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Cipolla; Francis Dayton; James Blanchard; Hak Kim Chan

Abstract: The present study was conducted to harness spray drying
technology as a novel method of producing Ciprofloxacin nanocrystals
inside liposomes (CNL) for inhalation delivery. Liposomal ciprofloxacin
dispersions were spray dried with sucrose as a lyoprotectant in different
mass ratios (0.5:1, 1:1 and 2:1 sucrose to lipids), along with 2% w/w
magnesium stearate and 5% w/w isoleucine as aerosolization enhancers.
Spray drying conditions were: inlet air temperature 50 °C, outlet air
temperature 33-35 °C, atomizer rate 742 L/h and aspirator 35 m3/h. After
spray drying, the formation of ciprofloxacin nanocrystals inside the
liposomes was confirmed by cryo- transmission electron microscopy. The
physiochemical characteristics of the spray dried powder (particle size,
morphology, crystallinity, moisture content, drug encapsulation
efficiency (EE), in vitro aerosolization performance and drug release)
were determined. The EE of the liposomes was found to vary between 44 and
87% w/w as the sucrose content was increased from 25 to 57% w/w. The
powders contained partially crystalline particles with a volume median
diameter of ~ 1 μm. The powders had low water content (~2 % wt.) and were
stable at high relative humidity. Aerosol delivery using the Osmohaler®
inhaler at a flow rate of 100 L/min produced an aerosol fine particle
fraction (% wt. < 5 μm) of 58 - 64%. The formulation with the highest
sucrose content (2:1 w/w sucrose to lipid) demonstrated extended
ciprofloxacin release from liposomes (80% released within 7 hours) in
comparison to the original liquid formulation (80% released within 2
hours). In conclusion, a stable and inhalable CNL powder with controlled
drug release was successfully prepared by spray drying.

COMMENTS TO AUTHOR

Manuscript Number: IJP-D-19-02297R1

Article type: Research Paper

Title: Formation of ciprofloxacin nanocrystals within liposomes by spray drying for controlled release via inhalation

Authors : Isra Khatib; Patricia Tang; Juanfang Ruan; David Cipolla; Francis Dayton; James Blanchard; Hak Kim Chan*

We thank the Reviewers for the valuable time and efforts in providing constructive comments. We have now addressed all the comments and accordingly implemented changes to the manuscript.

The comments were divided into two parts:

Part 1 is a table that contains all the comments but with only short responses to some of them.

Part 2 comes after the table which contains a detailed answers to the rest of the comments.

Colour Codes:

Reviewers' Comments: Black

Responses to the comments: Green

Corrections and Text insertions in the manuscript: Red

❖ **Part 1:**

No.	Page no.	Line no.	Reviewers' comments	Responses
1	Abstract	1	Please remove first sentence from the abstract, as it is not required for the present study.	Removed
2	Abstract	7	Please include units of Magnesium stearate and isoleucine.	Added
3	Abstract	8	Please include optimized spray drying parameters used to prepare dry powder formulations.	Added
4	Abstract	10	Please correct to: "The **physicochemical** characteristics **of the spray dried powder** (particle size,…	Modified
5	Abstract	14	Please include the unit of EE – 44-87% w/w or w/v? Please include the range of sucrose amount.	Added

6	Abstract	19-20	Please include drug:sucrose ratio or sucrose amount for the optimized formulation or formulation with highest sucrose concentration.	Added
7	Graphical Abstract	-	What is the solid state of the drug ciprofloxacin in liquid state/solution? The core of the liposomal drug looks similar to the core of liposomal drug nanocrystals. Please provide correct representation of drug in the core of liposome for solution and legend in the drug release graph.	Modified
8	2	4	Please include recent relevant citations: (i) (2018). Excipients used in oral nanocarrier-based formulations. In Fundamentals of Nanoparticles (pp. 279-342). Elsevier.	Added
9	2	7	Please include recent relevant citations: (i) (2017). Effects of membrane PEGylation on entry and location of antifungal drug itraconazole and their pharmacological implications. Molecular pharmaceutics, 14(4), 1057-1070. (ii) (2019). Formulation and evaluation of itraconazole liposomes for Hedgehog pathway inhibition. Journal of Liposome Research, 1-7.	Added
10	3	3-6	Please correct to: "In general, liposomes **undergo physicochemical degradation: fusion; aggregation, drug leakage, and conversion to micelles, oxidation, hydrolysis of lipids, upon storage**."	Modified
11	3	11	Please correct: **…….** during storage **and/**or…… (**resulting in robust and economical drug product**).	Modified
12	3	17	Please include recent relevant citations: (i) (2017). Pharmaceutical amorphous nanoparticles. Journal of pharmaceutical sciences, 106(1), 39-65. (ii) (2018). Nanoamorphous drug products– Design and development. International Journal of Pharmaceutics, 553(1-2), 238-260. (iii) (2019). Comprehensive quality by design approach for stable nanocrystalline drug products. International journal of pharmaceutics, 564,426-460.	Added

13	3	25-28	1) The novelty of the present study needs to be clearly provided. The previous studies also included freeze/spray drying of the liquid liposomal drug formulations, which is repeated in the present study. Please include 2-3 sentences clearly providing the novelty of the present research. 2) Please include physicochemical properties of the model drug – ciprofloxacin – aqueous solubility, pKa, log P, molecular weight, etc.	1) Refer to Part 2 - (1). 2) Refer to Table 1 in the revised manuscript.
14	4	1	Please include abbreviation of the drug name – Cf to refer throughout the manuscript. Include lipids used in the present study.	Added
15	4	5	Please correct to: **……**grade and **obtained form**….	Modified
16	4	2.2.1 (Title)	Please correct to: ……formulation (**liquid**)	Modified
17	4	14	Please correct to: "The **manufacturing process of** an aqueous dispersion of **encapsulated Cf** was previously reported.	Modified
18	4	19	Please include the particle size of the empty liposomes.	Added
19	4	19-21	The addition of drug and removal of free drug needs to be elaborated. Please include details of drug encapsulation in aqueous core or in lipid bilayer.	Required information was added
20	4	21-22	"After final……50mg/mL." – Please remove from methods and include in the results section.	This sentence refers to the final product we obtained from the supplier as specified in Section *2.1. Material*
21	4	23	Please explain the rationale for the selection of sucrose as the lyoprotectant for the present study.	Explanation was added to the revised manuscript
22	4	25	Please include the final drug concentration post dilution using sucrose solution and deionized water.	Added
23	5	1-2	Please include a table to clearly differentiate ingredients and quantity of each ingredient of three formulations.	Table is added
24	5	2	Please use consistent terminology throughout the manuscript. Either mass ratios or weight ratios.	All modified to mass ratio
25	5	4	Please correct to: **…..**at a feed **flow** rate…..	Modified

26	5	5	The present study uses cholesterol as one of lipid component to prepare liposomes. It is known that cholesterol is prone to oxidation. Please explain the rationale for using "air" as the drying gas during spray drying. Please correct to: …….atomizer rate 742 L/h…..	Refer to Part 2 - (2).
27	5	6-7	Did the authors used desiccators to store the spray dried formulations? What does dry container mean? Did the authors use glove box to collect the spray dried formulations to avoid exposure to atmospheric humidity?	Desiccator was used and the collection was inside the glove box (Text was modified to include that information)
28	5	2.3. (Title)	Please correct to: "Physical state of Cf/drug in liposomal Cf formulations"	Modified
29	5	11	Please correct to: "The **liposomal Cf formulation characterized were:** *(i)* **Cf liposomes (liquid) (diluted 10 times using DI water) and;** *(ii)* **spray dried Cf liposomes (reconstituted using saline to 1mg/mL).** instead of "A ten times…….1mg/mL"	Modified
30	5	13	Please include concentration of saline used. 0.9% or other??	Added
31	5	2.4. (Title)	Please correct to: "**Particle** size distribution of **liposomal Cf formulations**"	Modified
32	5	19	1) Please include rationale for the selection of saline. 2) What is the solubility of Cf/drug in saline?	1) Refer to Part 2 - (3). 2) Refer to table 1 in the revised manuscript.
33	5	22-23	Please include a table for PSD parameters.	Table is added
34	5	25	Please correct to: **…..**batches were **recorded.**	Modified
35	5	2.5. (Title)	Please correct to: "**Encapsulation efficiency (EE) of Cf/drug in liposomal Cf formulations**"	Modified
36	6	1	Please use consistent format throughout the manuscript. "mL" or "ml"	All adjusted to mL
37	6	2-3	Please correct to: "400µL of each sample **was** centrifuged for 18 min at 10,000 rpm (8100 g)."	Modified
38	6	3-4	Please correct to: "Free **drug/Cf** was **quantitatively evaluated** *via* **HPLC**." Instead of "The quantity…..with deionized water."	Modified
39	6	2-8	Please include dilution details in parenthesis in brief.	Modified

40	6	8-10	Please provide an equation of %encapsulated drug.	Added
41	6	2.6. (Title)	Merge title and section 2.6. with 2.4. Particle size distribution of different formulations and include sub titles 2.4.1. Particle size distribution of liposomal Cf formulations 2.4.2. Particle size distribution of liposomal Cf formulations (reconstituted) 2.4.3. Particle size distribution of liposomal Cf formulations (dry powders)	The following titles and subtitles were used to combine the two sections into one. 2.4.Particle size distribution of different formulations 2.4.1. Particle size distribution of liposomal Cf formulations (liquids) 2.4.2. Particle size distribution of liposomal Cf formulations (dry powders)
42	6	2.7. (Title)	Please correct to: "Moisture content **of liposomal Cf formulations (solid/dry powders)**	Modified
43	7	2.8. (Title)	Merge title and section 2.8. with 2.3. Structure of liposomal Cf formulations *via* microscopic techniques and include subtitles 2.3.1. Physical state of Cf/drug in liposomal Cf formulations *via* TEM 2.3.2. Surface morphology of liposomal Cf formulations *via* SEM	Modified
44	7	2.9. (Title)	Please correct to: "**Solid-state of spray dried liposomal Cf formulations**"	Modified
45	7	7	Please include PXRD parameters in a tabular format.	Table was added
46	7	8-13	Please correct to: "Spray dried liposomal Cf formulations, controls (empty liposomes, spray dried drug/Cf, and each individual excipients were evaluated" instead of "Powders obtained from….were tested."	Modified
47	7	2.10. (Title)	Please correct to: "Moisture sorption behavior of spray dried liposomal Cf formulations"	Modified
48	7	15	Please correct to: ………was utilized to explore…..	Modified
49	7	17	Please clarify the unit "sccm" for the gas flow.	sccm is standard cubic centimeters per minute (cm^3min^{-1})

50	8	17-19	Please correct to: "The spray dried liposomal formulations and a control (aqueous liposomal formulation) were reconstituted in saline for *in vitro* release experiments."	Modified
51	8	23	Please provide the rationale for the selection of non compendial apparatus (shaking water bath) for *in vitro* release studies.	Refer to Part 2 - (4).
52	8	24	Please correct to: "Samples were withdrawn at specific……"	Modified
53	9	6	Please include the range of f1 and f2 according to USP/FDA guidance for establishing difference or similarity between two release profiles.	The range of f1 and f2 was specified in the Section *2.12. Statistical analysis* and was referred to it in Section *2.10. In vitro assay of Cf release from liposomes*
54	9	2.13. (Title)	Please correct to: "Quantitative evaluation of Cf/drug"	Modified
55	9	9	Please correct to: "A previously reported Reverse Phase-HPLC was used for quantitative evaluation of Cf."	Modified
56	9	16-17	Please correct to: "Statistical analysis was performed on triplicate observations calculated as a mean±SD. One way ……..test were used for calculation of statistical differences."	Modified
57	9	Titles of Result section	Please arrange and edit according to the suggested section and subsection headings in 2. Materials and Methods section	Modified
58	9	25-26	Please correct to: "Control samples (liposomal Cf formulations (liquid)) showed uniform spherical vesicles with an approximate size of 90nm."	Modified
59	9,11	26, 1	Please correct to: "These vesicles represent a supersaturated solution of Cf encapsulated in liposomes."	Modified
60	11	1-2	Please correct to: "As a result of spray drying, elongated liposomes were formed with the rod-shaped structure (drug) in the core"	Modified
61	11	6-10	Please provide detailed explanation of the correlation between amount of sucrose and drug encapsulation in the core of liposomes.	Refer to Part 2 - (5).

62	11	11	Please correct to "The control (liposomal Cf formulations (liquid)) sample….."	Modified
63	11	15-17	1) Please explain how does sucrose maintain monodispersity at 2:1 w/w sucrose:lipid ratio. 2) Please include the particle size distribution of spray dried formulation at sucrose:lipid ratio of 0:1 w/w in the table to compare the liquid *vs.* solid samples.	1) Refer to Part 2 - (6). 2) Spray dried formulation with sucrose: lipid ratio 0:1 was added to Table 5.
64.	12	1-3	Please correct to: "According to previous literature reports (), 99% of Cf is encapsulated within the liposomal vesicles in a soluble form in liposomal Cf formulations (liquid)."	Modified
65	12	3-5	Please explain the rationale of decrease in encapsulated drug to 12% using optimum quantity of sucrose during spray drying.	Refer to Section 4. Discussion – Paragraph No. 2 in the revised manuscript
66	12	6-7	Please correct to: "All spray dried formulations showed a low moisture content of 1.5-2% w/w (Table 2).	Modified
67	13	2	Please highlight "protruding needle like structure" with colored arrows in Figure 2 and label them.	Arrows added
68	13	Fig. 2	Please use correct/consistent nomenclature of the formulations in the figure caption throughout the manuscript for all figures. Solid dry powders – Spray dried liposomal Cf formulations (solid, dry powder), Liquid – Liposomal Cf formulations (liquid)	Modified
69	14	3	Please clarify: There is no difference in the PXRD peak intensity at 26° for the 0:5 and 1:1 w/w sucrose:lipid.	Refer to Part 2 - (7).

70	14	Fig. 3.	1) Please include diffractograms overlay of – (i) neat crystalline drug, (ii) spray dried Mg stearate. Sucrose blinds the lipid peaks, indicating that PXRD is not a good tool to differentiate between different spray dried liposomal formulations. 2) Suggest use DSC, PLM, to further investigate solid-state.	1) Diffractogram of neat crystalline drug was added. However, spray dried Mg stearate is not possible to obtain (Refer to Part 2 - (8.1)). 2) DSC results were added in Section 3.4 in the revised manuscript, while PLM not appropriate for our powder (Refer to Part 2 - (8.2)).
71	15	4-5	Please include PXRD, PLM data to confirm the absence of moisture induced recrystallization in the spray dried formulations.	Please refer to DSC results in Section 3.4 in the revised manuscript. PXRD and PLM were not considered due to the reasons mentioned in Part 2 - (9).
72	17, 18	Fig. 6	1) The control formulation do not contain sucrose. The results of IVRT is interesting with control formulation showing a slightly slower drug release compared to spray dried formulation containing 0.5:1 w/w sucrose:lipid. Please explain the rationale for this observation. 2) Please include Spray dried formulation of control *i.e.* 0:1 w/w sucrose:lipid in figure 6 and table 5.	1) Refer to Part 2 - (10). 2) Spray dried control 0:1 w/w was added in figure 7 and table 9 in the revised manuscript.
73	17	15-17	Please restructure the sentence: "Producing……lung infections."	Refer to Section 4. Discussion in the revised manuscript
74	19	-	Please draft a succinct discussion section.	

❖ **Part 2** (the followings are the detailed answers for some comments in **Part 1**):

(1) Comment No. 13. Page: 3, lines: 25-28.

The novelty of the present study needs to be clearly provided. The previous studies also included freeze/spray drying of the liquid liposomal drug formulations, which is repeated in the present study. Please include 2-3 sentences clearly providing the novelty of the present research.

The last paragraph in the introduction section contains the following sentences: **[The nanocrystalline formulation was based on processes involving multiple steps. It required freeze-thaw of liposomes to create the nanocrystals of Cf followed by spray drying for conversion into a dry powder form (Khatib et al., 2019). None of the previous reports manifested a single-step method of preparing powders of Cf nanocrystals inside liposomes**

directly from the liquid liposomal dispersions of non-crystalline Cf. In the present study, we report direct spray drying without freeze-thaw as a new method of generating Cf nanocrystals in liposomes (CNL) in dry powder form for inhalation and controlled drug release]. These sentences indicate the novelty of our study, which is spray drying as a single process to generate nanocrystals of ciprofloxacin within liposomal dry powder from a non-crystalline liquid formulation.

(2) Comment No. 26., page: 5, line: 5.

The present study uses cholesterol as one of lipid component to prepare liposomes. It is known that cholesterol is prone to oxidation. Please explain the rationale for using "air" as the drying gas during spray drying.

Spray drying of liposomes using air as a drying gas can be justified by the low inlet and outlet temperature (50 and 33 – 35 ° C, respectively) that have been used in this study. Also, the short exposure to the heat during spray drying probably will not allow a major oxidation event to occur. Goldbach et al. (1993) found that lipids were not significantly hydrolyzed or oxidized during spray drying in air. Wiggenhorn (2007) found slight decreases of up to 3 % in the lipid content of a 10 mM liposome formulation when using high inlet temperatures of up to 220 °C and low trehalose content of 5 % (w/v). In addition, other studies investigated the effect of spray drying temperature on the oxidation of cholesterol-rich food, the lowest temperature studied was 75 ° C which produced 58 ppm of cholesterol oxidation products even if a very high level of nitrogen oxides as a strong oxidizing agent was used (300 ppm) (Morgan and Armstrong, 1992). Moreover, this study has shown the physical stability of the liposomes after spray drying when compared to the initial control liquid form, which indicates that the physicochemical properties of liposomes were protected during processing.

(3) Comment No. 32. Page: 5, line: 19.

Please include rationale for the selection of saline.

Liposomes self-assembly depends on many factors e.g. concentration of lipoplexes, ionic strength and the presence of surfactants. Sweeney et al. (2005) observed the highest encapsulation efficiency of ciprofloxacin drug inside liposomes when the spray freeze dried powder was reconstituted in isotonic saline. Hence, saline was chosen as the reconstitution solvent of spray dried liposomal powder to enhance liposomal assembly and drug encapsulation.

The sentence in red was added under the Section's headings listed below to include the above rationale:

2.3. Structure of liposomal Cf formulations via microscopic technique

2.3.1. Physical state of Cf/drug in liposomal Cf formulations via TEM

The liposomal Cf formulation characterized were: (i) Cf liposomes (liquid) (diluted 10 times using DI water) and; (ii) spray dried Cf liposomes (reconstituted to ~1 mg mL^{-1} using saline (0.9% w/v) as isotonic medium suitable for both liposomes assembly and drug entrapment (Sweeney et al., 2005)).

(4)   Comment No. 51. Page: 8, line: 23.

Please provide the rationale for the selection of non compendial apparatus (shaking water bath) for *in vitro* release studies.

There are no standard methods specified for liposomes either in the United States Pharmacopeia or the European Pharmacopeia, as well as no regulatory guidelines of binding recommendations released by the FDA or the European Medicines Agency. The current techniques that are most often used to assess *in vitro* drug release from liposomal products are the membrane diffusion techniques (dialysis, reverse dialysis, fractional dialysis, and microdialysis), the sample-and-separate approach, the in-situ method, the continuous flow, and the modified United States Pharmacopeia methods (USP I and USP IV). There are a few significant challenges involved in conducting and interpreting *in vitro* release studies for liposomes. Liposomes are found to rapidly associate with lipoprotein-like particles after incubation with serum and this correlates with huge destruction of the liposome structures and release of the drug. Lipoproteins and apolipoproteins are present in lung fluid. Thus, release study experiments that do not account for the presence of proteins in an *in vitro* setup might perform significantly different in in vivo conditions. Also, there are several different types of liposomes, and a universal release study method cannot be used for all formulations (Solomon et al., 2017). Hence, the *in vitro* assay method used in this study was based on a validated method that is specifically adjusted to liposomal formulations made with hydrogenated soy phosphatidylcholine and cholesterol encapsulating ciprofloxacin. We have investigated liquid and solid formulations derived from those liposomal formulations. In addition, this *in vitro* release method utilizes bovine serum as the dissolution medium to simulate the lung fluids. Cipolla et al. (2014) stated that the *in vitro* release method was also found to be discriminatory between formulations of liposomal ciprofloxacin that differed in composition. Hence, we considered this method to study the difference in release behavior between liposomal formulations differs in the physical form i.e. liquid and powder.

(5) Comment No. 61. Page: 11, lines: 6-10.

Please provide detailed explanation of the correlation between amount of sucrose and drug encapsulation in the core of liposomes.

We have explained the correlation between the amount of sucrose and drug encapsulation in two sections (**bold phrases** refer to the most relevant parts in the answer to the above question):

Section 3.3. Encapsulation efficiency (EE%) of Cf/drug in liposomal Cf formulations

Table 8 shows a highest EE% value with the highest sucrose to lipid ratio (2:1 w/w).

Section 4. Discussion – paragraph 2: [**When processing liposomes, the vesicle integrity must be maintained to avoid rupturing and subsequent drug leakage,** intact vesicles will also act as the rate-controlling barrier for the formation of single drug nanocrystals. However, spray drying involves atomization of the liposomal formulation with exposure to mechanical stress and generation of air-liquid interface. Furthermore, the vesicles are exposed to osmotic stress resulting from an increase in the concentration of the solutes during drying. Thus, some liposomal particles may rearrange into larger vesicles as a response to the limited distances between drying particles (Stark et al., 2010). **Sucrose as lyoprotectant can decrease the stresses of spray drying via hydrogen bonding with the lipids and/or reducing the lipid mobility in a vitrified state.** As a result, liposomes could maintain the initial uniform size distribution of the starting liposomal dispersion (Ingvarsson et al., 2011). **Moreover, sucrose has been shown to preserve most of the encapsulated drug inside liposomes (Wessman et al., 2010). Despite the possible instability of liposomes during spray drying, our previous report revealed that ~ 80% of Cf was encapsulated if a 2:1 w/w sucrose to lipids ratio was used during a combined process of freeze-thaw and spray drying (FT-SD) (Khatib et al., 2019). Our present data showed that spray drying liposomes with the same sucrose to lipids ratio produced Cf nanocrystals with a better encapsulation value of 87%, and the change in the size characteristics of the spray dried liposomes following rehydration was minimal].**

(6) Comment No. 63. Page: 11, lines: 15-17.

Please explain how does sucrose maintain monodispersity at 2:1 w/w sucrose:lipid ratio.

[Bold phrases refer to the most relevant parts in the answer to the above question]

Section 4. Discussion – Paragraph 2: [spray drying involves atomization of the liposomal formulation with exposure to mechanical stress and generation of air-liquid interface. Furthermore,

the vesicles are exposed to osmotic stress resulting from an increase in the concentration of the solutes during drying. **Thus, some liposomal particles may rearrange into larger vesicles as a response to the limited distances between drying particles (Stark et al., 2010). Sucrose as lyoprotectant can decrease the stresses of spray drying via hydrogen bonding with the lipids and/or reducing the lipid mobility in a vitrified state. As a result, liposomes could maintain the initial uniform size distribution of the starting liposomal dispersion (Ingvarsson et al., 2011).** Moreover, sucrose has been shown to preserve most of the encapsulated drug inside liposomes (Wessman et al., 2010). Despite the possible instability of liposomes during spray drying, our previous report revealed that ~ 80% of Cf was encapsulated if a 2:1 w/w sucrose to lipids ratio was used during a combined process of freeze-thaw and spray drying (FT-SD) (Khatib et al., 2019). Our present data showed that spray drying liposomes with the same sucrose to lipids ratio produced Cf nanocrystals with a better encapsulation value of 87%, **and the change in the size characteristics of the spray dried liposomes following rehydration was minimal**].

(7) Comment No. 69. Page: 14, line: 3.

Please clarify: There is no difference in the PXRD peak intensity at 26 ° for the 0:5 and 1:1 w/w sucrose:lipid.

The PXRD graph does not show that clearly due to a very small difference between the two formulations, as table 1 is indicating that the percentage of ciprofloxacin in the two formulations is 23.2% and 18.6%, respectively. However, a more focused graphs for the three formulations have been included below to show the difference.

12
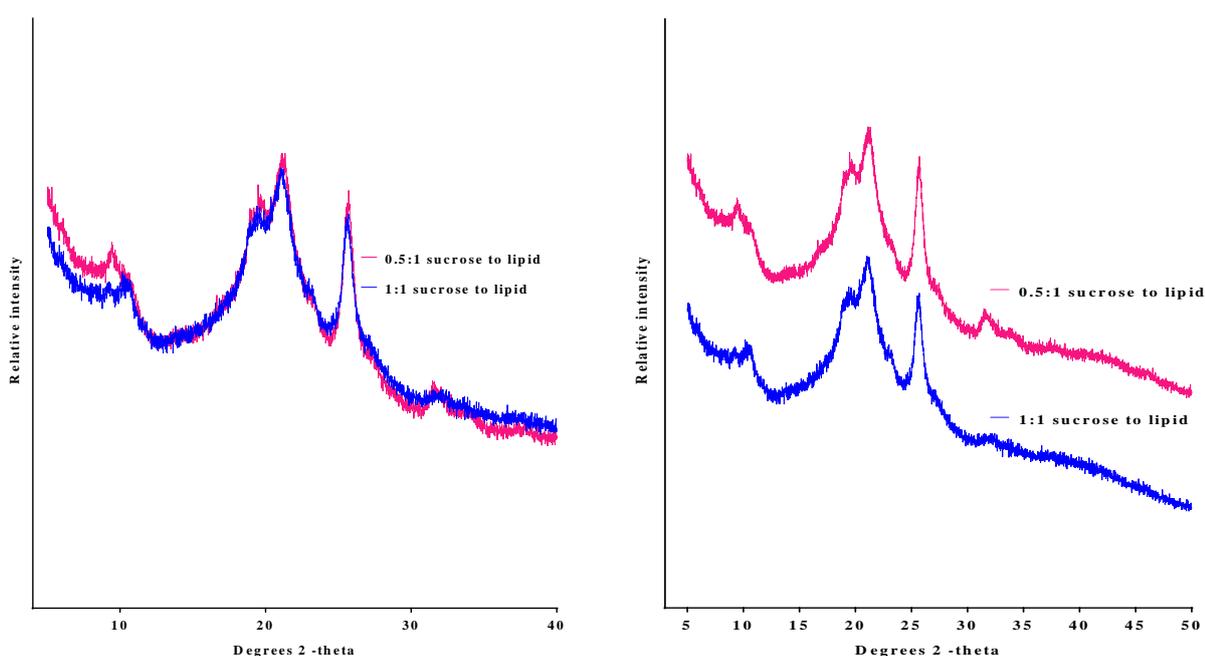
(8) Comment No. 70. Page: 14, Fig. 3.

(8.1) Please include diffractograms overlay of – (i) neat crystalline drug, (ii) spray dried Mg stearate. Sucrose blinds the lipid peaks, indicating that PXRD is not a good tool to differentiate between different spray dried liposomal formulations.

Spray-dried Mg stearate was not included due to the very low solubility of Mg stearate in the water (0.004 g/mL) and other solvents e.g. alcohol (0.02 g/mL). We considered the use of alcohol to aid the dissolution of Mg stearate but the resulted powder won't be the same as that in the spray-dried liposomal formulations. The contribution of 2% w/w Mg-stearate in the overall PXRD pattern of the spray-dried formulation is expected to be minor.

(8.2) Suggest use DSC, PLM, to further investigate solid-state.

We have considered the reviewer`s suggestion of the use of DSC as a complementary tool for the results of PXRD. Please refer to Section 3.4 in the revised manuscript. PLM was not considered because of the presence of ciprofloxacin in the nanocrystalline form.

(9) Comment No. 71. Page: 15, lines 4-5.

Please include PXRD, PLM data to confirm the absence of moisture induced recrystallization in the spray dried formulations.

The PXRD was not used because it requires a large amount of powder to produce reliable data while DVS requires a sample of 10 to 20 mg by max. PLM was not appropriate due to the initial crystalline characteristic of the powders as a result of the presence of ciprofloxacin nanocrystals. Samples of the three formulations were run in DVS, then were measured by DSC to confirm whether recrystallization happened or not (Section 3.4).

(10)   Comment No. 72. Pages: 17 and 18, Fig. 6.

The control formulation do not contain sucrose. The results of IVRT is interesting with control formulation showing a slightly slower drug release compared to spray dried formulation containing 0.5:1 w/w sucrose:lipid. Please explain the rationale for this observation.

The liquid control with zero sucrose content encapsulates almost 99% of the drug in the core of the liposomal vesicles. On the other hand, the spray-dried liposomal Cf formulation with sucrose to lipids ratio 0.5:1 w/w contains around 66% of the drug outside liposomes (Table 7 in the revised manuscript) due to drug leakage caused by the mechanical and osmotic stresses during spray drying. The *in vitro* release profile of the low sucrose-containing formulation displayed a burst release at the

beginning due to the unencapsulated drug.

References:

Goldbach, P., Brochart, H., Stamm, 1993. Spray-drying of liposomes for a pulmonary administration. I. Chemical stability of phospholipids. Drug development and industrial pharmacy 19, 2611-2622.

Ingvarsson, P.T., Yang, M., Nielsen, H.M., Rantanen, J., Foged, C., 2011. Stabilization of liposomes during drying. Expert Opinion on Drug Delivery 8, 375-388.

Khatib, I., Khanal, D., Ruan, J., Cipolla, D., Dayton, F., Blanchard, J.D., Chan, H.-K., 2019. Ciprofloxacin nanocrystals liposomal powders for controlled drug release via inhalation. International journal of pharmaceutics 566, 641-651.

Morgan, J., Armstrong, D., 1992. Quantification of cholesterol oxidation products in egg yolk powder spray-dried with direct heating. Journal of food science 57, 43-45.

Stark, B., Pabst, G., Prassl, R., 2010. Long-term stability of sterically stabilized liposomes by freezing and freeze-drying: Effects of cryoprotectants on structure. European Journal of Pharmaceutical Sciences 41, 546-555.

Sweeney, L.G., Wang, Z., Loebenberg, R., Wong, J.P., Lange, C.F., Finlay, W.H., 2005. Spray-freeze-dried liposomal ciprofloxacin powder for inhaled aerosol drug delivery. International journal of pharmaceutics 305, 180-185.

Wessman, P., Edwards, K., Mahlin, D., 2010. Structural effects caused by spray-and freeze-drying of liposomes and bilayer disks. Journal of pharmaceutical sciences 99, 2032-2048.
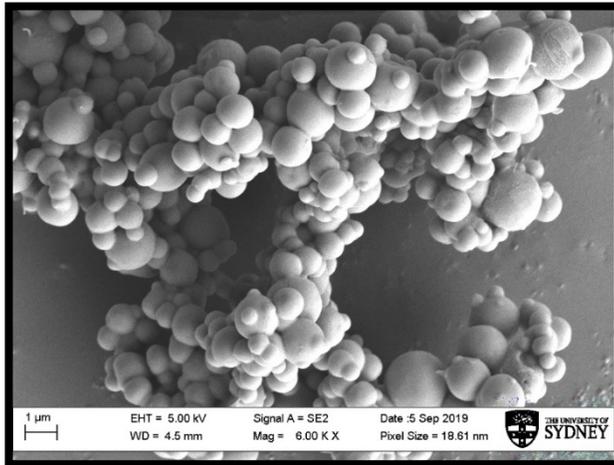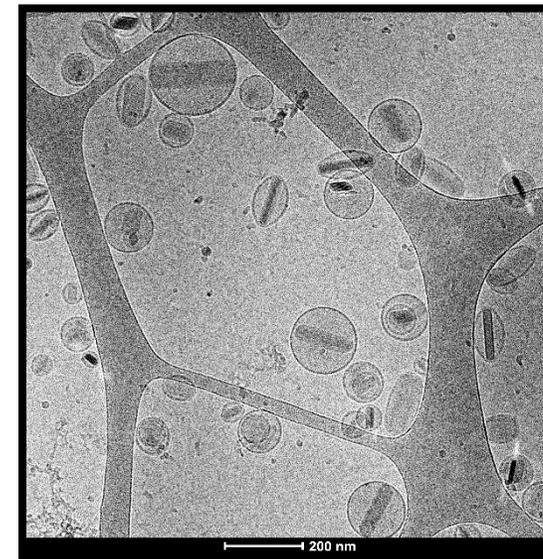
Wiggenhorn, M., 2007. title., lmu.

Drug in liquid form
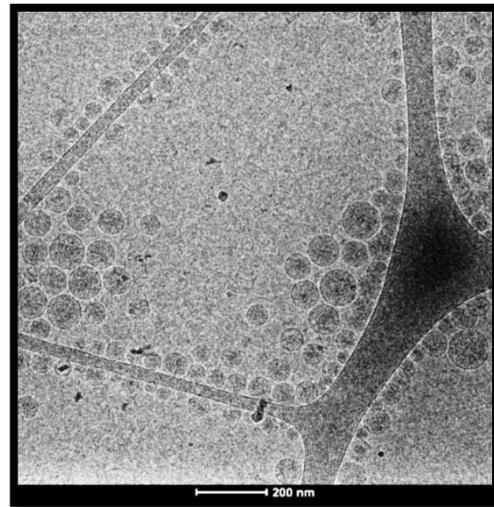
Liposomal ciprofloxacin
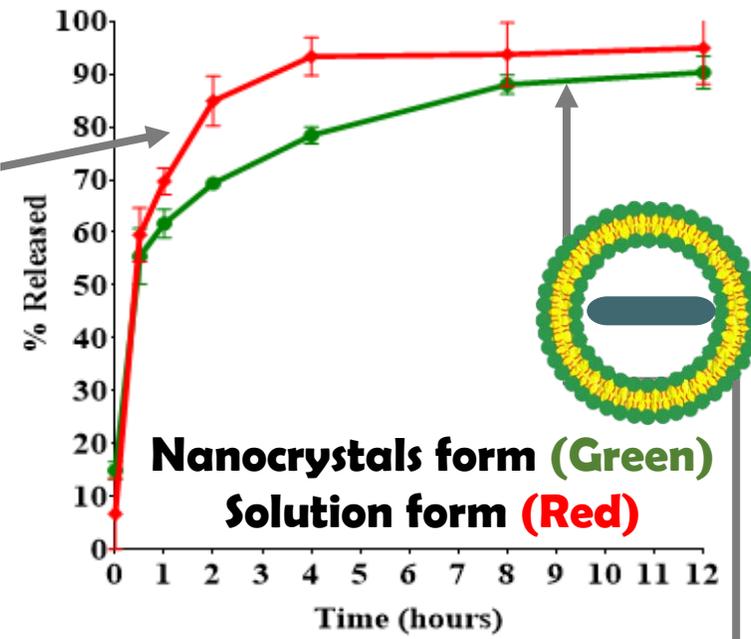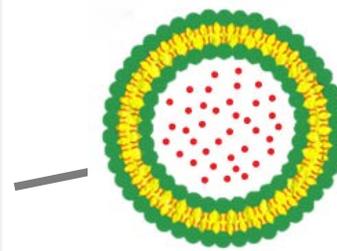
Spray Drying

Drug in powder form

Reconstitution in Saline

Nanocrystals form (Green)
Solution form (Red)

Liposomal ciprofloxacin nanocrystals

Research paper

Formation of ciprofloxacin nanocrystals within liposomes by spray drying for controlled release via inhalation

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Abstract

The present study was conducted to harness spray drying technology as a novel method of producing Ciprofloxacin nanocrystals inside liposomes (CNL) for inhalation delivery. Liposomal ciprofloxacin dispersions were spray dried with sucrose as a lyoprotectant in different mass ratios (0.5:1, 1:1 and 2:1 sucrose to lipids), along with 2% w/w magnesium stearate and 5% w/w isoleucine as aerosolization enhancers. Spray drying conditions were: inlet air temperature 50 °C, outlet air temperature 33-35 °C, atomizer rate 742 L/h and aspirator 35 m^3/h. After spray drying, the formation of ciprofloxacin nanocrystals inside the liposomes was confirmed by cryo- transmission electron microscopy. The physiochemical characteristics of the spray dried powder (particle size, morphology, crystallinity, moisture content, drug encapsulation efficiency (EE), *in vitro* aerosolization performance and drug release) were determined. The EE of the liposomes was found to vary between 44 and 87% w/w as the sucrose content was increased from 25 to 57% w/w. The powders contained partially crystalline particles with a volume median diameter of ~ 1 μm. The powders had low water content (~2 % wt.) and were stable at high relative humidity. Aerosol delivery using the Osmohaler® inhaler at a flow rate of 100 L/min produced an aerosol fine particle fraction (% wt. < 5 μm) of 58 – 64%. The formulation with the highest sucrose content (2:1 w/w sucrose to lipid) demonstrated extended ciprofloxacin release from liposomes (80% released within 7 hours) in comparison to the original liquid formulation (80% released within 2 hours). In conclusion, a stable and inhalable CNL powder with controlled drug release was successfully prepared by spray drying.

Keywords

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

Abbreviations

Cf, ciprofloxacin; 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-SD, freeze-thaw and spray dry; HBS, HEPES buffered saline; HPMC, hydroxypropyl methylcellulose; HSPC, hydrogenated soy phosphatidylcholine; LC, loading capacity; CNL, ciprofloxacin nanocrystals in liposomes; 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

Liposomes are widely used as drug nano carriers in the pharmaceutical industry. They are colloidal spheres with outer phospholipid bilayers and an internal aqueous centre. These carriers are utilized for targeted drug delivery applications as well as to minimize drug toxicity (Allen and Cullis, 2013; Jog and Burgess, 2018a). The encapsulated drug inside liposomes occurs in different physical forms, either as a supersaturated solution (e.g. cisplatin, ciprofloxacin), as an amorphous precipitate (e.g. vinorelbine, vincristine) or as a nanocrystalline precipitate (e.g. doxorubicin, topotecan, idarubicin) (Dzieciuch-Rojek et al., 2017; Li et al., 2018; Pace et al., 2019). Doxil® is the first FDA approved liposomal formulation containing doxorubicin nanocrystals. The active loading of the drug using transmembrane ammonium sulfate gradient was believed to be responsible for the spontaneous formation of doxorubicin sulfate nanocrystals inside liposomes (Li et al., 1998; Schilt et al., 2016). Although ciprofloxacin (Cf) is actively loaded into liposomes using the same technique, the drug remains in the soluble form with concentrations well above its aqueous solubility shown in Table 1 (Maurer et al., 1998; Webb et al., 1998). Intra-liposomal entrapment of the drug in crystalline form can enhance formulation stability by improving both drug loading and its retention inside liposomes (Barenholz, 2012). Furthermore, the presence of the crystalline form of the drug can modify the pharmacokinetics resulting in a prolonged drug release profile (Maurer et al., 1998). As a consequence, a new approach for *in vitro* crystallization inside liposomes was developed for the antibiotic Cf. This approach harnesses a freeze-thaw process to convert the soluble drug loaded inside liposomes into nanocrystalline form. The presence of ice crystals inside the vesicles during the freeze-thaw process is believed to provide nucleation sites for drug crystallization (Cipolla et al., 2016b). These liposomal formulations containing

Cf nanocrystals produced by freeze-thaw retained reasonable integrity upon storage and after nebulization (Cipolla et al., 2015).

Table 1
The physicochemical properties of Ciprofloxacin hydrochloride.

Property name	Property value
Solubility (water)	10 - 30 mg/ml at 25 °C
Dissociation constants (pKa)	pKa_1 = 6.09 (carboxylic acid group)
	pKa_2 = 8.74 (nitrogen on piperazinyl ring)
Partition coefficient (log P): n-octanol/pH 7.0 buffered solution	-0.94 at 37 °C, -1.45 (temperature not given) and -1.70 at 25 °C and pH 7.2
Molecular weight	367.8 g/mol

In general, liposomes in liquid dispersions undergo physiochemical degradation: fusion, aggregation, drug leakage, conversion to micelles, oxidation and hydolysis of lipids upon storage (Ingvarsson et al., 2011). Liquid formulations intended for inhalation usually utilize a nebulization device for delivery into the airways. Air – jet nebulization will expose the formulations to shear and air-liquid interface, which may affect liposomal integrity leading to drug leakage (Cipolla et al., 2013; Niven and Schreier, 1990; Taylor et al., 1990). In comparison, a solid-state form of liposomal formulations may have a better product shelf life and abolish the need for 'cold-chain' during storage and/or distribution (resulting in robust and economical drug product). Powder formulations intended for respiratory delivery can be delivered using a small and portable dry powder inhaler (DPI) and the associated short administration time may lead to improved patient adherence to treatment when compared to nebulization delivery (Cipolla et al., 2013; Willis et al., 2012). Freeze drying, spray drying, and spray freeze drying are common techniques to convert liquid liposomal dispersions or suspensions into a dry powder form (Ingvarsson et al., 2011; Jog and Burgess, 2017, 2018b, 2019). Liquid-formulated liposomes without (Goldbach et al., 1993; Ingvarsson et al., 2011; Kim, 2001; Misra et al., 2009; Patel et al., 2009; Skalko-Basnet et al., 2000) or with nanocrystals (Khatib et al., 2019) were re-processed successfully into dry powder forms using the above mentioned techniques. However, the nanocrystalline formulation was based on processes involving multiple steps. It required freeze-thaw of liposomes to create the nanocrystals of Cf followed by spray drying for conversion into a dry powder form (Khatib et al., 2019). None of the previous reports manifested a single-step method of preparing powders of Cf nanocrystals inside liposomes directly from the liquid liposomal dispersions of non-

crystalline Cf. In the present study, we report direct spray drying without freeze-thaw as a new method of generating Cf nanocrystals in liposomes (CNL) in dry powder form for inhalation and controlled drug release.

2. Materials and methods

2.1. Materials

Empty and Ciprofloxacin hydrochloride (Cf HCl) (50 mg mL^{-1}) containing liposomes (hydrogenated soy phosphatidylchocline (HSPC) and cholesterol) in aqueous solution at pH 6.0 histidine buffer were manufactured by Exelead (Indianapolis, IN, USA and Northern Lipids Incorporated (Burnaby, BC, Canada) and provided by Aradigm Corporation (Hayward, CA, USA). Isoleucine, magnesium stearate, sucrose, sodium chloride, triethylamine (TEA) and adult bovine serum were all of analytical grade and obtained from Sigma-Aldrich (Castle Hill, New South Wales, Australia), deionized water from Modulab Type II Deionization System (Continental Water System, Sydney, Australia), Nanosep Omega centrifugal filtration devices, 10k molecular weight from Pall Australia Pty Ltd, (Victoria, Australia) and HEPES, free acid from Dojindo, China. An Osmohaler$^{®}$ inhaler was obtained from Pharmaxis Ltd. (Frenches Forest, Australia) and size 3 hydroxypropyl methylcellulose (HPMC) capsules from Capsugel (West Ryde, Australia).

2.2. Preparation of powder formulations of Cf nanocrystals in liposomes (CNL)

2.2.1. Preparation method of liquid liposomal Cf formulations

The manufacturing process of an aqueous dispersion of encapsulated Cf inside liposomes was previously reported (Ong et al., 2014; Webb et al., 1998). Liposomes composed of HSPC and cholesterol in a 7:3 ratio (by weight). Multilamellar liposomes were produced and then converted to unilamellar vesicles by extrusion through 80 nm polycarbonate membrane, followed by solvent removal by diafiltration to generate empty liposomes with approximate size of 80 nm in diameter. The hydrophilic Cf HCl was added to the empty liposomes at 50 ° C and the mixture was agitated to enhance active loading of Cf into the aqueous core of the liposomal vesicles. After loading, the excess Cf was removed by diafiltration with 25 mM histidine, 145 mM NaCl, pH 6.0 buffer. After final processing, more than 99% of the Cf was encapsulated at a concentration of 50 mg mL^{-1}.

2.2.1. Spray drying of liquid liposomal Cf formulations to produce CNL powders

In all spray drying trials, sucrose was used as a lyoprotectant for liposomes (Chougule et al., 2008; Lo et al., 2004). Sucrose was successfully used to protect liposomes composed of HSPC and cholesterol that encapsulating ciprofloxacin during single process i.e. freeze-thaw (Cipolla et al., 2016b) or even with multiple processes such as combined freeze-thaw and spray dry (FT-SD) (Khatib et al., 2019). Liposomal Cf HCl at 50 mg mL^{-1} was diluted with 25, 50 or 100 mg mL^{-1} sucrose solution and then with deionized water to produce liposomes containing Cf at 12.5 mg mL^{-1}. Magnesium stearate and isoleucine were added to the liposome dispersion as moisture protectants and aerosolization enhancers prior to spray drying (Chan and Chew, 2003; Yu et al., 2017). Three liposomal formulations were prepared at 3 mg mL^{-1}, with solute comprising sucrose and lipid at three different mass ratios (0.5:1, 1:1, or 2:1), 2 % w/w Mg stearate and 5 % w/w isoleucine (Table 2). The liposome formulations were under continous stirring while being pumped into a spray dryer (B-290 mini spray-dryer, Büchi Falwil, Switzerland) at a feed flow rate of 1.4 mL/min. Spray drying conditions were: inlet air temperature 50 °C and outlet air temperature 33-35 °C, atomizer rate 742 L/h, aspirator 35 m^3/h. After spray drying, the powder was collected inside glove box with controlled humidity (<15% RH). The collected powder was then placed in a desiccator and protected from light at ambient temperature (~ 23°C).

Table 2
The ingredients and the quantity of each ingredient in the three spray dried formulations.

Formulation Name	Ingredients (mg%)				
Sucrose: lipids (w/w)	Sucrose	Lipids	Ciprofloxacin	Isoleucine	Mg stearate
0.5:1	23.2	46.4	23.2	5	2
1:1	37.2	37.2	18.6	5	2
2:1	53.1	26.6	13.3	5	2

2.3. Structure of liposomal Cf formulations via microscopic technique

2.3.1. Physical state of Cf/drug in liposomal Cf formulations via TEM

The formation of Cf nanocrystals was confirmed by imaging liposomal Cf formulations before and after spray drying using cryo-TEM. A Talos Arctica transmission electron microscope (Thermo Fisher Scientific, USA) operating at 200 kV was utilized. The liposomal Cf formulation characterized were: (i) Cf liposomes (liquid) (diluted 10 times using DI water) and; (ii) spray dried Cf liposomes (reconstituted to ~1 mg mL^{-1} using saline (0.9% w/v) as

isotonic medium suitable for both liposomes assembly and drug entrapment (Sweeney et al., 2005)). Using a chamber controlled at 4 °C and 85 % RH, 4 µL of the samples were loaded into a glow discharged Lacey formvar/carbon grid (TED PELLA, USA). The grids were blotted once with filter paper for 3 s followed by plunging into liquid ethane to vitrify the samples using a Leica EM GP device (Lecia Microsystem, Germany). The vitrified samples were stored in a liquid nitrogen dewar prior to analysis by cryo-TEM.

2.3.2. *Surface morphology of liposomal Cf formulations via SEM*

Scanning electron microscopy (SEM, Carl Zeiss SMT AG, Oberkochen, Germany) was used to visualize the surface morphology of the particles. The powders were spread on an SEM stub and sputter coated with gold (15 nm thick) using a K550X sputter coater (Quorum Emitech, Kent, UK). The SEM was operated under 5 kV to obtain the images.

2.4. *Particle size distribution of different formulations*

2.4.1. *Particle size distribution of liposomal Cf formulations (liquids)*

Control liquid of Cf liposomes were diluted and dry powder formulations were reconstituted in saline to yield samples with a Cf concentration of ~ 0.1 mg mL^{-1}. The particle size distribution of the liposomes was measured using a Malvern Zetasizer Nano ZS (Malvern, UK) with the instrument parameters in Table 3. The mean and relative standard deviation (RSD %) of the liposome size distribution of three measurements of three batches were recorded.

Table 3
Parameters of Malvern Zetasizer Nano ZS used for measuring particle size distribution of liquid liposomal Cf formulations.

Parameter	Unit	Value	Parameter	Unit	Value
Temperature	° C	23	Channel width	µs	10
Viscosity	cP	0.887	Scattering angle	°	90
Refractive index	-	1.34	Run time	min	5
Intensity set point	kHz	300			

2.4.2. *Particle size distribution of liposomal Cf formulations (dry powders)*

Malvern Mastersizer 2000® laser diffractometer combined with a dry sampling accessory (Scirocco 2000, Malvern Instruments, Malvern UK) was used to measure the particle size distribution of the powders. The real and imaginary refractive indices (RI) were set at 1.40

and 0.10, respectively. These values were found to achieve low residuals (<1.5 %) when calculating the particle size distribution, indicating that the optical model using these RI values were suitable. Pressure titration was applied during the measurement to ensure powder de-agglomeration but not fragmentation of the primary particles. The D50 decreased as the pressure increased from 0.5 to 3.5 bar and reached a plateau at 4 bar which was consequently used for all the measurements. To achieve signals above the background, a 100 % vibration feed rate and 12 s of measurement time were applied. The values of D10, D50, and D90 (i.e. the diameter at 10%, 50% and 90% undersize, respectively) and span (i.e. the difference between D10 and D90 divided by D50) were reported. The values of mean and SD of the size distribution were obtained from three measurements of three batches.

2.5. Encapsulation efficiency (EE%) of Cf/drug in liposomal Cf formulations

The unencapsulated drug was separated from the encapsulated drug using Nanosep Omega centrifugation devices with modified polyethersulfone membrane filters of 10,000 molecular weight cut-offs (Cipolla et al., 2014). Samples of Cf at a concentration of ~ 1 mg mL^{-1} were prepared by reconstituting 10 mg of the liposomal dry powder in 5 mL saline solution. A 400 µL of each sample was centrifuged for 18 min at 10,000 rpm (8100 g). Free drug/Cf was quantitatively evaluated in the diluted filtrate (20 times) via HPLC. The quantity of total Cf (the encapsulated and free drug) in the samples was determined after diluting (10 times) with 80% methanol to dissolve liposomes. Samples were centrifuged for 15 min at 13,400 rpm, then filtrates were diluted (4 times) with deionized water and analysed by HPLC. The percent of encapsulated drug was calculated using the following equation:

EE% = [(Total drug amount – Free drug amount) / Total drug amount] ×100%

2.6. Thermal analysis of of liposomal Cf formulations (dry powders)
2.6.1. Moisture content of liposomal Cf formulations via TGA

Thermogravimetric analysis (TG/SDTA 851e, Mettler-Toledo, Germany) was used to determine the residual moisture content of the spray dried powders. Alumina pan was loaded with 5-10 mg of the dry powder formulation and heated from 30 to 150 °C at a heating rate of 10 °C/min under a nitrogen atmosphere. The percent drop in mass between 30 to 100 °C was taken as the sample water content. The reported data were the mean and SD of three observations.

2.6.2. Thermal properties of liposomal Cf formulation via DSC

Differential scanning calorimetry (DSC) (Mettler-Toledo, Germany) was applied to investigate the thermal properties of liposomal Cf dry powder. The analyzed samples were (i) Spray-dried powder stored in desiccator; (ii) Spray-dried powder after dynamic vapor sorption (Section *2.8. Moisture sorption behavior of spray-dried liposomal Cf formulations*) and; (iii) Spray-dried controls (empty liposomes, drug/Cf, and sucrose) and neat crystalline Cf., about 5 mg of the samples were spread into an aluminum crucible with a perforated lid crimpled on top, and then heated from 25 to 400 ° C at 10 °C/min under a continuous flow of N2 gas. Thermal data were recorded and analyzed through STARe software V.9.0x (Mettler Toledo, Greifensee, Switzerland).

2.7. Solid-state of spray dried liposomal Cf formulations

The crystallinity of the spray dried powders was determined by powder X-ray diffraction (PXRD) (Shimadzu XRD-6000, Shimadzu Corporation, Kyoto, Japan) using the instrument parameters in Table 4. Spray dried liposomal Cf formulations, controls (spray dried empty liposomes and drug/Cf), and each individual excipient were evaluated.

Table 4
Parameters of powder X-ray diffraction used for measuring particle size distribution of solid liposomal Cf formulations.

Parameter	Unit	Value
Cu-Ka radiation	kV	40
Current	mA	30
2θ range	°	5-50
Scan speed	°/min	2

2.8. Moisture sorption behavior of spray dried liposomal Cf formulations

Dynamic vapor sorption system (DVS-1, Surface Measurement Systems Ltd., London, UK) was utilized to explore the moisture sorption behavior of the spray dried powders. The sample pan was filled with about 10-20 mg of powder and positioned in the equipment chamber with continuous nitrogen gas flow of 200 cm^3min^{-1} at 25 °C. The relative humidity (RH) of the chamber was changed from 0 to 90% with 10% increment or decrement steps for both the sorption and desorption cycles, respectively. Moisture equilibrium of the sample was considered reached when the change in the sample weight per time (dm/dt) was below 0.002%.

2.9. In vitro aerosol performance

The aerosol performance of the powder formulations was determined using a next generation impactor (NGI, Copley, Nottingham, UK) coupled to a mouthpiece adapter and a dry USP induction port. Size 3 HPMC capsules were filled with 30 ± 1 mg of the powder and dispersed using an Osmohaler® device under ambient conditions. At a flow rate of 100 L min^{-1} and within 2.4 s, four liters of air were drawn through the inhaler, which resulted in a pressure drop of about 4 kPa across the device. Using an Aerolizer®, which is essentially the same as an Osmohaler®, an air flow of 105 L/min can be produced using a comfortable inspiratory effort of 40 cm H_2O (i.e., 4 kPa) by patients with compromised lung functions (Chew and Chan, 2001). The dispersed powders which deposited on the capsule, inhaler, adapter, throat, and stages 1–8 of the NGI were dissolved with 80% methanol and then collected for HPLC analysis. The total mass of the deposited powders on the capsule, inhaler, adapter, throat, and stages 1–8 of the NGI was considered as the total recovered mass. The percent mass of powder exiting the device relative to the total recovered mass represented the percent emitted dose (ED). For each stage of the impactor, the percent mass of deposited powder in comparison to the total recovered mass was determined. The percent of the total recovered mass with aerodynamic diameter ≤ 5 µm was calculated to obtain the fine particle fraction (FPF). The mass of Cf obtained from stages with a particle size ≤ 5 µm was considered as the fine particle dose (FPD). Mass median aerodynamic diameter (MMAD) is the diameter at which 50% of the particles by mass are larger and 50% are smaller. According to the British Pharmacopeia 2018 (Appendix XII C. Consistency of Formulated Preparations), FPD and MMAD were calculated from plots of the cumulative fraction or mass of Cf versus the cut-off diameter of impactor stages.

2.10. In vitro assay of Cf release from liposomes

Ciprofloxacin release from liposomes was measured according to a previously established method (Cipolla et al., 2014). The spray dried liposomal formulations and a control (aqueous liposomal formulation) were reconstituted in saline for *in vitro* release experiments. HEPES buffered saline (HBS: 20 mM HEPES, 145 mM NaCl, pH 7.4) was used to dilute the formulations to contain around 50 µg mL^{-1} Cf. They were further diluted with an equal volume of chilled (2–8°C) adult bovine serum (Sigma-Aldrich, Castle Hill, New South Wales, Australia). Samples from each formulation were withdrawn just prior to loading into a shaking water bath (Labec J-SWB60, Marrickville, Australia) at 37°C and 150 rpm. Samples were

withdrawn at specific time points (30, 60, 120, 240, 480 and 720 min) and loaded immediately in an ice-water bath. To eliminate additional release of Cf, chilled (2–8°C) HBS was added to the samples in one-to-one dilution. The released Cf was separated from the encapsulated drug by centrifuging 400 μL of the sample in the Nanosep centrifugal device at 10,000 rpm for 18 min. The filtrate was analysed using HPLC to quantify the released Cf. To compensate for the loss of the drug in the filter in the presence of the serum, a 0.93 correction factor was used to normalize the calculated values (Cipolla et al., 2014). The total Cf amount was determined by following the method described in Section 2.5. The percent drug release was the amount of the released drug relative to the total drug content in the sample. A normalized percentage of release was calculated due to the presence of free drug at T0min; hence, the release of (T30min-T0min) was divided by the total possible release (100-T0min) and then converted to a percentage: 100* (T30-T0)/ (100-T0) (Cipolla et al., 2016a). Subsequently, the similarity factor (f2) and difference factor (f1) were calculated using the normalized percentage in order to compare the release profiles of the different formulations as described in Section 2.12.

2.11. Quantitative evaluation of Cf/drug

A previously reported Reverse Phase-HPLC was used for quantitative evaluation of Cf. (Ong et al., 2014). Briefly, a Phenosphere-Next C-18 column (5 mm, 4.6 × 150 mm, Phenomenex, USA) at 35 °C was used as the stationary phase. A mixture of 0.5 % TEA in water, pH 3.0 and 100 % methanol (78: 22 v/v) was used as the mobile phase with isocratic elution at a flow rate of 0.9 mL min^{-1}. The detector was set at 277 nm wavelength to quantify Cf. The concentration of Cf was expressed in terms of Cf HCl.

2.12. Statistical analysis

Statistical analysis was performed on triplicate observations calculated as a mean±SD. One-way analysis of variance (ANOVA) and Tukey's multiple comparisons test were used for calculation of statistical differences. The statistical differences were deemed significant if the probability values were < 0.05. Difference factor (f1) and similarity factor (f2) were calculated to compare the *in vitro* release profiles. The FDA has recommended the calculation of the f2 and f1 factors for modified release solid oral dosage forms (FDA, 1997) with f2 values greater than 50 suggesting similarity while f1 values less than 15 suggesting no difference (Riley et al., 2012). Only similarity factor values were reported because in all cases where the f2 values indicated similarity (f2 > 50), the f1 values indicated no difference (f1 < 15).

3. Results

3.2. Structure of liposomal Cf formulations via microscopic technique

3.1.1. Physical state of Cf/drug in liposomal Cf formulations via TEM

Control samples (liposomal Cf formulations (liquid)) showed uniform spherical vesicles with an approximate size of 90nm. These vesicles represent a supersaturated solution of Cf encapsulated in liposomes (Fig. 1. A). As a result of spray drying, elongated liposomes were formed with the rod-shaped structure (drug) in the core (Fig. 1. C - E). In comparison, spray dried empty liposomes did not show these elongated features (Fig. 1. B). These rod-shaped structures were formed due to in situ crystallization of Cf within the drying liposomal vesicles. Cf crystals were mostly between 100 and 200 nm in length and resided inside intact liposomal vesicles. However, the percent of encapsulated Cf nanocrystals was dependant on the amount of sucrose present in the formulation; empty vesicles were seen in the formulations with low sucrose content (Fig. 1. C). This highlights the importance of including sucrose in the proper amount to protect the integrity of liposomes during the process of spray drying.

3.1.2. Surface morphology of liposomal Cf formulations via SEM

The spray dried powders showed agglomerates of smooth spherical particles which are mostly below 2 µm (Fig. 2. A, B and C). In addition, needle like structures protruding from the particles were also observed in the formulation with the lowest sucrose content (white arrows in Fig. 2. C) which may be related to the emergence of the encapsulated Cf from the liposomes.

3.3. Particle size distribution of different formulations

3.3.1. Particle size distribution of liposomal Cf formulations (liquids)

The control (liposomal Cf formulations (liquid)) sample contained monodisperse liposomes with a mean particle diameter of approximately 100 nm (Table 5). On the other hand, spray-dried liposomal Cf formulation without sucrose (solid control) contained very large liposomal particles after reconstitution. The liposomes particle size of the spray-dried samples with three varying sucrose amounts increased by about 18 to 33 nm from the liquid control, while the polydispersity index did not change from 0.1 (in the liquid control) for the formulation with the most sucrose but increased to 0.6 in the lowest sucrose-containing formulation. Thus, in order to maintain monodisperse liposomes with minimal deviation in size

from the starting aqueous liposomal dispersion, sucrose must be present in an amount greater than that of lipids in the formulation.
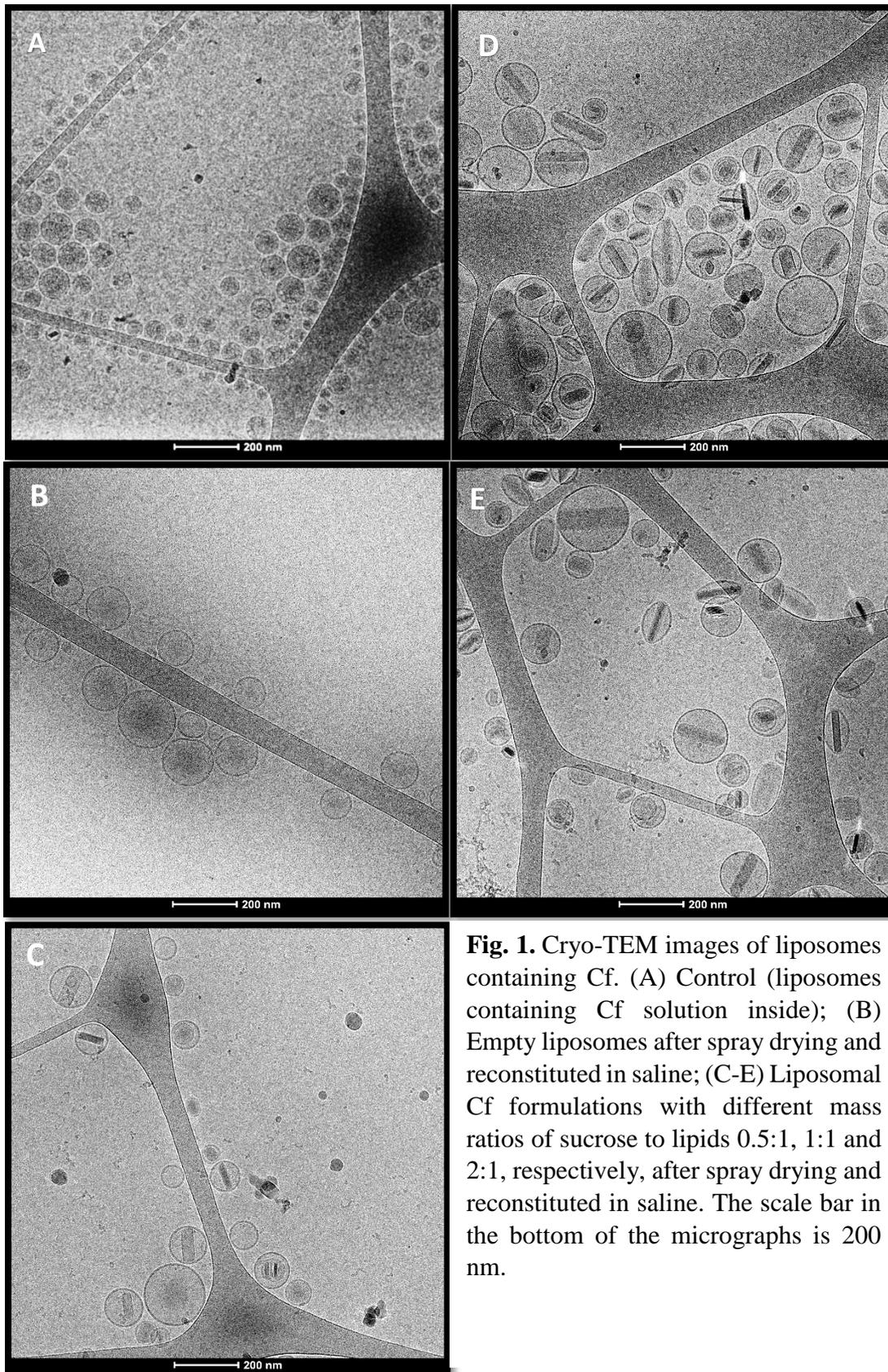

Fig. 1. Cryo-TEM images of liposomes containing Cf. (A) Control (liposomes containing Cf solution inside); (B) Empty liposomes after spray drying and reconstituted in saline; (C-E) Liposomal Cf formulations with different mass ratios of sucrose to lipids 0.5:1, 1:1 and 2:1, respectively, after spray drying and reconstituted in saline. The scale bar in the bottom of the micrographs is 200 nm.
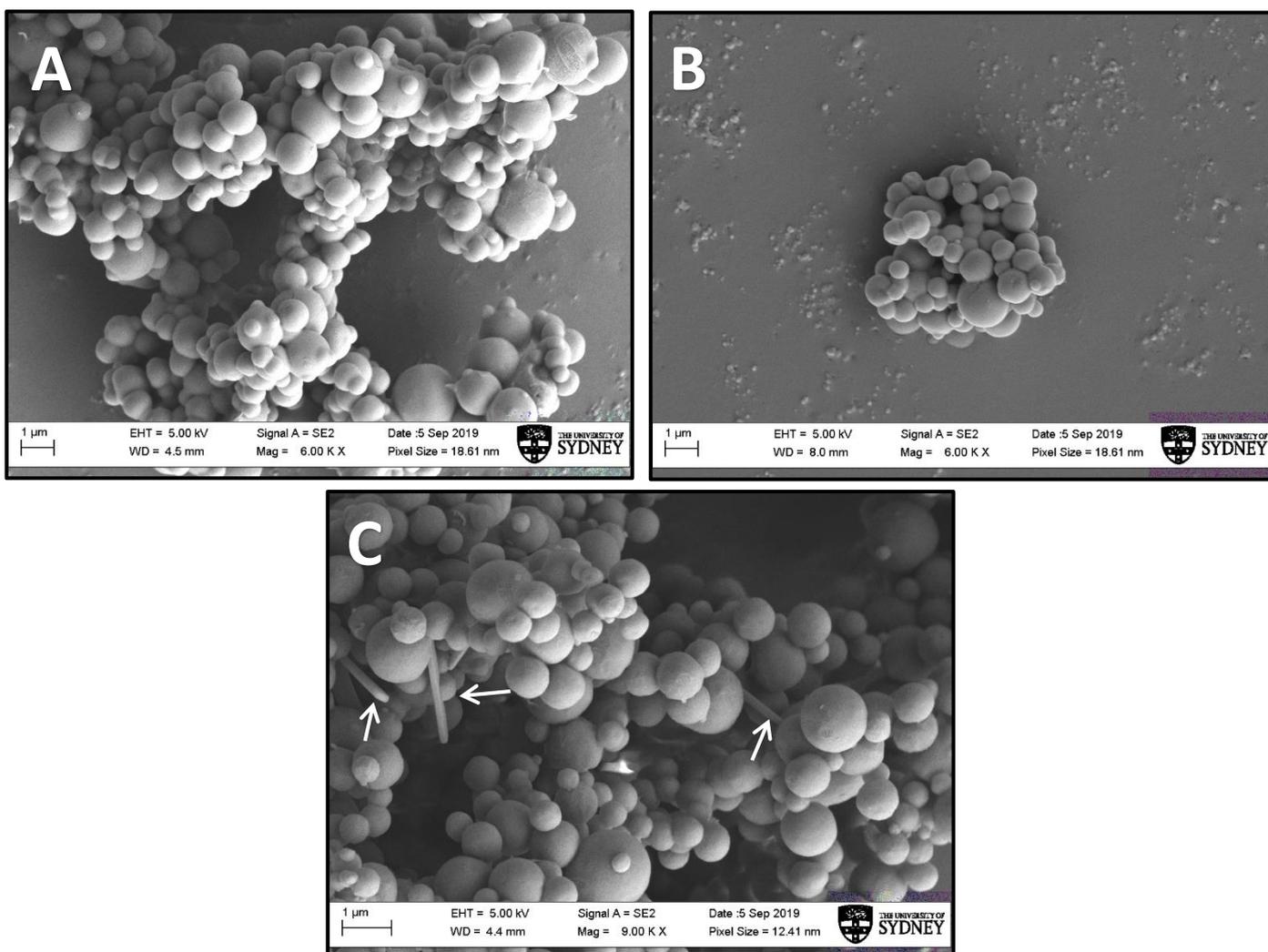
Fig. 2. SEM micrographs of Cf nanocrystal liposomal dry powders containing different mass ratios of sucrose to lipids: (A) 2:1; (B) 1:1 and; (C) 0.5:1(white arrows refer to needle-like structures of Cf).

Table 5
Size distribution of liposomes by dynamic light scattering for original (liquid control), spray dried original (solid control) and spray dried samples with sucrose (n=3).

Sample type (sucrose: lipid) (w/w)	Particle size (nm) Mean [RSD%]	Polydispersity index Mean [SD]
Liquid control (0:1)	105 [3.2]	0.1 [0.04]
Solid (0:1)	1203 [9.7]	0.4 [0.05]
Spray dried (0.5:1)	123 [2.9]	0.6 [0.03]
(1:1)	138 [3.7]	0.2 [0.02]
(2:1)	123 [2.7]	0.1 [0.04]

3.3.2. Particle size distribution of liposomal Cf formulations (dry powders)

The laser diffraction results revealed the presence of respirable particles with a D_{90} less than 3 µm and a narrow size distribution in the three different formulations (Table 6). These data are in good agreement to that previously reported for spray-dried liposomal formulations for inhalation (Lo et al., 2004; Tang et al., 2015).

Table 6
Particle size distribution of Cf nanocrystal liposomal dry powders. Mean [SD], n=3.

Sucrose: lipid (w/w)	D_{10} (µm)	D_{50} (µm)	D_{90} (µm)	Span
0.5:1	0.37 [0.006]	0.72 [0.02]	1.59 [0.095]	1.68 [0.07]
1:1	0.39 [0.002]	0.77 [0.005]	1.74 [0.01]	1.76 [0.003]
2:1	0.47 [0.03]	1.04 [0.06]	2.31 [0.08]	1.78 [0.13]

3.4. Encapsulation efficiency (EE%) of Cf/drug in liposomal Cf formulations

According to previous literature reports (Cipolla et al., 2016b), 99% of Cf is encapsulated within the liposomal vesicles in a soluble form in liposomal Cf formulations (liquid). After spray drying to produce the encapsulated Cf nanocrystals, the decrease in encapsulated Cf was 12% when the optimum amount of sucrose was used during drying (Table 7).

Table 7
Encapsulation efficiency (EE) and moisture content of Cf nanocrystal liposomal dry powders. Mean [SD], n=3.

Sucrose: lipid (w/w)	EE (%)	Moisture Content (wt. %)
0.5:1	44.1 [4.4]	1.5 [0.3]
1:1	80.8 [3.5]	1.8 [0.3]
2:1	87.8 [1.3]	2.0 [0.5]

3.5. Thermal analysis of of liposomal Cf formulations (dry powders)

3.5.1. Moisture content of liposomal Cf formulations via TGA

All spray dried formulations showed a low moisture content of 1.5-2% w/w (Table 7). Despite the variation in the sucrose content in the three formulations, their residual moisture contents were not significantly different.

3.5.2. Thermal properties of liposomal Cf formulation via DSC

DSC thermograms of the raw and spray dried Cf are shown in Fig. 3A. The first endothermic peak around 150°C for the raw Cf powder was attributed to dehydration of Cf HCl monohydrate. The characteristic melting peak of Cf appeared as the second endothermic peak between 315°C to 330°C. For the spray dried Cf, the endothermic melting peak was observed around 320°C which was preceded by the exothermic peak around 198°C as indicative of a phase transition of amorphous Cf (Shetty et al., 2018). In the thermograms of the three formulations (Fig. 3B), the endothermic peak around 158 °C can be ascribed to the dehydration of Cf HCl monohydrate nanocrystals within the liposomal particles. A small exothermic peak was observed around 189 °C in the formulation with mass ratio 0.5:1 sucrose to lipids but not in the other two formulations. This peak is indicative of a phase transition of the higher amount of amorphous Cf leaked out from the liposomal vesicles during spray drying due to insufficient lyoprotectant. Spray dried sucrose exhibits an initial step change corresponding to the glass transition (Tg) close to 50 °C (Fig. 3A), which agrees with the Tg range reported in literature (Jawad et al., 2018). Above the glass transition, the mobility of sucrose molecules increases sufficiently to initiate nucleation and subsequent growth into crystals, with a re-crystallisation exothermic peak appeared at ~124 °C. The sucrose sample started to melt at ~185–200 °C followed by degradation between 220 and 250 °C (Jawad et al., 2018). This confirms the formation of a wholly amorphous spray dried sucrose sample after spray drying. In the three formulations (Fig. 3B), an exothermic broad peak appeared at ~128 °C which was corresponding to re-crystallization of amorphous sucrose. The endothermic peak at ~ 170 – 190 °C showed the melting of the sucrose component, with the peak area decreasing with the sugar content in the formulation. After exposure to 90% RH in the DVS, the three formulations still showed similar thermal events of amorphous sucrose and crystalline ciprofloxacin observed in the samples prior to moisture exposure. However, there was an extra endothermic peak starting at ~113 °C which was ascribed to water loss as supported by the mass drop at that temperature in the TGA. Overall, the formulations absorbed moisture when exposed to high RH but did not show recrystallization.
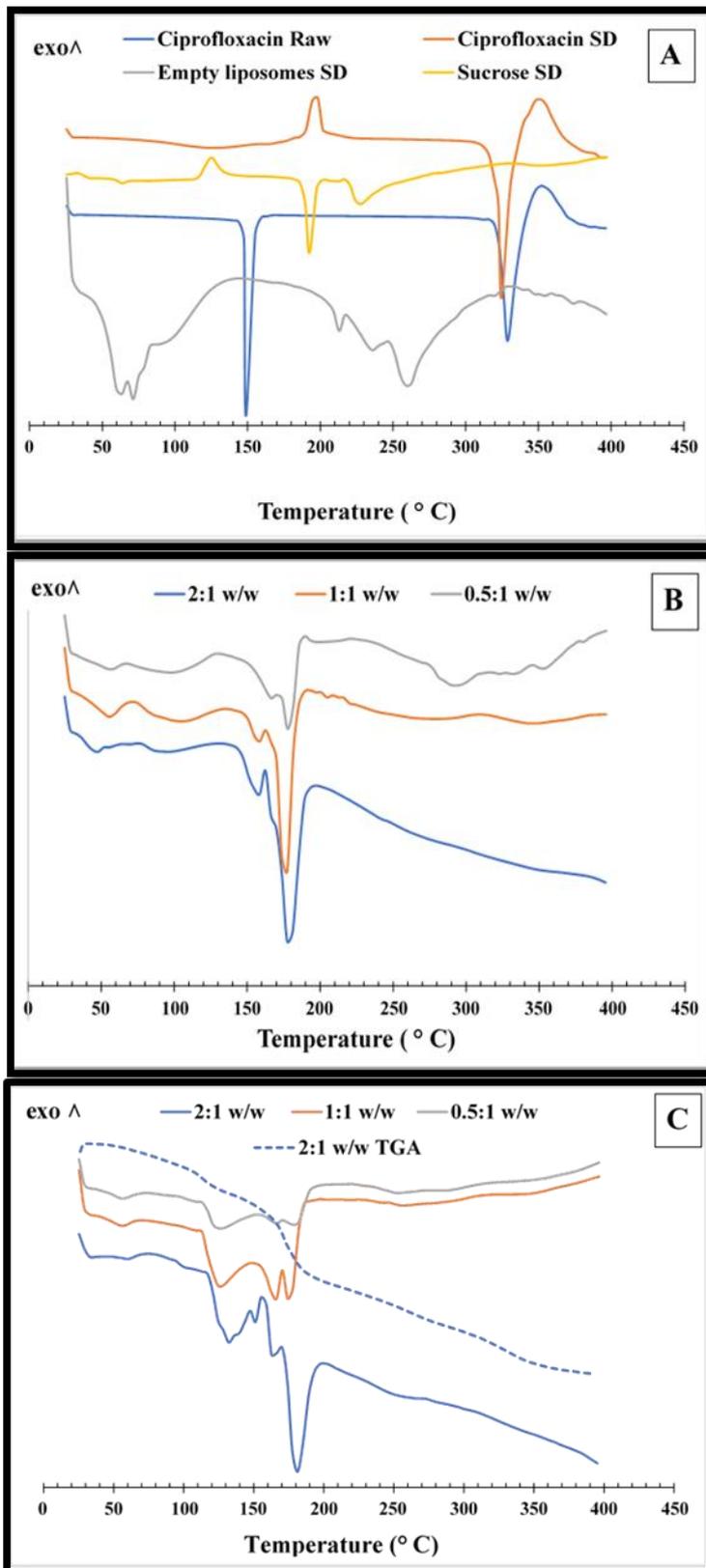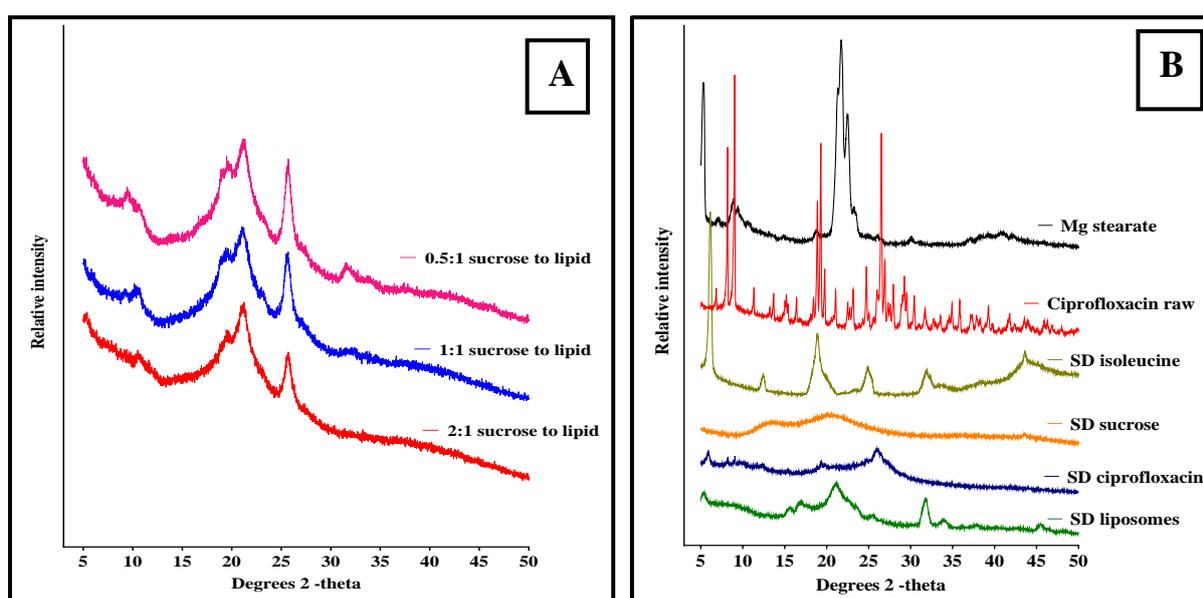
Fig. 3. DSC of: (A) Cf nanocrystal liposomal dry powders containing different sucrose to lipids mass ratios (0.5:1, 1:1, 2:1) stored in desiccator; (B) spray dried raw materials (sucrose, empty liposomes, Cf) and neat crystalline ciprofloxacin (raw) were included for comparison; (C) Cf nanocrystal liposomal powders containing different sucrose to lipids mass ratios (0.5:1, 1:1, 2:1) after DVS, TGA of Cf nanocrystals liposomal powder of mass ratio 2:1 after DVS.

3.6. *Solid-state of spray dried liposomal Cf formulations*

The X-ray diffraction patterns of the CNL dry powder formulations, the spray dried empty liposomes and the Mg stearate all showed a peak at 2θ around 21° (Fig. 4. A and B). This peak in the CNL powder formulations is attributed to liposomes (HSPC and cholesterol) as Mg stearate was present at only about 2% wt. in the powder. The diffraction peak observed in the three formulations at around 26° is attributed to the spray dried Cf; it showed a higher intensity in the lowest sucrose containing formulation. The overall diffraction pattern of all three formulations revealed a partially crystalline state despite the different amounts of amorphous sucrose.

Fig. 4. X-ray powder diffraction patterns of (A) Cf nanocrystal liposomal dry powders containing different sucrose to lipids mass ratios (0.5:1, 1:1, 2:1); (B) spray dried raw materials (isoleucine, sucrose, empty liposomes, Cf), Mg stearate and neat crystalline ciprofloxacin (raw) were included for comparison.

3.7. *Moisture sorption behavior of spray dried liposomal Cf formulations*

In the three formulations, the sample weight increased gradually with the increase in the RH (Fig. 5). At the highest RH (90%), the powder with the highest sucrose content showed a maximum mass increase of 48%. A reversible moisture sorption trend was observed in all formulations. Furthermore, no moisture-induced recrystallization occurred in any of the formulations as also confirmed by the DSC results in Section 3.4.

3.8. In vitro aerosol performance

When the formulations were dispersed by the Osmohaler, the fine particle fraction (FPF %) and percent emitted dose (ED %) were in the range of 58.7 – 64.4 % and 85.0 – 90.7 %, respectively (Table 8). The fine particle dose (FPD) of Cf ranged from 2.6 to 4.1 mg per capsule containing 30 mg powder of which only 4.5 to 7.0 mg represented Cf. The powders showed a high potential to reach the lower airways and the alveolar region, as the MMAD of the formulations ranged between 2.6 and 2.9 μm. No significant differences ($P > 0.05$) in the aerosol performance were observed between the different formulations despite the varying sucrose content (Fig. 6).
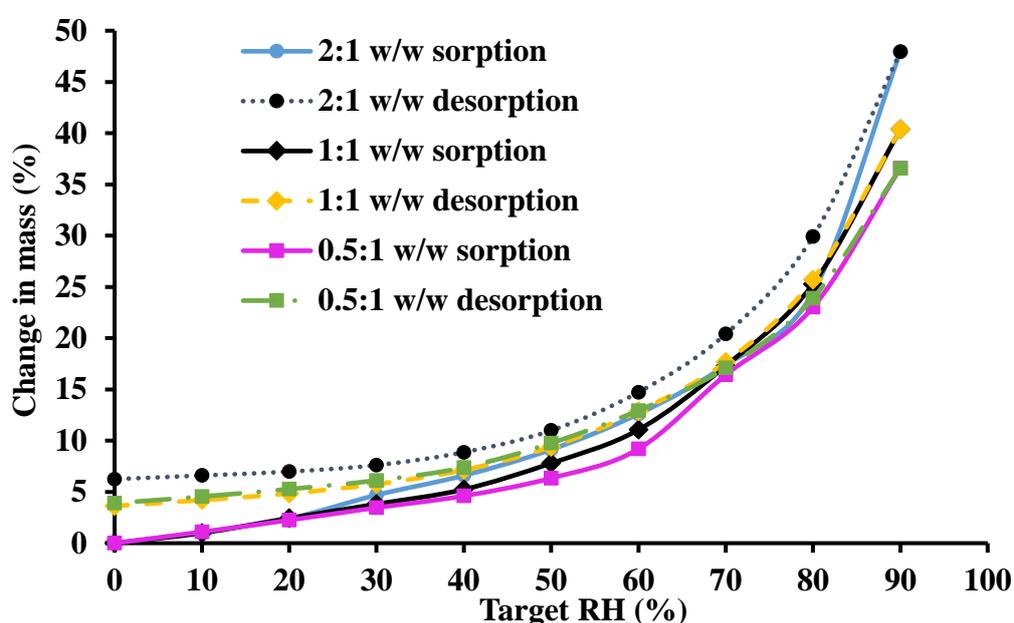
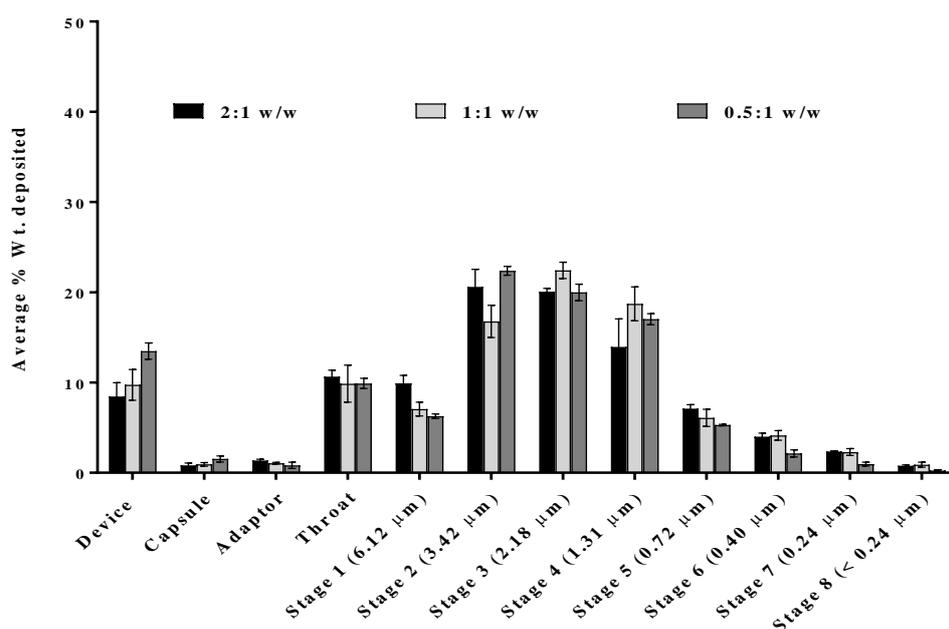**Fig. 5.** Dynamic water sorption behavior of Cf nanocrystals liposomal dry powders containing different mass ratios of sucrose to lipid: 0.5:1, 1:1 and 2:1.

Table 8
Aerosol performance of Cf nanocrystal liposomal dry powders. Mean [SD], n=3.

Sucrose: lipid (w/w)	ED[a]%	MMAD[b] (μm)	FPF[c]%	FPD[d] (mg)
0.5:1	85.0 [0.9]	2.90 [0.03]	58.7 [1.1]	4.13 [0.2]
1:1	89.3 [1.9]	2.57 [0.13]	64.4 [1.8]	3.87 [0.2]
2:1	90.7 [1.3]	2.88 [0.19]	60.3 [2.5]	2.63 [0.3]

ED[a]: Emitted dose.   FPF[c]: Fine particle fraction.
FPD[d]: Fine particle dose.   MMAD[b]: Mass median aerodynamic diameter.

3.9. In vitro release of Cf from liposomes

The drug release profiles of the three formulations (Fig. 7) revealed 90% of the drug was released in the first hour for the lowest sucrose containing formulation (0.5:1 w/w sucrose to lipids). In contrast, the powder formulation with a 2:1 w/w sucrose to lipid ratio showed a slower release rate in comparison to the liquid control formulation after the first 30 minutes. The powder formulation with a 1:1 w/w sucrose to lipids ratio also showed a slower release in comparison to the liquid control after the first hour.

Fig. 6. The aerosolization performance of Cf nanocrystals liposomal dry powders containing different mass ratios of sucrose to lipid: 0.5:1, 1:1 and 2:1. Mean with SD, n=3.

Similarity factor (f2) analysis was implemented to obtain quantitative data for comparing the differences in release rates (Table 9). The f2 of the dry powder formulation with the highest sucrose amount was 38, which is considerably below the cut-off value of 50, indicating a statistically significant difference between the release profile of this formulation and the control liquid formulation. The release profile of the dry powder formulation (0.5:1 w/w sucrose to lipids) was similar (f2 >50) to the controls release profile. On the other hand, the dry powder formulation with 1:1 w/w sucrose to lipids ratio was not similar to the liquid control with an f2 value of 48, just reaching significance. These results suggest that a sucrose amount comparable to or exceeding the lipids content should be considered during spray drying to achieve a prolonged release of Cf.

Table 9
Similarity factor analysis (f2) for powder formulations of liposomal Cf nanocrystals with different amount of sucrose versus formulations of liposomal Cf without nanocrystals (liquid and powder controls).

Test (sucrose: lipid) (w/w)	Reference (sucrose: lipid) (w/w)	
	Liquid control (0:1)	Powder control (0:1)
Spray dried (0.5:1)	71	54
(1:1)	48	51
(2:1)	38	49

4. Discussion

We have demonstrated a simple process of spray drying to convert Cf liposomal dispersions into dry powders which generate single drug nanocrystals inside the liposomes when reconstituted in saline as confirmed by the Cryo-TEM. Cipolla et al (2016b) reported the use of a freeze-thaw cycle to generate Cf nanocrystals in an aqueous liposome preparation. Subsequently, freeze-thaw followed by spray drying (FT-SD) was utilized to develop dry powder formulations of Cf nanocrystals in liposomes (Khatib et al., 2019). The present study showed it is feasible to obtain similar dry powders without the need for the freeze-thaw step, thus simplifying the production into a single-step process.

When processing liposomes, the vesicle integrity must be maintained to avoid rupturing and subsequent drug leakage, intact vesicles will also act as the rate-controlling barrier for the formation of single drug nanocrystals. However, spray drying involves atomization of the liposomal formulation with exposure to mechanical stress and generation of air-liquid interface. Furthermore, the vesicles are exposed to osmotic stress resulting from an increase in the concentration of the solutes during drying. Thus, some liposomal particles may rearrange into larger vesicles as a response to the limited distances between drying particles (Stark et al., 2010). Sucrose as lyoprotectant can decrease the stresses of spray drying via hydrogen bonding with the lipids and/or reducing the lipid mobility in a vitrified state. As a result, liposomes could maintain the initial uniform size distribution of the starting liposomal dispersion (Ingvarsson et al., 2011). Moreover, sucrose has been shown to preserve most of the encapsulated drug inside liposomes (Wessman et al., 2010). Despite the possible instability of liposomes during spray drying, our previous report revealed that ~ 80% of Cf was encapsulated if a 2:1 w/w sucrose to lipids ratio was used during a combined process of freeze-thaw and spray drying (FT-SD) (Khatib et al., 2019). Our present data showed that spray drying

liposomes with the same sucrose to lipids ratio produced Cf nanocrystals with a better encapsulation value of 87%, and the change in the size characteristics of the spray dried liposomes following rehydration was minimal.
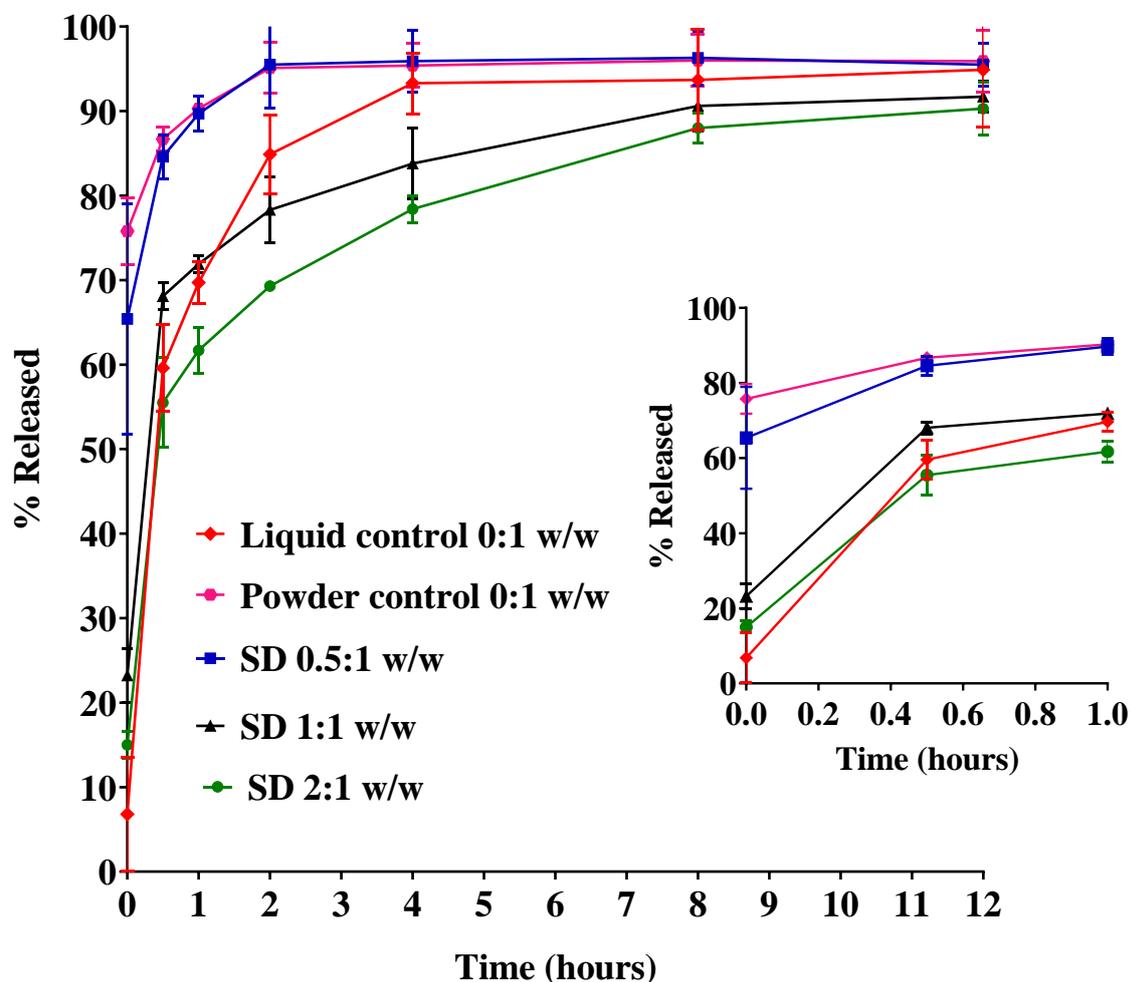

Fig. 7. *In vitro* release profiles of spray dried liposomal formulations containing different mass ratios of sucrose to lipid (0.5:1, 1:1 and 2:1) in comparison to the original sample (liquid and powder controls). Mean with SD, n=3. The inset magnifies the drug release during the first hour.

Modulating Cf release is the core objective of developing the crystalline form of the drug inside the liposomes. Previous studies showed a slower dissolution rate of Cf if it was in the nanocrystalline form within the liposomal vesicles (Cipolla et al., 2016b; Fugit et al., 2015). The *in vitro* release data revealed a significantly prolonged release of Cf from liposomal powders (at 2:1 w/w sucrose to lipids ratio) produced by spray drying. Similar results were reported for the dry powder formulation of liposomal Cf nanocrystals developed by the combined FT-SD cycle. The nanocrystal powder formulation produced by the present single-step process (spray drying) exhibited improved control on the drug release, as almost complete

release (90%) was achieved after 12 hours in comparison to 9 hours for the formulation produced by FT-SD (Khatib et al., 2019). In this *in vitro* release method, the interaction of the liposomal membrane bilayer with the serum lipoproteins and apolipoproteins in the dissolution medium, which are also present in lung fluid, is thought to result in drug release over time (Cipolla et al., 2014).

A dry powder formulation of CNL produced by spray drying was characterized to determine its suitability for pulmonary drug delivery. Residual moisture can affect the storage stability of powders, as water acts as a plasticizer to decrease the glass transition temperature (Tg) of the amorphous contents in the spray dried powder, leading to recrystallization and poor dispersion of the powder as an aerosol for inhalation. The TGA results indicated a low moisture content of *ca* 2 % wt. in the spray dried powders. No signs of recrystallization were observed as the spray dried powders were exposed to elevated RH. This is probably due to the presence of Mg stearate and isoleucine which both prefer to migrate to and occupy the surface of the particles during drying. Consequently, the thermodynamically unstable regions containing amorphous sucrose are less likely to be exposed on the surface and thus the shelf-life stability may be improved (Chan and Chew, 2003; Li et al., 2016; Yu et al., 2017). All the spray dried powders showed fine particle size in the inhalable range (D90 < 3 μm) which was due to the low total solid concentration (~3 mg mL^{-1}) of the starting formulation. The formulations comprise the fine particles in the form of agglomerates as observed in the SEM images. Agglomeration of particles can negatively impact the dispersion performance of a dry powder formulation. The dispersability will be dependent on the agglomerate strength, so weak agglomerates will be easier to be dispersed into fine particles (Adi et al., 2011). The aerosol performance of the three powder formulations showed FPF values exceeding 50%. Our data are in line with previous reports showing better aerosolization for liposomes spray dried with sucrose than with other lyoprotectants such as lactose and trehalose (Lo et al., 2004). Moreover, powder dispersability and flowability were enhanced by the incorporation of Mg stearate and isoleucine in the formulations (Chan and Chew, 2003; Yu et al., 2017).

In previous studies, soluble Cf was converted into nanocrystals inside liposomes by a freeze-thaw cycle. Concentrated Cf remained soluble inside the liposomes, but during freezing ice crystals act as nucleation sites for the transformation of the drug to nanocrystals (Cipolla et al., 2016b; Lasic et al., 1995; Maurer et al., 1998). In the present study, CNL were produced by spray drying which does not involve any ice crystal nuclei. We hypothesize that as Cf in the spray droplets becomes increasingly concentrated during drying, the supersaturation level is

increased to the threshold of self-association into nuclei followed by crystal growth. Crystal formation likely has been initiated from a single nucleus inside every liposome, as only one nanocrystal was observed in the majority of them. As the growing crystals become elongated, the liposomes still remain intact and are able to accommodate the elongation due to the flexibility of the lipid membrane; otherwise, the liposomes will rupture and crystallization will occur outside the vesicles as was evident in the SEM images of the formulation containing the lowest amount of sucrose. In the latter case, needle-like structures were seen protruding from the spray dried particles, possibly due to the inter-liposomal and/or intra-liposomal growth of Cf nanocrystals.

The main advantage of CNL in treating lung infections will be a convenient once-daily inhaled DPI dose regimen. The non-crystalline nebulized formulation administered once daily showed significant reduction in *Pseudomonas aeruginosa* colonization *in vivo* (Cipolla et al., 2015; Haworth et al., 2019; Serisier et al., 2013). *In vitro* drug release experiments revealed a slower release of the drug in the nanocrystalline form compared with the non-crystalline form (Cipolla et al., 2016b; Khatib et al., 2019). As described by Khatib et al. (2019), two once-daily inhalations of the CNL produced by the combined FT-SD method would be expected to be effective based on the calculation of the dose delivered to the lung and the drug concentrations needed to exceed the pathogen MIC. The *in vitro* aerosol performance of CNL powder produced by spray drying is similar to those produced by the combined FT-SD method. Thus, a once-daily treatment scheme should also be applicable with the CNL produced by spray drying. This paradigm should be at least as convenient to patients as compared to the four inhalations required twice-daily using the TOBI Podhaler (VanDevanter and Geller, 2011). In addition, a liposomal formulation containing drug nanocrystals may retain sufficient dose of the drug inside liposomes prior to being phagocytosed by macrophages (Blanchard et al., 2018). A higher uptake by alveolar macrophages was reported for the amikacin liposomal formulation (Arikayce[®]) than for free drug alone (Zhang et al., 2018). Consequently, the nanocrystalline form of the drug inside liposomal vesicles may be useful for improved eradication of intracellular non-tuberculous mycobacteria.

5. Conclusions

Nanocrystallization of drug within liposomes was successfully induced by a simple one step process of spray drying. A dry powder preparation of liposomes (containing 2:1 w/w sucrose to lipids ratio) was produced in which a single drug nanocrystal is located inside each

vesicle after reconstitution in saline. The stability of liposomes as well as the retention of Cf nanocrystals inside liposomes were controlled using sucrose as a lyoprotectant. The spray dried powder was inhalable and suitable for aerosol delivery. It showed a prolonged drug release over 12 hours in the IVR assay which suggests that it may be amenable to a once daily treatment regimen enhancing patient adherence to treatment.

References

Adi, S., Adi, H., Chan, H.-K., Finlay, W.H., Tong, Z., Yang, R., Yu, A., 2011. Agglomerate strength and dispersion of pharmaceutical powders. Journal of Aerosol Science 42, 285-294.

Allen, T.M., Cullis, P.R., 2013. Liposomal drug delivery systems: from concept to clinical applications. Advanced drug delivery reviews 65, 36-48.

Barenholz, Y.C., 2012. Doxil®—the first FDA-approved nano-drug: lessons learned. Journal of controlled release 160, 117-134.

Blanchard, J.D., Elias, V., Cipolla, D., Gonda, I., Bermudez, L.E., 2018. Effective Treatment of Mycobacterium avium subsp. hominissuis and Mycobacterium abscessus Species Infections in Macrophages, Biofilm, and Mice by Using Liposomal Ciprofloxacin. Antimicrobial agents and chemotherapy 62, e00440-00418.

Chan, H.-K., Chew, N.Y., 2003. Novel alternative methods for the delivery of drugs for the treatment of asthma. Advanced Drug Delivery Reviews 55, 793-805.

Chew, N.Y., Chan, H.-K., 2001. In vitro aerosol performance and dose uniformity between the Foradile® Aerolizer® and the Oxis® Turbuhaler®. Journal of aerosol medicine 14, 495-501.

Chougule, M., Padhi, B., Misra, A., 2008. Development of spray dried liposomal dry powder inhaler of dapsone. Aaps Pharmscitech 9, 47-53.

Cipolla, D., Gonda, I., Chan, H.-K., 2013. Liposomal formulations for inhalation. Therapeutic delivery 4, 1047-1072.

Cipolla, D., Wu, H., Eastman, S., Redelmeier, T., Gonda, I., Chan, H.-K., 2016a. Tuning Ciprofloxacin Release Profiles from Liposomally Encapsulated Nanocrystalline Drug. Pharmaceutical research 33, 2748-2762.

Cipolla, D., Wu, H., Eastman, S., Redelmeier, T., Gonda, I., Chan, H.K., 2014. Development and characterization of an in vitro release assay for liposomal ciprofloxacin for inhalation. Journal of Pharmaceutical Sciences 103, 314-327.

Cipolla, D., Wu, H., Gonda, I., Chan, H.-K., 2015. Aerosol performance and stability of liposomes containing ciprofloxacin nanocrystals. Journal of aerosol medicine and pulmonary drug delivery 28, 411-422.

Cipolla, D., Wu, H., Salentinig, S., Boyd, B., Rades, T., Vanhecke, D., Petri-Fink, A., Rothin-Rutishauser, B., Eastman, S., Redelmeier, T., Gonda, I., Chan, H.-K., 2016b. Formation of drug nanocrystals under nanoconfinement afforded by liposomes. Rsc Advances 6, 6223-6233.

Dzieciuch-Rojek, M., Poojari, C., Bednar, J., Bunker, A., Kozik, B., Nowakowska, M., Vattulainen, I., Wydro, P., Kepczynski, M., Róg, T., 2017. Effects of membrane PEGylation on entry and location of antifungal drug itraconazole and their pharmacological implications. Molecular pharmaceutics 14, 1057-1070.

FDA, S., 1997. Guidance for Industry: SUPAC-MR: Modified Release Solid Oral Dosage Forms; Scale-Up and Post-Approval Changes: Chemistry, Manufacturing and Controls. Vitro Dissolution Testing, and In Vivo Bioequivalence Documentation.

Fugit, K.D., Xiang, T.-X., Choi, D.H., Kangarlou, S., Csuhai, E., Bummer, P.M., Anderson, B.D., 2015. Mechanistic model and analysis of doxorubicin release from liposomal formulations. Journal of Controlled Release 217, 82-91.

Goldbach, P., Brochart, H., Stamm, 1993. Spray-drying of liposomes for a pulmonary administration. I. Chemical stability of phospholipids. Drug development and industrial pharmacy 19, 2611-2622.

Haworth, C.S., Bilton, D., Chalmers, J.D., Davis, A.M., Froehlich, J., Gonda, I., Thompson, B., Wanner, A., O'Donnell, A.E., 2019. Inhaled liposomal ciprofloxacin in patients with non-cystic fibrosis bronchiectasis and chronic lung infection with Pseudomonas aeruginosa (ORBIT-3 and ORBIT-4): two phase 3, randomised controlled trials. The Lancet Respiratory Medicine 7, 213-226.

Ingvarsson, P.T., Yang, M., Nielsen, H.M., Rantanen, J., Foged, C., 2011. Stabilization of liposomes during drying. Expert Opinion on Drug Delivery 8, 375-388.

Jawad, R., Elleman, C., Martin, G.P., Royall, P.G., 2018. Crystallisation of freeze-dried sucrose in model mixtures that represent the amorphous sugar matrices present in confectionery. Food & function 9, 4621-4634.

Jog, R., Burgess, D.J., 2017. Pharmaceutical amorphous nanoparticles. Journal of pharmaceutical sciences 106, 39-65.

Jog, R., Burgess, D.J., 2018a. Excipients used in oral nanocarrier-based formulations, Fundamentals of Nanoparticles. Elsevier, pp. 279-342.

Jog, R., Burgess, D.J., 2018b. Nanoamorphous drug products–Design and development. International Journal of Pharmaceutics 553, 238-260.

Jog, R., Burgess, D.J., 2019. Comprehensive quality by design approach for stable nanocrystalline drug products. International journal of pharmaceutics 564, 426-460.

Khatib, I., Khanal, D., Ruan, J., Cipolla, D., Dayton, F., Blanchard, J.D., Chan, H.-K., 2019. Ciprofloxacin nanocrystals liposomal powders for controlled drug release via inhalation. International journal of pharmaceutics 566, 641-651.

Kim, J.-C.K., Jong-Duk, 2001. Preparation by spray drying of amphotericin B-phospholipid composite particles and their anticellular activity. Drug delivery 8, 143-147.

Lasic, D., Čeh, B., Stuart, M., Guo, L., Frederik, P., Barenholz, Y., 1995. Transmembrane gradient driven phase transitions within vesicles: lessons for drug delivery. Biochimica et Biophysica Acta (BBA)-Biomembranes 1239, 145-156.

Li, L., Sun, S., Parumasivam, T., Denman, J.A., Gengenbach, T., Tang, P., Mao, S., Chan, H.-K., 2016. l-Leucine as an excipient against moisture on in vitro aerosolization performances of highly hygroscopic spray-dried powders. European Journal of Pharmaceutics and Biopharmaceutics 102, 132-141.

Li, T., Cipolla, D., Rades, T., Boyd, B.J., 2018. Drug nanocrystallisation within liposomes. Journal of Controlled Release 228, 96-110.

Li, X., Hirsh, D.J., Cabral-Lilly, D., Zirkel, A., Gruner, S.M., Janoff, A.S., Perkins, W.R., 1998. Doxorubicin physical state in solution and inside liposomes loaded via a pH gradient. Biochimica et Biophysica Acta (BBA)-Biomembranes 1415, 23-40.

Lo, Y.-l., Tsai, J.-c., Kuo, J.-h., 2004. Liposomes and disaccharides as carriers in spray-dried powder formulations of superoxide dismutase. Journal of Controlled Release 94, 259-272.

Maurer, N., Wong, K.F., Hope, M.J., Cullis, P.R., 1998. Anomalous solubility behavior of the antibiotic ciprofloxacin encapsulated in liposomes: a 1H-NMR study. Biochimica et Biophysica Acta (BBA)-Biomembranes 1374, 9-20.

Misra, A., Jinturkar, K., Patel, D., Lalani, J., Chougule, M., 2009. Recent advances in liposomal dry powder formulations: preparation and evaluation. Expert opinion on drug delivery 6, 71-89.

Niven, R.W., Schreier, H., 1990. Nebulization of liposomes. I. Effects of lipid composition. Pharmaceutical research 7, 1127-1133.

Ong, H.X., Benaouda, F., Traini, D., Cipolla, D., Gonda, I., Bebawy, M., Forbes, B., Young, P.M., 2014. In vitro and ex vivo methods predict the enhanced lung residence time of liposomal ciprofloxacin formulations for nebulisation. European Journal of Pharmaceutics and Biopharmaceutics 86, 83-89.

Pace, J.R., Jog, R., Burgess, D.J., Hadden, M.K., 2019. Formulation and evaluation of itraconazole liposomes for Hedgehog pathway inhibition. Journal of Liposome Research, 1-7.

Patel, G., Chougule, M., Singh, M., Misra, A., 2009. Nanoliposomal dry powder formulations. Methods in enzymology 464, 167-191.

Riley, T., Christopher, D., Arp, J., Casazza, A., Colombani, A., Cooper, A., Dey, M., Maas, J., Mitchell, J., Reiners, M., 2012. Challenges with developing in vitro dissolution tests for orally inhaled products (OIPs). AAPS PharmSciTech 13, 978-989.

Schilt, Y., Berman, T., Wei, X., Barenholz, Y., Raviv, U., 2016. Using solution X-ray scattering to determine the high-resolution structure and morphology of PEGylated liposomal doxorubicin nanodrugs. Biochimica et Biophysica Acta (BBA)-General Subjects 1860, 108-119.

Serisier, D.J., Bilton, D., De Soyza, A., Thompson, P.J., Kolbe, J., Greville, H.W., Cipolla, D., Bruinenberg, P., Gonda, I., investigators, O.-. 2013. Inhaled, dual release liposomal ciprofloxacin in non-cystic fibrosis bronchiectasis (ORBIT-2): a randomised, double-blind, placebo-controlled trial. Thorax 68, 812-817.

Shetty, N., Zeng, L., Mangal, S., Nie, H., Rowles, M.R., Guo, R., Han, Y., Park, J.H., Zhou, Q.T., 2018. Effects of moisture-induced crystallization on the aerosol performance of spray dried amorphous ciprofloxacin powder formulations. Pharmaceutical research 35, 7.

Skalko-Basnet, N., Pavelic, Z., Becirevic-Lacan, M., 2000. Liposomes containing drug and cyclodextrin prepared by the one-step spray-drying method. Drug development and industrial pharmacy 26, 1279-1284.

Stark, B., Pabst, G., Prassl, R., 2010. Long-term stability of sterically stabilized liposomes by freezing and freeze-drying: Effects of cryoprotectants on structure. European Journal of Pharmaceutical Sciences 41, 546-555.

Sweeney, L.G., Wang, Z., Loebenberg, R., Wong, J.P., Lange, C.F., Finlay, W.H., 2005. Spray-freeze-dried liposomal ciprofloxacin powder for inhaled aerosol drug delivery. International journal of pharmaceutics 305, 180-185.

Tang, Y., Zhang, H., Lu, X., Jiang, L., Xi, X., Liu, J., Zhu, J., 2015. Development and evaluation of a dry powder formulation of liposome-encapsulated oseltamivir phosphate for inhalation. Drug Delivery 22, 608-618.

Taylor, K., Taylor, G., Kellaway, I., Stevens, J., 1990. The stability of liposomes to nebulisation. International journal of pharmaceutics 58, 57-61.

VanDevanter, D.R., Geller, D.E., 2011. Tobramycin administered by the TOBI® Podhaler® for persons with cystic fibrosis: a review. Medical devices (Auckland, NZ) 4, 179.

Webb, M.S., Boman, N.L., Wiseman, D.J., Saxon, D., Sutton, K., Wong, K.F., Logan, P., Hope, M.J., 1998. Antibacterial efficacy against an in vivo Salmonella typhimurium infection model and pharmacokinetics of a liposomal ciprofloxacin formulation. Antimicrobial agents and chemotherapy 42, 45-52.

Wessman, P., Edwards, K., Mahlin, D., 2010. Structural effects caused by spray-and freeze-drying of liposomes and bilayer disks. Journal of pharmaceutical sciences 99, 2032-2048.

Willis, L., Hayes, D., Mansour, H.M., 2012. Therapeutic liposomal dry powder inhalation aerosols for targeted lung delivery. Lung 190, 251-262.

Yu, J., Chan, H.-K., Gengenbach, T., Denman, J.A., 2017. Protection of hydrophobic amino acids against moisture-induced deterioration in the aerosolization performance of highly hygroscopic spray-dried powders. European Journal of Pharmaceutics and Biopharmaceutics 119, 224-234.

Zhang, J., Leifer, F., Rose, S., Chun, D.Y., Thaisz, J., Herr, T., Nashed, M., Joseph, J., Perkins, W.R., DiPetrillo, K., 2018. Amikacin Liposome Inhalation Suspension (ALIS) Penetrates Non-tuberculous Mycobacterial Biofilms and Enhances Amikacin Uptake Into Macrophages. Frontiers in Microbiology 9.

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09 January, 2020

Professor. J. Siepmann

Editor-In-Chief, International Journal of Pharmaceutics

Dear Professor Siepmann,

Thank you for reviewing our manuscript entitled **"Formation of ciprofloxacin nanocrystals within liposomes by spray drying for controlled release via inhalation".** We thank for the reviewer's comment. We have made changes according to the comments.

Yours sincerely,

Professor Hak-Kim Chan (Corresponding author)

*Conflict of Interest

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:

This work is related to a provisional patent for the university of Sydney entitled with "Formation of ciprofloxacin nanocrystals within liposomal vesicles by spray drying for controlled drug release via inhalation" (CDIP Ref. Number IP [2019-024]) created by the originators Prof. Hak-Kim Chan and Ms. Isra Khatib.

Authorship Statement

Manuscript title: Formation of ciprofloxacin nanocrystals within liposomes by spray drying for controlled release via inhalation.

All authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript. Furthermore, each author certifies that this material or similar material has not been and will not be submitted to or published in any other publication before its appearance in the *International Journal of Pharmaceutics*.

Authorship contributions

Kim Chan: Initiation, conception and design of study, supervision of the project and revising the manuscript critically for important intellectual content.

Isra Khatib: Conception and design of the study, acquisition of data (conducted the core of most experiments), analysis and interpretation of data, drafting the manuscript.

Patricia Tang: Acquisition of data (conducted SEM experiment).

Juanfang Ruan: Acquisition, analysis and interpretation of data (conducted TEM experiment).

David Cipolla: Design of study, revising the manuscript critically for important intellectual content.

Francis Dayton: Revising the manuscript critically for important intellectual content.

James D. Blanchard: Revising the manuscript critically for important intellectual content.

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