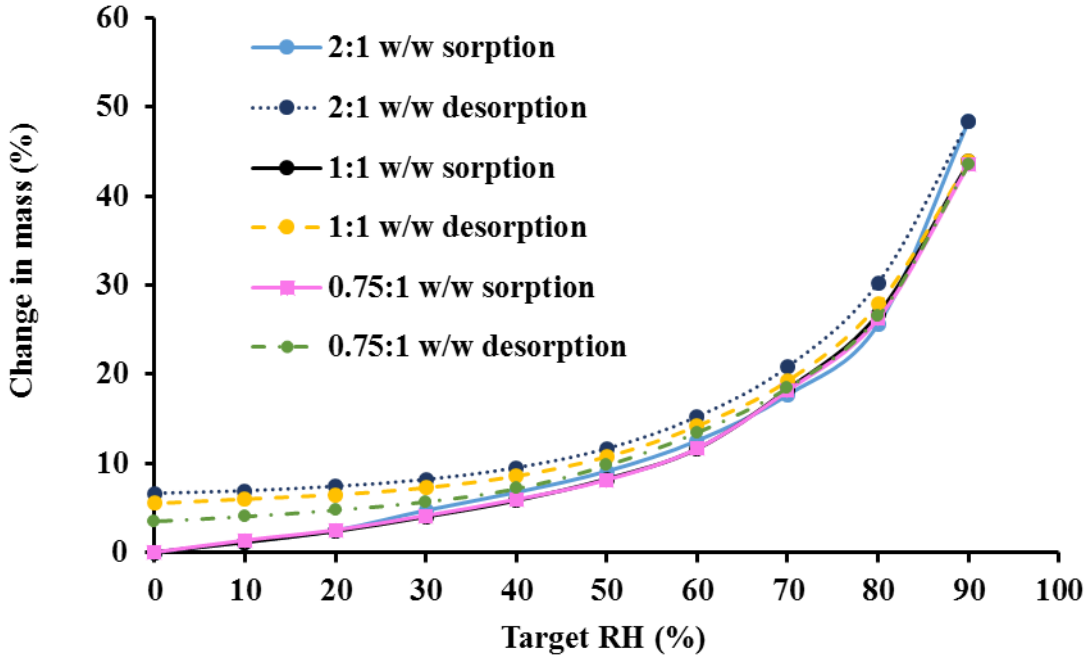

Fig. 4. Dynamic water sorption behaviour of ciprofloxacin nanocrystals liposomal dry powders containing different mass ratios of sucrose to lipid: 0.75:1, 1:1 and 2:1.

3.9. In vitro aerosol performance

The fine particle fraction (FPF %) and emitted dose (ED %) of all formulations using the Osmohaler inhaler were very promising, ranging from 65.8 – 69.7 % and 86.7 – 93.5 %, respectively (Table 4). The fine particle dose (FPD) of ciprofloxacin ranged from 2.9 to 4.7 mg from one 30 mg capsule of each of the three different formulations. The mass median aerodynamic diameter (MMAD) of the formulations ranged between 2.3 and 2.7 µm, showing a high potential to reach the lower airways and alveoli. Despite the varying sucrose content, there were no significant differences ($P > 0.05$) in the aerosol performance between the three different formulations (Fig. 5).

Table 4
Aerosol performance of ciprofloxacin nanocrystals liposomal dry powders. Mean [SD], n=3.

Sucrose: lipid (w/w)	ED[a]%	MMAD[b] (µm)	FPF[c]%	FPD[d] (mg)
0.75:1	89.9 [2.43]	2.61 [0.14]	66.9 [2.85]	4.67 [0.29]
1:1	93.5 [1.14]	2.66 [0.05]	65.8 [1.15]	4.03 [0.15]
2:1	86.7 [1.54]	2.30 [0.21]	69.7 [2.89]	2.93 [0.05]

ED[a] : Emitted dose. FPF[c]: Fine particle fraction.

FPD[d] : Fine particle dose. MMAD[b]: Mass median aerodynamic diameter.

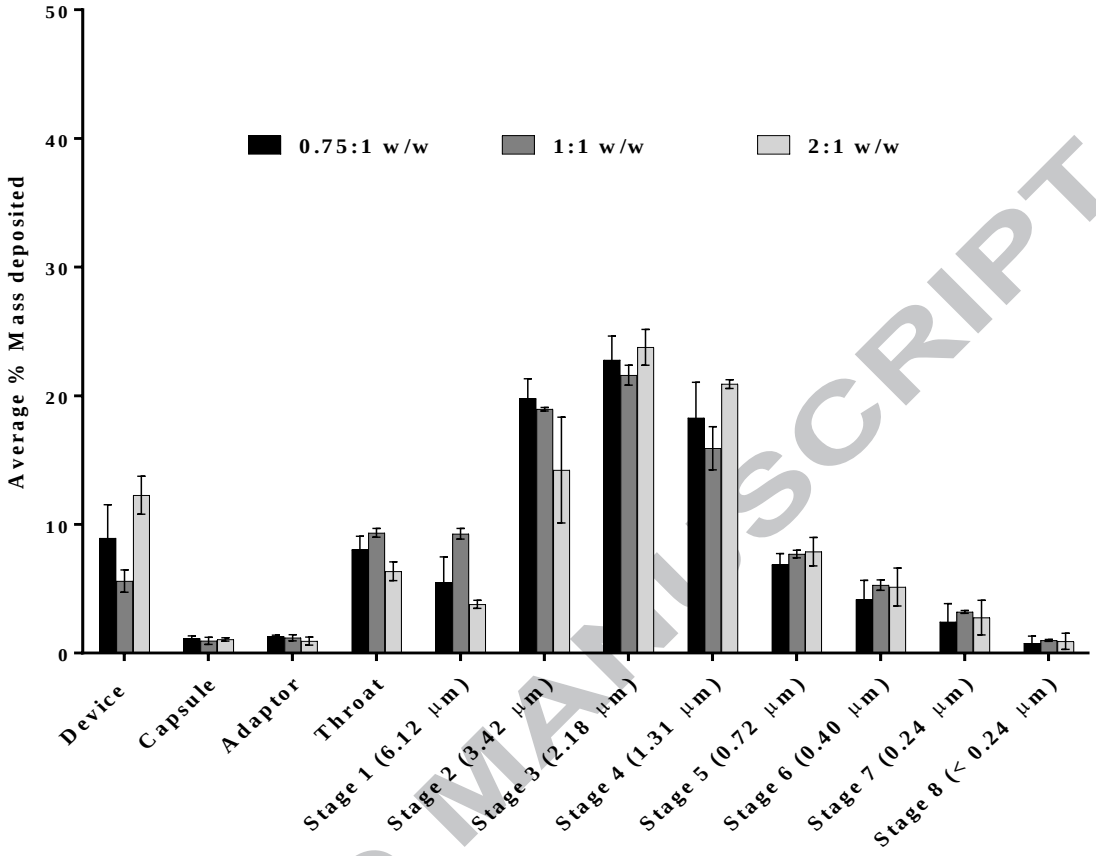

Fig. 5. The aerosolization performance of ciprofloxacin nanocrystals liposomal dry powders containing different mass ratios of sucrose to lipid: 0.75:1, 1:1 and 2:1. Mean with SD, n=3.

3.10. In vitro release of ciprofloxacin from liposomes

Fig. 6 shows the drug release profiles of all formulations. The freeze-thawed liquid formulations had the slowest release profile, with around 47-51% of the drug released in the first hour as reported previously (Cipolla et al., 2016c). Dry powder formulations with 0.75:1 and 1:1 w/w sucrose to lipid ratios released 87 % and 83 % of the drug in the first hour, respectively, suggesting instability of the liposomes in the powders. In contrast, the powder formulation with a 2:1 w/w sucrose to lipid ratio showed a comparable release rate to the control liquid formulation in the first hour, and then the release became slower over the next seven hours until reaching a plateau of nearly complete release at the 12 hour time point. Similarity analysis (f2) was performed to produce quantitative values for the apparent differences in release rates (Table 5); the dry powder formulation with the highest sucrose amount showed a similarity factor of 38, much less than the critical value of 50, confirming that there is a real difference between the release profile of this formulation and the control

liquid formulation. On the other hand, the similarity factor for the same formulation was around 50 when compared with the freeze-thawed liquid formulation indicating a possible similarity between the two release profiles. The release profiles of the other two dry powder formulations (0.75:1 and 1:1 w/w sucrose to lipids) were similar (f2 >50) to the control release profile. This result is due to the poor stability of the spray dried liposomes in the presence of an insufficient amount of sucrose, so they were incapable of further delaying the release rate compared to the control liquid formulation.

Table 5
Similarity Factor Analysis (f2) for powder formulations containing liposomes of ciprofloxacin nanocrystals with different sucrose amount versus liquid formulations containing liposomes with ciprofloxacin nanocrystals or without nanocrystals (control).

Test (sucrose: lipid) (w/w)	Reference (sucrose: lipid) (w/w)		
	Control (0:1)	FT (1:1)	FT (2:1)
FTSD (0.75:1)	50	-	-
(1:1)	53	33	-
(2:1)	38	-	50

4. Discussion

The retention of ciprofloxacin nanocrystals inside intact liposomes after spray drying to produce an inhalable powder is a novel outcome, which has never been reported previously. In this study, we discovered that ciprofloxacin nanocrystals which were formed by freeze-thaw were preserved intact within the liposomal vesicles after spray drying; however, the stability of the liposomes during both processes (freeze-thaw and spray dry) was dependent on the amount of lyoprotectant in the formulation. An important indicator of the stability of a liposome preparation after spray drying is the retention of its particle size following rehydration; the mean vesicle size increased in all of the dry powder formulations, but this increment was the lowest for the one with the highest sucrose content. The change in the particle size of the rehydrated liposomes was attributed to freezing, spraying and drying stresses: freezing introduces a mechanical stress because of ice crystal formation, accompanied by an osmotic stress due to a steep increase in the concentration of the solutes. Consequently, liposomal particles may come into closer contact with each other, enhancing the chance for liposomes to rearrange into larger structures, as observed in the literature for the thawed pegylated liposomes (Stark et al., 2010).

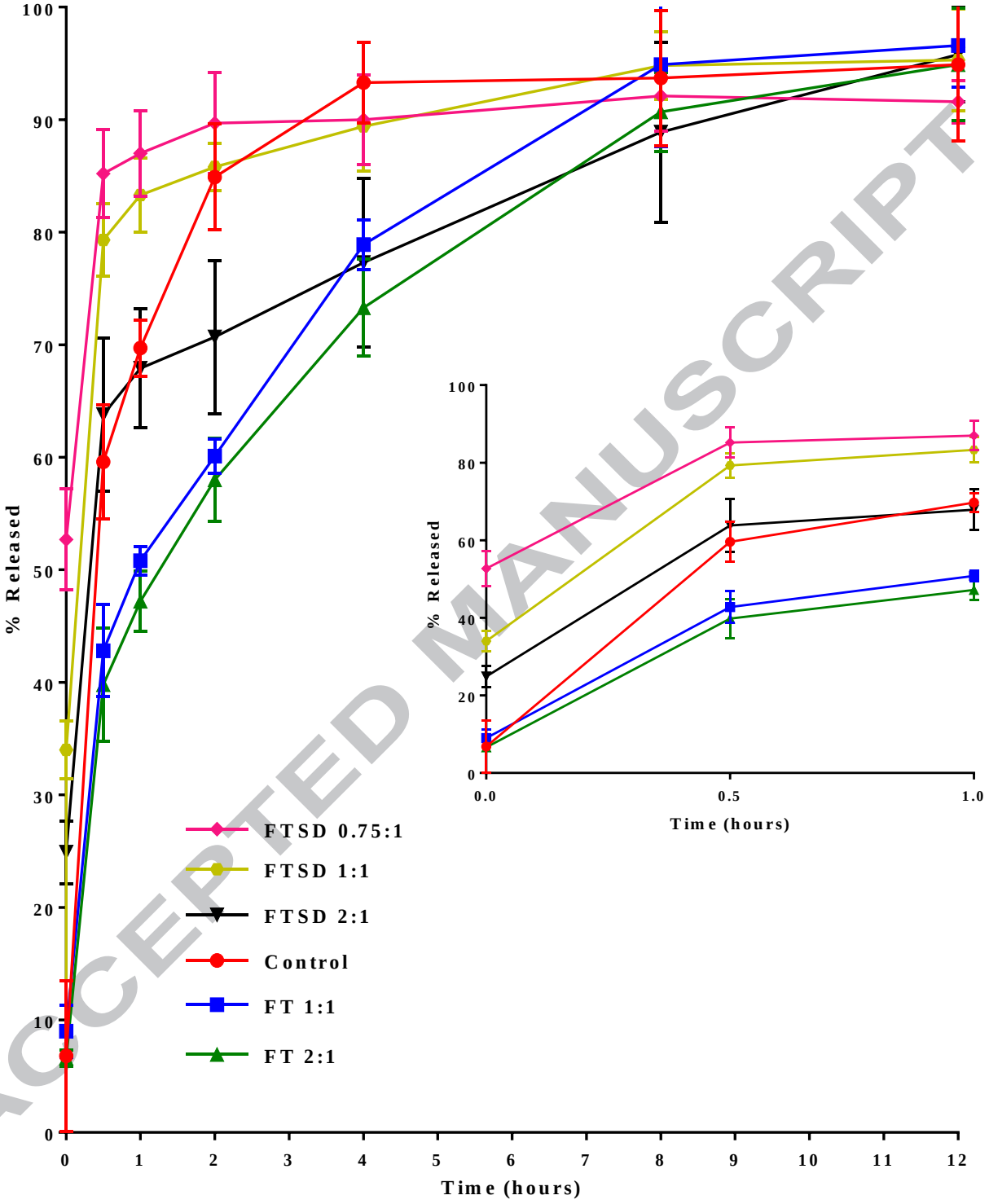

Fig. 6. *In vitro* release profiles of freeze-thawed then spray dried (FTSD) liposomal formulations containing different mass ratios of sucrose to lipid (0.75:1, 1:1 and 2:1) in comparison to freeze-thawed (FT) and non-freeze-thawed (control) liquid formulations. Mean with SD, n=3 (the small graph to magnify the drug release during the first hour).

Likewise, spray drying introduces additional mechanical stress to the liposomes as the liquid is being atomized, and the drying causes an osmotic stress. These extra stresses cause the large size change in the liposomes upon rehydration of the powders, but the deleterious effect can be minimized by using sucrose as a protectant (Wessman et al., 2010). Furthermore, stable liposomes will retain most of the encapsulated drug nanocrystals; the encapsulation efficiency values suggest that ~20 % of the drug leaked out during both freeze-thaw and spray drying processes if sufficient amount of sucrose as a lyoprotectant was utilized. Although atomization can disturb the membrane integrity of liposomes, the presence of sucrose will likely preserve the bilayer integrity during the spray drying process (Hauser and Strauss, 1987). As a consequence, the two formulations with the higher sucrose amount retained most of the entrapped ciprofloxacin nanocrystals.

The physical characterization of the dry powders demonstrated a small particle size, which is attributed to the low total solid concentration (~3 mg mL^{-1}) used in the spray dried suspensions. Small particle size is a requirement in powders intended for inhalation as well as a low moisture content to prevent particle agglomeration. The TGA results showed a low moisture content in all of the spray dried formulations. High residual moisture can impact the shelf life stability of powders. The amorphous nature of spray dried compounds, particularly those containing sugars, weakens the powder physical stability (Chan et al., 2004). The presence of water acts as a plasticizer to lower the glass transition temperature (Tg) and may induce recrystallization resulting in poor aerosol performance of the powder. A low moisture content will help maintain the Tg above the storage temperature to improve stability (White et al., 2005).

The dry powder morphology was dependent on the dominant component in the formulations. In the formulation containing the least sucrose content, liposomes represented the majority of the formulation mass and being surface active materials, they reduced the particle surface energy during drying. Additionally, the liposomes may have diffused more slowly from the droplet surface during spray drying, leading to the liposomes preferentially accumulating on the exterior of the powder particles and resulting in a wrinkled or corrugated appearance (Lo et al., 2004). Furthermore, isoleucine, a hydrophobic amino acid, would also tend to accumulate at the surface of the atomized droplets during spray drying, which may also contribute to the forming of corrugated surfaces on the dried particles (Yu et al., 2017).

The PXRD showed a partially crystalline powder, while the DVS revealed no sign of recrystallization on exposure to high relative humidity. These data demonstrate that the spray dried powder formulations of liposomes encapsulating ciprofloxacin are stable even when exposed to moisture. Sucrose functions as a lyoprotectant to stabilize liposomes in the dry state. However, spray dried sucrose alone is amorphous which is thermodynamically unstable leading to poor powder storage stability, particularly at elevated humidity. The presence of the hydrophobic excipients Mg stearate and isoleucine would protect the powders from moisture as these excipients tend to migrate to and dominate on the surface of the particles during spray drying (Chan and Chew, 2003; Li et al., 2016; Yu et al., 2017), protecting the sensitive sucrose amorphous regions during powder storage and extending the shelf life.

Dry powders encapsulating ciprofloxacin nanocrystals showed superior aerosol performance, with more than 50% of the particles having an aerodynamic diameter less than 5 µm. These results are consistent with previous reports showing better dispersibility of liposomes spray dried with sucrose than with other carriers like lactose and trehalose (Lo et al., 2004). Sucrose was the best cryoprotectant for liposomes encapsulating ciprofloxacin during freeze-thaw (Cipolla et al., 2016c). As a consequence, it is also preferred as a lyoprotectant as it would minimize the number of excipients and enhance the loading capacity of the drug in the powder formulation. The amount of sucrose used did not affect the aerosol performance of the dry powder, as no significant difference in the FPF values was observed between the three different spray dried formulations. Furthermore, the presence of Mg stearate and isoleucine were reported to have a significant positive influence on the powder dispersibility and flowability (Chan and Chew, 2003; Yu et al., 2017).

As reported previously, freeze-thaw of the liposome formulation created nanocrystals of the drug, which further slows the release of the drug from the liposomes as a result of the slow dissolution rate of ciprofloxacin nanocrystals inside a non-sink environment within the liposomal vesicle (Cipolla et al., 2016c; Fugit et al., 2015). In our study, we showed that nanocrystals were preserved in all three dry powder formulations; however, a prolonged release was observed only in the formulation containing the highest amount of sucrose. The in vitro release assay was designed to simulate body conditions and lung fluids; serum was used in this study as it contains lipoproteins and apolipoproteins which are also present in lung fluid. These components will affect the membrane integrity of the liposomes due to the exchange of phospholipid with high-density lipoprotein (Allen, 1981); as a consequence, drug release from liposomes will occur (Cipolla et al., 2014b). Based on the in vitro release

values, the presence of a sufficient amount of lyoprotectant protects the liposomal bilayers and ensures that the vesicles remain intact during nanocrystal formation, which introduces a slow dissolution rate and prolonged drug release.

One important caveat to note is that while the IVR assay utilizes a biological release media to simulate components found in the lung, it was not designed to actually model the timescale of drug release *in vivo* (Cipolla et al., 2014b). Instead, it was originally designed as a quality control tool that could be conducted over an 8-hour workday to enable comparison of the drug release profiles of various batches. The IVR assay has been validated and confirms that batches manufactured under identical process conditions possess similar IVR properties while batches that are known to be different possess different IVR properties; e.g., the presence of surfactant in the liposome membrane causes faster release (Cipolla et al., 2014b) while the presence of nanocrystalline drug causes slower release. In the IVR assay half of the encapsulated drug is released within an hour while in the in vivo setting the drug half-life in the lung is about ten hours (Antonela Antoniu, 2012; Cipolla et al., 2016a). Thus, while the nanocrystalline formulations produced from freeze-thaw or spray drying possess significantly slower release profiles *in vitro*, it is difficult to predict the prolongation in residence time in the lung without conducting *in vivo* experiments.

A once-daily dose of inhaled Apulmiq® (previously termed Linhaliq® and Pulmaquin® containing 30% free ciprofloxacin and 70% liposomal ciprofloxacin without nanocrystals) was well tolerated for the treatment of non-CF BE patients infected with *Pseudomonas aeruginosa* and resulted in a statistically significant decrease in PA colonization (Cipolla et al., 2015; Haworth et al., 2019; Serisier et al., 2013). However, patients with intracellular infections (e.g., NTM) may require a liposomal ciprofloxacin formulation with a delayed drug release to give time for the liposomes to be taken up by the macrophages, the site of the intracellular infections (Blanchard et al., 2018). Thus, the formulation of drug nanocrystals encapsulated within the liposomal vehicle has the potential to be a more effective treatment for those patients with intracellular infections in the lung.

An important consideration when treating infections is the relationship between the antibiotic concentration and the minimum inhibitory concentration (MIC) for the specific pathogen that is being targeted. Thus, we assessed whether this new dry powder formulation and delivery system would deliver an effective dose in a convenient number of administration events. Fluoroquinolone antibiotics, like aminoglycosides, are considered concentration-dependent antibiotics whose effectiveness is based upon the extent that the drug

concentration exceeds the MIC (Wright et al., 2000). The best predictor of antimicrobial effect is thus when the drug concentration exceeds the MIC by at least 10-fold (Wright et al., 2000). In an open label extension in the Phase 3 human clinical trials, the 210 mg nebulizer dose of Apulmiq yielded sputum concentrations of ciprofloxacin at its nadir (mean C_{min} ~170 µg/g sputum), just prior to the next administration event, that exceeded the MIC of *P. aeruginosa* (4 µg/g sputum) by a factor of ~45 (2018a). Assuming that the nebulizer had a lung delivery efficiency of ~10-20% (Cipolla et al., 2013), i.e., 21 to 42 mg, the minimum effective lung dose would be 4-8 mg of encapsulated ciprofloxacin to achieve drug concentrations exceeding the MIC of *P. aeruginosa* by at least 10-fold throughout the 24 hour dosing period. In the present study a 30 mg powder dose of the optimum sucrose formulation contains only 4.2 mg ciprofloxacin with approximately 78% representing encapsulated drug, comparable to Apulmiq. With a fine particle dose of ~2.9 mg from one capsule, the anticipated lung dose of encapsulated ciprofloxacin from two inhalations falls within the 4-8 mg requirement. Even if three or four doses were given once daily to provide further assurance of an effective lung dose, this would be a more convenient paradigm in comparison to the TOBI Podhaler which requires administration of 4 capsules twice daily to CF patients (VanDevanter and Geller, 2011).

For intracellular infections, e.g., those within the alveolar macrophages, a slower releasing nanocrystalline formulation may retain a greater dose of drug in the liposomes prior to being phagocytized. The local drug concentrations within the macrophages may be very high, exceeding the average drug concentration in sputum, but it is difficult to model. However, an inhaled liposomal formulation of amikacin (Arikayce®), recently approved in patients with refractory NTM infection, shows 5- to 8-fold greater uptake into macrophages than the free drug alone over 2, 6, and 24 hours post dose (Zhang et al., 2018), and resulted in ~30% conversion in a phase 3 clinical trial (2018b). Thus, a nanocrystalline formulation of liposomal ciprofloxacin may also prove beneficial in treating both biofilm and intracellular lung infections. In conclusion, the liposomal nanocrystal formulation of ciprofloxacin has been optimized in a dry powder inhaler format with comparable *in vitro* release characteristics to the nebulized solution formulation of a nanocrystalline liposomal ciprofloxacin produced from freeze-thaw and may represent a promising therapy for treating lung infections.

5. Conclusions

Dry powders of ciprofloxacin nanocrystal liposomal powders were successfully produced by freeze-thaw of liposomal liquid formulations followed by spray drying. Sucrose as a lyoprotectant stabilized both the liposomes and the encapsulated ciprofloxacin nanocrystals during the processing. These liposomal powders were highly respirable as well as physically stable upon moisture exposure. Prolonged drug release in an IVR assay was achieved for the spray dried formulation containing sucrose and lipids in a weight ratio of 2:1, with a slower release rate than the control liposomal formulation without nanocrystals. This formulation delivered in the Osmohaler produces an *in vitro* estimate of ciprofloxacin in the lung (based on the fine particle dose) that is designed to exceed the MIC by a factor of 10 over a 24-hour period, consistent with a once-daily treatment schedule.

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: