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Storage stability of inhalable, controlled-release powder formulations of ciprofloxacin nanocrystal-containing liposomes

--Manuscript Draft--

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Abstract:	Novel inhalable and controlled release powder formulations of ciprofloxacin nanocrystals inside liposomes (CNL) were recently developed. In the present study, the storage stability of CNL powders consisting of lyoprotectant (i.e. sucrose or lactose), lipids, ciprofloxacin (CIP), and magnesium stearate or isoleucine was investigated. These powders were produced by spray drying, collected in a dry box at < 15% relative humidity (RH), then stored at room temperature and either 4 or 20 %RH. Liposomal integrity, CIP encapsulation efficiency (EE), in vitro drug release (IVR), aerosol performance, and solid-state properties were examined over six months. Sucrose CNL powder exhibited consistent liposomal integrity, aerosol performance, and controlled release of CIP over six months of storage at 4 %RH. However, storage of the powder at 20 %RH for the same period caused sucrose crystallization and consequently a significant drop in EE and aerosol performance (p-values <0.05), along with the IVR of CIP becoming similar to that of the non-crystalline CIP liposomal dispersions (f2 > 50). Lactose CNL maintained superior aerosol performance over the six months irrespective of the storage RH. However, liposomal instability occurred at both RHs within the first month of storage with a significant drop in EE and an increase in liposome size (p-values <0.05). Moreover, the IVR assay of CIP from lactose CNL showed a less controlled release and a substantial difference (f2 < 50) from its initial value after six months regardless of the storage RHs. In conclusion, dry powder inhalers of CNL were physiochemically stable in sucrose lyoprotectant when stored below 4 %RH at room temperature for six months.

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January 28, 2021

Professor Jürgen Siepmann
Editor-in-Chief, International Journal of Pharmaceutics

Dear Professor Siepmann

We would be grateful if you would consider the publication of this manuscript entitled **'Storage stability of inhalable, controlled-release powder formulations of ciprofloxacin nanocrystal-containing liposomes'** in International Journal of Pharmaceutics.

In this manuscript, we described the storage stability of ciprofloxacin nanocrystals inside liposomes (CNL) powders consisting of lyoprotectant (i.e. sucrose or lactose), lipids, ciprofloxacin (Cf), and magnesium stearate or isoleucine. Liposomal dry powders intended for inhalation, especially those with high amorphous sugar content, are vulnerable to the destabilizing effects of temperature and humidity upon storage. The physical instability of such powder affects not only their aerosol performance but also liposomal integrity. The only liposomal formulations encapsulating nanocrystalline Cf investigated for storage stability was the aqueous dispersion of CNL produced by FT. To date, there is no report on the storage stability of CNL powders for inhalation purposes and controlled drug release applications. In this study, we have explored the stability of CNL powders stored at room temperature (RT) with relative humidity (RH) below 23%, and their possible shelf-life without refrigeration. The main finding of this study was that inhalable CNL powder with sucrose but not lactose as the stabilizing excipient possesses at least six-month shelf-life when stored at RT at 4% RH.

Thank you for handling our manuscript.

Yours sincerely,

Professor Hak-Kim Chan (Corresponding author)

COMMENTS TO AUTHOR

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Title: Storage stability of inhalable, controlled-release powder formulations of ciprofloxacin nanocrystal-containing liposomes

Authors: Isra Khatib, Wei-Ren Ke, David Cipolla, Hak-Kim Chan

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

Colour Codes:

Reviewers' Comments: Black

Responses to the comments: Green

Corrections and Text insertions in the manuscript: Red

Reviewer #2:

1- Not convinced. At least such as thorough discussion and stating that the crystallization probably takes place upon reconstitution (and the discussion of your results as presented as an answer) must humbly be included in the text of the manuscript itself.

a) SEM may be a hint, but as you state, these crystals are outside the liposomes and accordingly no proof for CNL!

b) DSC interpretation (3.2.3 B) is poor. The crystalline state cannot be proved with your argumentation. Otherwise, the crystallinity would redurch from day 0 to month 6, where there is no physico-chemical explanation for this behavior! The humble conclusion for your results can only be that it cannot be proved with this technique as different effect overlay (such as dehydration of sugars etc.). A direct representation of pure liposomes and pure API must be included to provide the reader with a chance to not only BELIEVE your interpretation!

c) XRD does not prove the partial (its partiality should be proved by measuring physical mixtures and comparing their diffractograms with those of actual samples, also taking mass variations into account) crystallinity. At least pure (dried) liposomes and pure API must be provided as a direct comparison!

Thank you for the comments again which are related to original comment No. 2 (i.e., 'I do not

see evidence for (nano)crystalline ciprofloxacin (CIP) in the dry product. This must be reasonably explained. If it is just the case that CIP recrystallizes upon reconstitution of the liposomes (as I understand it from the authors, this must be clarified and the in that case.'). We do appreciate the reviewer's comments regarding direct evidence of CIP nanocrystals inside the liposomes in the dry state. To be clear, we have now included the following statements in the revised manuscript.

The nanocrystalline CIP formed during the spray drying step is believed to present in the dry powder form (solid-state). This hypothesis has been based on the SEM, DSC, and X-ray data obtained from our previous publications (Khatib et al., 2021; Khatib et al., 2019; Khatib et al., 2020). First, the images obtained by the SEM showed needle-like structures for liposomal samples spray-dried using the least amount of protectant (sucrose). These structures were representing the growth of CIP nanocrystals outside the liposomal vesicles due to the poor protection because of the suboptimal sugar amount (Khatib et al., 2020). Second, the DSC thermogram of CNL powder revealed a CIP monohydrate peak, this is a typical peak in the DSC thermogram of the crystalline CIP. Finally, the powder x-ray diffraction patterns of CNL powders either formed solely by spray drying or by freeze-thaw (to generate CIP nanocrystals) followed by spray drying (FT-SD) were similar (Khatib et al., 2019; Khatib et al., 2020), and both showed the CIP monohydrate peak. However, these data only provided indirect evidence for our hypothesis. All discussions of CIP nanocrystals were based on data that involved the powder reconstitution step in liquid before testing by Cryo-TEM or measuring EE and IVR. CIP nanocrystals appeared in cryo-TEM images as elongated cylindrical structures within the core of liposomal vesicles (Khatib et al., 2021; Khatib et al., 2019; Khatib et al., 2020). EE was an indirect indicator of the presence of CIP inside the liposomal vesicles. Moreover, the IVR assay revealed that samples with low protectant amounts, which had a low EE and many empty liposomes in the Cryo-TEM images, had an immediate release of CIP (Khatib et al., 2020). In contrast, samples of proper protectants types and amounts had a controlled and delayed CIP release compared to the control (non-nanocrystalline CIP inside liposomes). Slow dissolution and release of crystalline structures entrapped inside a closed environment (liposomes) were evidenced with other crystallized drugs inside liposomes (Cipolla et al., 2016; Li et al., 2018). Taken together, these data did provide strong indirect evidence to show the location of CIP nanocrystals being inside the liposomes. However, these are not direct evidence as such data are currently unavailable due to the lack of analytical techniques that can visualize nanocrystals within liposomes at the nanoscale in the solid-state.

To confirm this hypothesis, we have also attempted further analyses using advanced atomic force microscopy-based infrared spectroscopy (AFM-IR) and focused ion beam (FIB) SEM on the powders to investigate the nanocrystals location in the dry product (**Figures 1 and 2**). Although both techniques confirmed the presence of CIP in the dry samples, they could not inform the location of CIP crystals in relation to the liposomal vesicles.

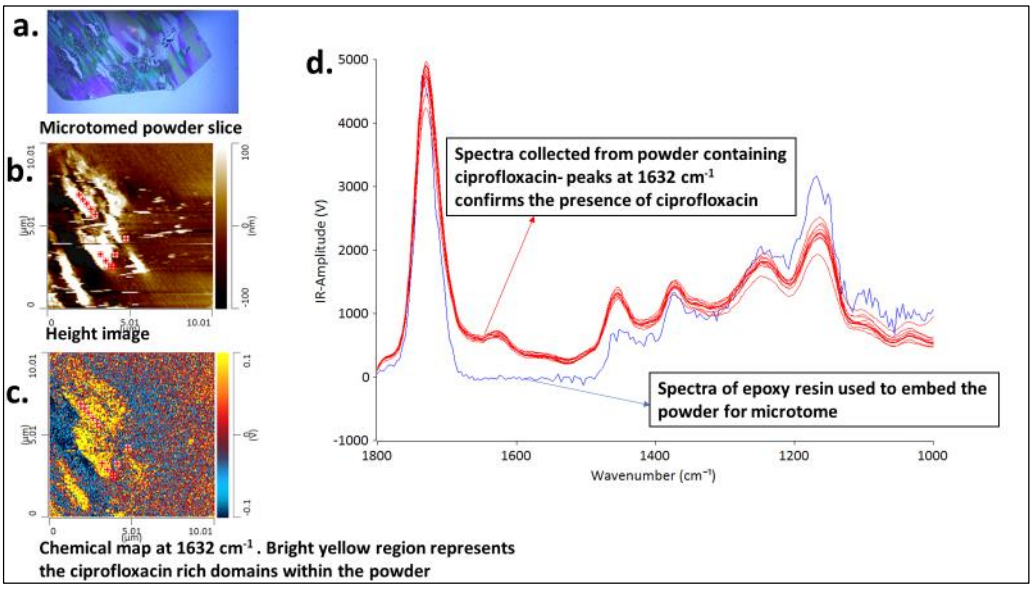

Figure 1. AFM-IR to evaluate the presence of nanocrystalline ciprofloxacin on a microtomed spray dried sample.

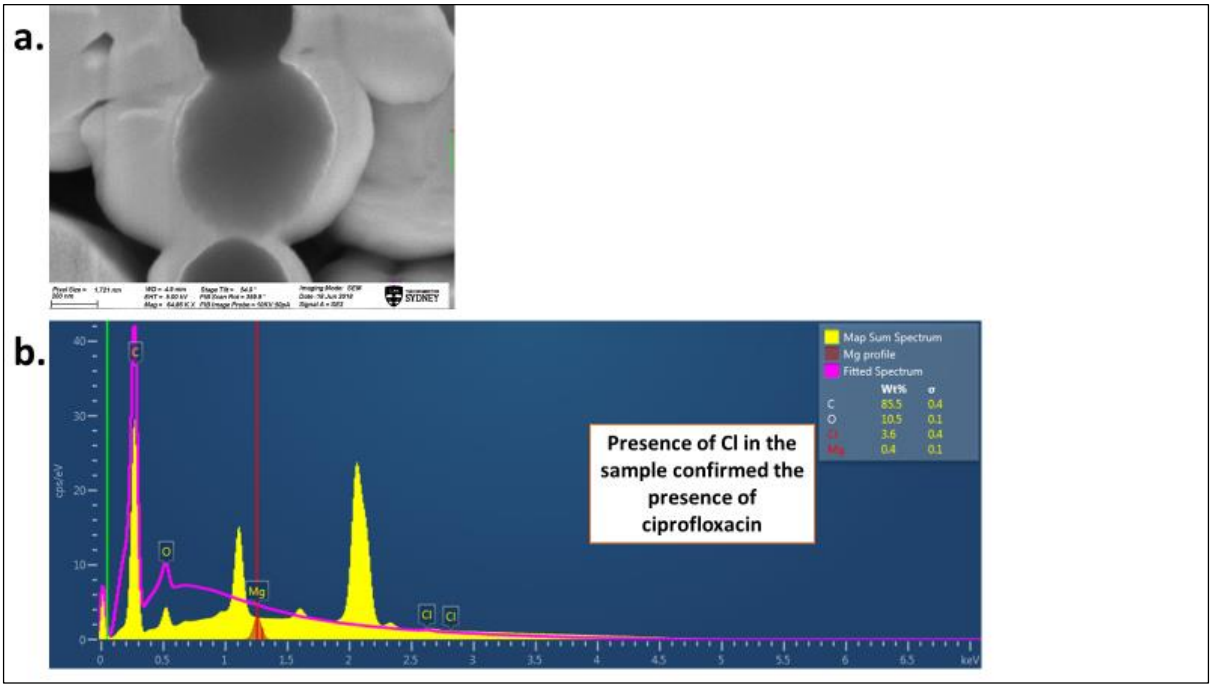

Figure 2. a) FIB SEM on spray dried powder to evaluate the presence of nanocrystalline ciprofloxacin, b) Energy Dispersive Spectroscopy map on the sliced sample to evaluate the elemental composition.

This manuscript objective was on the storage stability of the dry powder of liposomal ciprofloxacin nanocrystalline in terms of suitability for respiratory delivery and controlled drug release purposes. Discussing the mechanism of nanocrystals formation is important but outside the scope of the present study objective.

Regarding the XRD and DSC data, the crystallinity of the formulations compared to the raw materials, i.e. excipients or API have already been discussed thoroughly in our previous paper (Khatib et al., 2020) which was referred to in this manuscript. For example, **Figure 3** is the X-ray powder diffraction patterns of CIP nanocrystal liposomal dry powders containing different sucrose to lipids mass ratios (0.5:1, 1:1, 2:1) and spray dried raw materials (isoleucine, sucrose, empty liposomes, CIP), Mg stearate and neat crystalline ciprofloxacin (raw) which were included for comparison (Khatib et al., 2020). 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°. 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 CIP; 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. In this manuscript, we discussed the change in the crystallinity from time 0 to 6 months, in alignment with our study objective on storage stability.

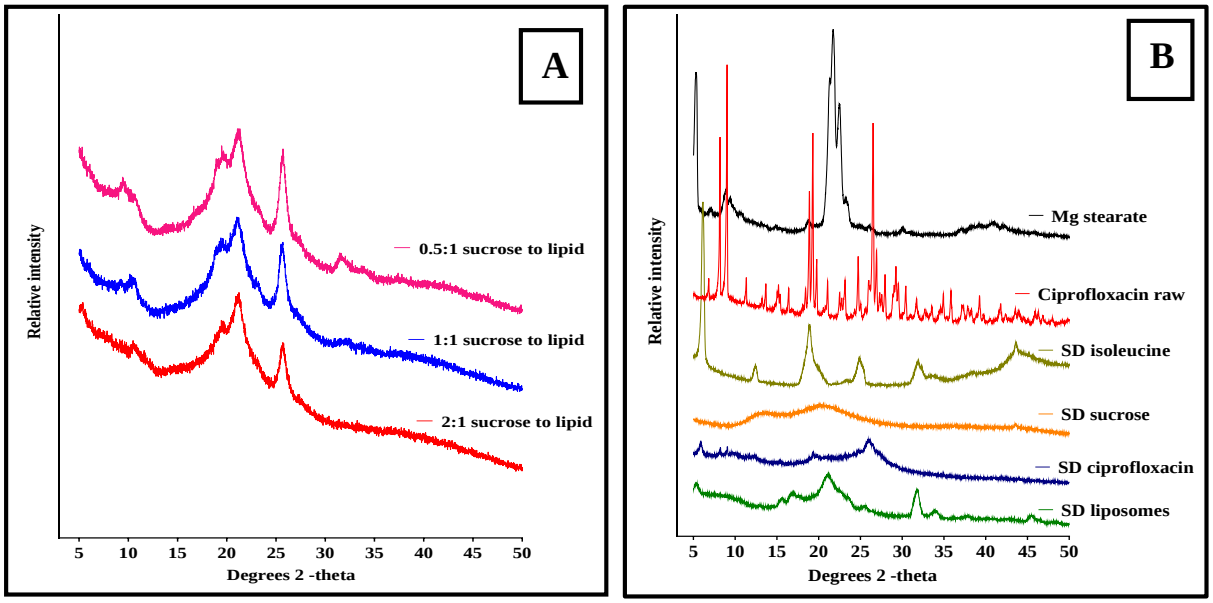

Figure 3. X-ray powder diffraction patterns of (A) CIP nanocrystal liposomal dry powders containing different sucrose to lipids mass ratios (0.5:1, 1:1, 2:1); (B) spray dried raw materials (isoleucine, sucrose, empty liposomes, CIP, Mg stearate and neat crystalline ciprofloxacin (raw) were included for comparison (Khatib et al., 2020).

2- The graphical abstract was partially improved, however, it is still hardly readable and not - abstract - enough.

We have revised the graphical abstract to improve visual communication as suggested (**Figure 4**). The graph legend must include details to allow the viewer to understand the abbreviations used in the graph.

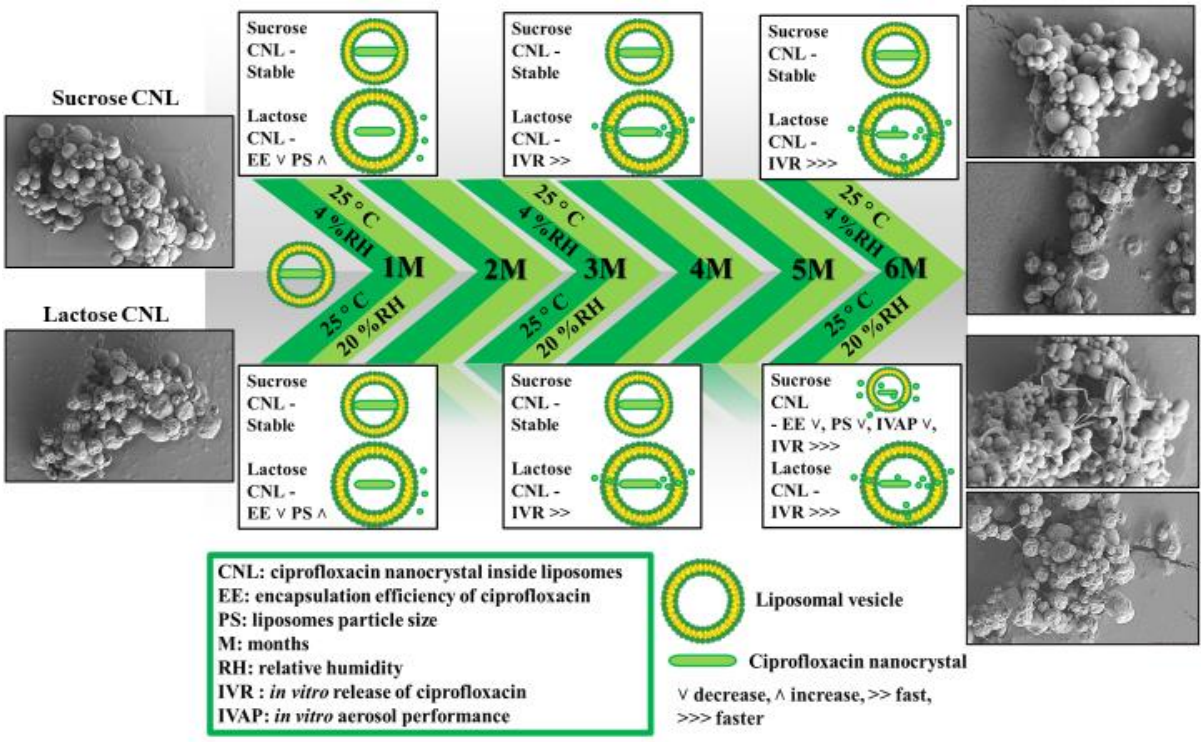

Figure 4. Revised version of graphical abstract.

3- See above, data must directly be stated and displayed in figures from the samples that were investigated in this study!

The previous comment was: Include data for pure liposomes and those with CIP before spray drying for particle size and zeta potential.

The following sentence was modified to provide information about the size of original liposomes: The size of the original liposomes (before SD) is about 96.8±2.1 nm for empty liposomes and 101.5±3.0 nm for liposomes encapsulating CIP as reported previously (Cipolla et al., 2016; Khanal et al., 2020; Khatib et al., 2021).

The following sentence was modified to include a short sentence about the zeta potential of original liposomes: Surface zeta potential value, which is about -9.93±0.09 mV for original liposomes (Khatib et al., 2021), remained within a narrow range over the storage period at

both RHs.

The data of original liposomes size and zeta potential were obtained from samples belong to the batch used to prepare the powders investigated in this manuscript. The figures were obtained after testing at least three samples of the same batch.

4- Additionally, a conclusion as a seperate paragraph is required.

5. Conclusion

Dry powder inhaler of CNL with sucrose lyoprotectant remained physiochemically stable when stored under a dry condition (4% RH) at controlled RT. When stored at 20% RH, sucrose began to recrystallize at six months of storage, and consequently, liposome EE decreased along with CNL dry powder aerosol performance. Although lactose as lyoprotectant was an efficient protectant for liposomes during SD, it failed to maintain liposomal stability for six months of storage. Within one month, the EE declined while the liposome particle size increased. Moreover, after six months of storage, the CIP release from CNL powder was different and less controlled than the CIP release of the freshly prepared powder.

Overall, this research suggests that an inhalable CNL powder formulation with sucrose as a stabilizing excipient when stored under a relatively low humidity environment of 4 %RH and controlled RT storage possesses at least a six-month shelf-life.

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Khanal, D., Khatib, I., Ruan, J., Cipolla, D., Dayton, F., Blanchard, J.D., Chan, H.-K., Chrzanowski, W., 2020. Nanoscale Probing of Liposome Encapsulating Drug Nanocrystal Using Atomic Force Microscopy-Infrared Spectroscopy. Analytical chemistry 92, 9922-9931.

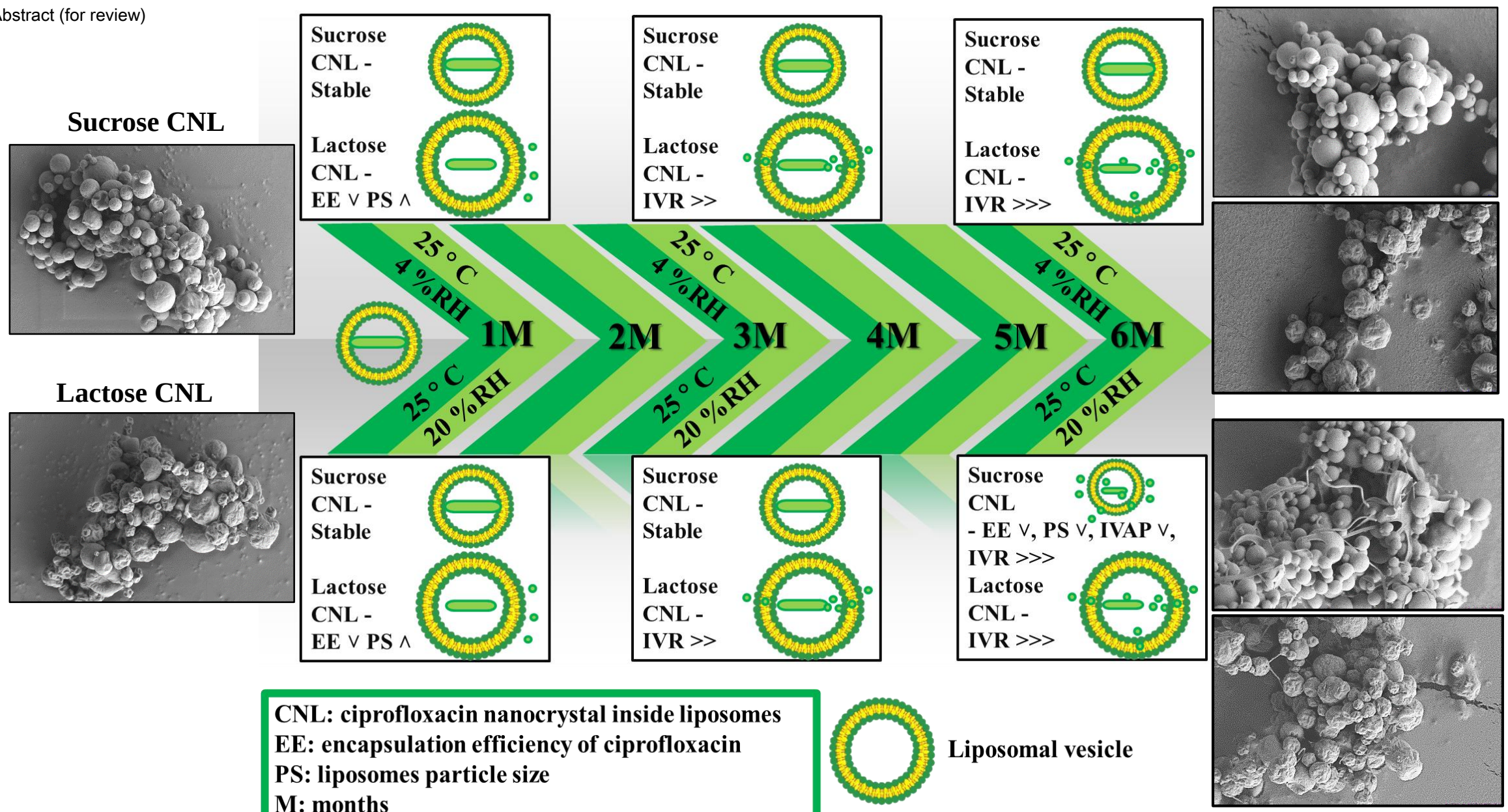
Khatib, I., Chow, M.Y.T., Ruan, J., Cipolla, D., Chan, H.K., 2021. Modeling of a spray drying method to produce ciprofloxacin nanocrystals inside the liposomes utilizing a response surface methodology: Box-Behnken experimental design. Int J Pharm 597, 120277.

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.

Khatib, I., Tang, P., Ruan, J., Cipolla, D., Dayton, F., Blanchard, J.D., Chan, H.-K., 2020. Formation of ciprofloxacin nanocrystals within liposomes by spray drying for controlled release via inhalation. International Journal of Pharmaceutics 578, 119045.

Li, T., Cipolla, D., Rades, T., Boyd, B.J., 2018. Drug nanocrystallisation within liposomes. Journal of Controlled Release 288, 96-110.

Graphical Abstract (for review)



Research paper

Storage stability of inhalable, controlled-release powder formulations of ciprofloxacin nanocrystal-containing liposomes

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Abstract

Novel inhalable and controlled release powder formulations of ciprofloxacin nanocrystals inside liposomes (CNL) were recently developed. In the present study, the storage stability of CNL powders consisting of lyoprotectant (i.e. sucrose or lactose), lipids, ciprofloxacin (CIP), and magnesium stearate or isoleucine was investigated. These powders were produced by spray drying, collected in a dry box at < 15% relative humidity (RH), then stored at room temperature and either 4 or 20 %RH. Liposomal integrity, CIP encapsulation efficiency (EE), *in vitro* drug release (IVR), aerosol performance, and solid-state properties were examined over six months. Sucrose CNL powder exhibited consistent liposomal integrity, aerosol performance, and controlled release of CIP over six months of storage at 4 %RH. However, storage of the powder at 20 %RH for the same period caused sucrose crystallization and consequently a significant drop in EE and aerosol performance (p-values <0.05), along with the IVR of CIP becoming similar to that of the non-crystalline CIP liposomal dispersions (f2 > 50). Lactose CNL maintained superior aerosol performance over the six months irrespective of the storage RH. However, liposomal instability occurred at both RHs within the first month of storage with a significant drop in EE and an increase in liposome size (p-values <0.05). Moreover, the IVR assay of CIP from lactose CNL showed a less controlled release and a substantial difference (f2 < 50) from its initial value after six months regardless of the storage RHs. In conclusion, dry powder inhalers of CNL were physiochemically stable in sucrose lyoprotectant when stored below 4 %RH at room temperature for six months.

Keywords:

Spray drying; ciprofloxacin nanocrystals; liposomes; dry powder inhaler; storage stability; controlled release

Abbreviations

ANOVA, analysis of variance; BE, bronchiectasis; CIP, ciprofloxacin; CF, cystic fibrosis; CNL, ciprofloxacin nanocrystals inside liposomes; DLS, dynamic light scattering; DPI, dry powder inhaler; DSC, differential scanning calorimetry; ED, emitted dose; EE, encapsulation efficiency; FPD, fine particle dose; FPF, fine particle fraction; FT, freeze-thaw; HBS, HEPES buffered saline; HPMC, hydroxypropyl methylcellulose; HSPC, hydrogenated soy phosphatidylcholine; IVR, *in vitro* release; LC, liposomal ciprofloxacin; MMAD, mass median aerodynamic diameter; NCF, non-cystic fibrosis; NGI, Next-Generation Impactor; NTM, non-tuberculous mycobacteria; PDI, polydispersity index; PXRD, powder X-ray Diffraction; RH, relative humidity; RT, room temperature; SD, spray drying or spray-dried; SEM, scanning electron microscopy; T_g, glass transition temperature; TGA, thermogravimetric analysis; T_s, storage temperature.

1. Introduction

Inhaled liposomal ciprofloxacin (LC) has the potential to treat respiratory infections in cystic fibrosis (CF) or non-cystic fibrosis (NCF) bronchiectasis (BE) patients (Bruinenberg et al., 2010; Serisier et al., 2013) and in animal models of nontuberculous mycobacteria (NTM) (Bermudez et al., 2015). We recently developed two inhalable LC controlled-release formulations by transforming the physical state of the encapsulated ciprofloxacin (CIP) into nanocrystals by either freeze-thaw (FT) (Cipolla et al., 2016) or spray-drying (SD) (Khanal et al., 2020). The presence of CIP nanocrystals inside liposomes (CNL) introduces a rate-limiting dissolution step that delays the diffusion of CIP across the liposomal membrane bilayers. The aqueous dispersion of CNL produced by FT retained its physicochemical properties after aerosolization by mesh nebulizer and after 3-months frozen storage. For optimum stability outcomes, CNL aqueous dispersion must be kept frozen and thawed just before anticipation for use by the patient (Cipolla et al., 2015b).

Powder CNL produced by SD exhibited *in vitro* physicochemical properties suitable for respiratory delivery and controlled drug release (Khanal et al., 2020; Khatib et al., 2019b). It is generally recognized that the development of powder liposomal formulations is potentially beneficial as they can be more stable than the aqueous dispersion (Ingvarsson et al., 2011). However, the dispersibility of dry powders intended for inhalation is affected by the storage temperature and humidity. Storage of CNL powder at improper conditions (e.g., high humidity) may lead to physical instability which will affect not only the aerosol performance but also

liposomal integrity (Shetty et al., 2020; Sun et al., 1996). Thus, the storage stability of CNL dry powder must be examined to identify conditions that provide a sufficient product shelf-life for clinical application. To date, there is no report on the storage stability of CNL powders for inhalation purposes and controlled drug release applications.

Published data showed that sugars such as sucrose and lactose protect liposomal ciprofloxacin against degradation during the SD process (Ingvarsson et al., 2011; Khanal et al., 2020; Khatib et al., 2021b). In addition, the glassy state of sugars is necessary to stabilize liposomes in the solid-state during storage as it prevents liposome fusion and drug leakage (Sun et al., 1996). According to the vitrification hypothesis, sugars hinder molecular mobility and constitute a barrier between the neighbouring liposomal vesicles by building a highly viscous glassy matrix around them (Koster et al., 1994). However, the capacity of lyoprotectants to construct a glassy matrix is reliant on their T_g above which the glassy matrix will be distorted due to increased molecular mobility and decreased viscosity (Ingvarsson et al., 2011). Therefore, the half-life of drug retention in dry liposomes is dependent on storing the powders at temperatures (T_s) below their glass transition temperature (T_g) and maintaining a high T_g value for the powder (Franzé et al., 2018; Sun et al., 1996).

Most of the sugar becomes amorphous after SD leading to increased moisture absorption of liposome dry powders which ultimately affects their stability (Ye et al., 2017). Sun et. al. (1996) noticed a significant drop in the T_g of dry liposomal powders due to moisture absorption after storage at high relative humidity (RH) which ultimately led to a dramatic drug leakage. Besides, the decrease in T_g because of the absorbed water, which acts as a plasticizer and increases the molecular mobility of amorphous powders, usually leads to recrystallization and poor dispersion of the powders to aerosols suitable for inhalation (Yu et al., 2017b). Research showed that SD amorphous lactose and sucrose had undergone an apparent moisture uptake at all tested RHs in the study but without any recrystallization or change in the physical form after 30 days of storage at 23% RH (Naini et al., 1998). Another study found that storage RH must be controlled strictly below 30% to preserve the amorphous SD lactose in suitable physical form for dry powder inhalers (DPIs) development (Wu et al., 2014). Furthermore, SD CIP formulations with excipients, i.e. sucrose, lactose and trehalose, maintained an amorphous form and excellent *in vitro* aerosol performance when stored at 20% RH (Shetty et al., 2018). Our CNL powders contain more than 50% (w/w) amorphous sugar (i.e. sucrose or lactose). Thus, CNL powder storage at or below 20 %RH is sensible to prolong the shelf-life of the product by maintaining the powder T_g above the T_s.

A long-term storage stability study of dry liposomal formulations revealed an increased degradation rate of lipids as the T_s and lipid unsaturation level increased (Payton et al., 2014). Thus, the SD CNL powders which contain saturated lipid, i.e. hydrogenated soy phosphatidylcholine (HSPC), may exhibit reasonable storage stability at room temperature (RT) compared to other unsaturated lipids (van Hoogevest and Fahr, 2019). In the literature, many dry liposomal formulations were stored at RT or refrigerated for investigation of their storage stability. Freeze-dried doxorubicin-loaded liposomes with residual content <1% (w/w) did not show significant physical instability or chemical degradation when stored at a temperature up to 30 °C over six months (Van Winden and Crommelin, 1997). A DPI formulation of lyophilized liposomal entrapped budesonide exhibited a shelf-life of one year when stored refrigerated (2 - 8 °C) (Joshi and Misra, 2001). Rifampicin liposomal dry powder displayed better stability when kept at 4 °C for six weeks (Changsan et al., 2009). Dry powder of liposome-encapsulated recombinant secretory leukocyte protease inhibitor was found stable at RT for five months (Gibbons et al., 2010). Clarithromycin liposomal DPI formulations showed no significant change of physical characters after 3-month storage at 25 °C and 60 %RH (Ye et al., 2017). Based on these research outcomes, CNL powders may exhibit reasonable stability at both RT or refrigeration. However, one of the main objectives of producing CNL in dry powder form is to explore the possible shelf-life of CNL without refrigeration which is a necessity for the freeze-thawed aqueous CNL dispersions. Hence, the feasibility of product distribution without 'cold chain' can be explored.

In the present study, we investigated the storage stability of CNL powders produced by spray drying with two different lyoprotectants, i.e. sucrose or lactose. The optimized conditions, which were reported in the previous study, were implemented for the CNL powders production (Khatib et al., 2021a). The powders stored at RT (~25 °C) and two RHs, one of them around 20% by using saturated lithium chloride solution, and the other RH was around 0% by using P_2O_5 and silica gel. The stored powders were examined for their liposomal integrity (liposomes particle size, polydispersity, surface zeta potential and drug retention (or EE)), *in vitro* drug release, aerosol performance, and solid-state properties (crystallinity, moisture content, particle size and morphology) over six months of storage. The obtained data were analyzed to identify the most stable formulation composition (i.e. lyoprotectant type) and the shelf-life stability of the CNL powders at the study storage conditions.

2. Materials and Methods

2.1. Materials

Liposomes either empty or encapsulating 50 mg mL^{-1} ciprofloxacin hydrochloride (CIP HCl) as aqueous dispersion in a pH 6.0 histidine buffer, were produced by Exelead (Indianapolis, IN, USA) and Northern Lipids Incorporated (Burnaby, BC, Canada) and provided by Aradigm Corporation (Hayward, CA, USA). Analytical grade sucrose, isoleucine, magnesium stearate, sodium chloride, triethylamine (TEA) and adult bovine serum were purchased from Sigma-Aldrich (Castle Hill, New South Wales, Australia), lactose was sourced from DFE Pharma (Goch, Germany) and HEPES, free acid from Dojindo, China. Deionized water was generated through Modulab Type II Deionization System (Continental Water System, Sydney, Australia). Nanosep Omega centrifugal filtration devices, 10k molecular weight were supplied from Pall Australia Pty Ltd, (Victoria, Australia), Osmohaler® inhalers 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 CIP nanocrystals inside liposomes (CNL)

Previous studies conducted by Khatib et al. revealed that sucrose and lactose are efficient lyoprotectants for LC during spray drying (Khatib et al., 2021a). Aqueous LC dispersion of 50 mg mL^{-1} CIP HCl was mixed with sucrose or lactose solutions of 100 mg mL^{-1} and deionized water to produce 12.5 mg mL^{-1} CIP HCl dispersion. Two excipients, isoleucine and magnesium (Mg) stearate, were added to the dispersions prior to spray drying to serve as moisture protectants and aerosolization enhancers (Chan and Chew, 2003a; Chan and Chew, 2003b; Yu et al., 2017a). Two liposomal dispersions were prepared at 3 mg mL^{-1} total solid concentration (**Table 1**). Lactose dispersions incorporated only isoleucine because a significant drop in the EE of CIP occurred when Mg stearate was added. The dispersions were pumped into a spray dryer (B-290 mini spray-dryer, Büchi Falwil, Switzerland) operating with the settings outlined in **Table 2**. These settings were chosen following the optimization study reported previously (Khatib et al., 2021a).

2.3. Storage stability

Spray-dried CNL powders, either containing sucrose or lactose, were divided into 1g samples into 100 mL beakers. All samples were stored at controlled RT (~25 °C). One sample of each of the CNL powders was stored in a desiccator over P_2O_5 and silica gel maintaining RH close to 0%. The other sample was stored in another desiccator over a saturated lithium

chloride solution maintaining RH of ~15%. Actual RH values were measured using a temperature/humidity data logger USB-502 (Measurement Computing Corporation, MA, USA). The actual RH values were 4±1% and 20±3%. The desiccators were stored protected from light in an incubator at a controlled temperature (~25 °C). The physicochemical characteristics of CNL powders after 0, 1, 3, and 6 months of storage were measured as described below.

Table 1. Composition of spray-dried CNL powders.

Dispersion	Ingredients (%w/w)				
	Lyoprotectant	Lipids	Ciprofloxacin	Isoleucine*	Mg stearate*
Sucrose CNL	53.1	26.6	13.3	5	2
Lactose CNL	54.3	27.1	13.6	5	-

***** Moisture protectants and aerosolization enhancers.

Table 2. Spray dryer settings for producing sucrose and lactose CNL powders.

Dispersion	Parameters			
	Feed rate (mL min^{-1})	Inlet temperature (°C)	Atomizer rate (L hr^{-1})	Aspirator (m^3 hr^{-1})
Sucrose CNL	1.5	80	742	35
Lactose CNL	1.5	65	742	35

2.4. Physicochemical characterization of CNL powders (after reconstitution in liquid)

2.4.1. Encapsulation efficiency (EE) by Nanosep device and HPLC

CNL powders were reconstituted in a saline solution to determine the EE of CIP inside the liposomes. The free drug in samples with a total CIP concentration of 1 mg mL^{-1} was separated using Nanosep Omega centrifugation devices as per the previously established method (Cipolla et al., 2014). These devices are composed of modified polyethersulfone membrane filters of 10,000 molecular weight cut-offs. The samples were transferred in 400 µl aliquots into the Nanosep devices and centrifuged for 18 min at 10,000 rpm (6,700 × g). The filtrates were diluted (20 times) with deionized water before assaying by HPLC for free CIP quantification. The total amount of CIP (the encapsulated and free drug) in the samples was determined by dissolving the liposomes in the samples with 80% methanol to release the encapsulated CIP. Then, samples were centrifuged for 15 min at 13,400 rpm (12,100 × g), and their filtrates were analyzed on HPLC. The percentage of the encapsulated drug was calculated for three measurements using the following equation:

EE (%) = [(Total drug amount – Free drug amount) / Total drug amount] ×100% (**Eq. 1**)

2.4.2. Particle size distribution and surface zeta potential of liposomes: Dynamic light scattering (DLS)

CNL powders were transformed into liquid dispersions by reconstituting 10 mg of the powder in 5 mL saline solution. The samples were diluted to 10 mM NaCl concentration before particle size and zeta potential measurement. Malvern Zetasizer Nano ZS (Malvern, UK) with disposable folded capillary cell (DTS1070) was used to measure the samples at the following instrument parameters: temperature 25 °C, viscosity 0.887 cP, refractive index 1.34, backscatter angle 173°, and a run time of 5 min. Smoluchowski model was used for zeta potential measurements and Henry`s function (F(ka)) value was set at 1.5. Three reconstituted samples of CNL powders were examined and the reported data were the D50 ± SD.

2.4.3. In vitro assay of CIP release from liposomes (IVR assay)

Ciprofloxacin release from liposomes was measured for CNL powders after reconstitution in saline solution and compared to CIP release of aqueous LC dispersion (control). Following the previously validated method (Cipolla et al., 2014), samples were diluted with HEPES buffered saline (HBS: 20 mM HEPES, 145 mM NaCl, pH 7.4) to obtain dispersions with CIP concentrations around 50 µg mL^{-1}. Chilled (2–8°C) adult bovine serum was added to each sample in a 1:1 v/v ratio and then a 300 µl sample was withdrawn for analysis. The rest of the sample was incubated in a shaking water bath (Labec J-SWB60, Marrickville, Australia) at 37°C and 150 rpm. After t_n = 30, 60, 120, 240, 480, and 600 min of incubation, samples were withdrawn and immersed immediately in an ice-water bath to halt the CIP release. Moreover, samples were mixed in equal volumes with chilled (2–8°C) HBS. The amount of released CIP (free CIP) at t_n and the total CIP (free and encapsulated) amount in the sample were determined following the method reported in **Section 2.4.1**. Drug loss in the filter because of the serum was compensated for by a correction factor (0.93) for normalizing the calculated value (Cipolla et al., 2014). The amount of released CIP at t_n relative to the total CIP amount in the sample is the percent of CIP released at t_n. Because of the burst drug release at t_0, the release percentage normalization is needed. The release of (t_n - t_0) was divided by the total probable release (100 -t_0) and then changed to a percentage: 100 * (t_n - t_0)/(100 - t_0) (Cipolla et al., 2016). The similarity factor (f2) and the difference factor (f1) were determined (as in **Section 2.7**) from the normalized percentages. These factors provide

quantitative comparisons between the *in vitro* release profiles of the CNL powders at different storage time points and for the control.

2.5. Physicochemical characterization of CNL powder (solid form)

2.5.1. Particle size and morphology: Scanning electron microscopy (SEM)

The surface morphology and size of the CNL particles were visualized during the stability study via scanning electron microscopy at 2 kV beam accelerating voltage (SEM, Carl Zeiss SMT AG, Oberkochen, Germany). The powder was scattered on a carbon tape attached on an SEM stub followed by coating before imaging with 15 nm thick of gold using a K550X sputter coater (Quorum Emitech, Kent, UK).

2.5.2. Crystallinity: Powder X-ray Diffraction (PXRD)

The crystallinity of CNL powders was evaluated by powder X-ray diffraction (Shimadzu XRD-6000, Shimadzu Corporation, Kyoto, Japan). The followings were the used operating parameters: 45 kV Cu-Kα radiation, current of 40 mA, 2θ range of 5-40° and a scan speed of 2 ° min^{-1}.

2.5.3. Thermal analysis:

A- Moisture content: Thermogravimetric analysis (TGA)

The percent of residual moisture in the stored CNL powders was evaluated using Thermogravimetric analysis (TG/SDTA 851e, Mettler Toledo, Greifensee, Switzerland). Powder samples of 5-10 mg weight were transferred to an alumina pan. The starting temperature of 30 °C was increased at a heating rate of 10 °C min^{-1} under nitrogen atmosphere until reaching 150 °C. Sample water content was considered as the percent drop in mass between 30 to 100 °C. The reported data were the mean ± SD of three measurements.

B- Thermal properties: Differential scanning calorimetry (DSC)

The stored CNL powders were analyzed via differential scanning calorimetry (DSC) (Mettler Toledo, Greifensee, Switzerland) to explore their thermal properties. About 5 mg of the samples were mounted into an aluminum crucible and covered with a perforated lid. All measurements were performed under a nitrogen atmosphere over a temperature range of 25 – 250 °C at a heating rate of 5 or 10 °C min^{-1} for lactose or sucrose CNL powders, respectively. STARe software V.9.0x (Mettler Toledo, Greifensee, Switzerland) was applied for thermal data analysis.

2.5.4. In vitro aerosol performance

The aerosol performance of the CNL powders was evaluated using a Next-Generation Impactor with a USP induction port (NGI, Copley, Nottingham, UK) following a previously reported method (Khanal et al., 2020). The dispersion of aerosol was conducted under ambient conditions and the collection plates of the NGI stages were coated with silicone (Dry Film Silicone Lubricant; LPS, Tucker, GA, USA) to avoid particle bounce. An HPMC capsule filled with 30 ± 1 mg of the CNL powder was dispersed within 2.4 s using an Osmohaler® device at a flow rate of 100 L min^{-1}. These conditions were selected because airflow of 105 L min^{-1} using an Aerolizer® (which has a similar resistance to airflow) can be produced with a comfortable inspiratory effort of 40 cm H_2O (i.e., 4 kPa) even with patients with compromised lung function (Chew and Chan, 2001). The aerodynamic cut-off diameters of the individual stages of the NGI at this flow rate are 0.24, 0.4, 0.72, 1.3, 2.2, 3.4, and 6.1 μm from stage 7 to 1, respectively. The mass of CIP in the powder deposited on the capsule, inhaler, adapter, throat, and NGI stages was determined using HPLC after being dissolved with 80% methanol. The total recovered mass is the mass of CIP deposited on the capsule, inhaler, adapter, throat, and NGI stages. The percent emitted dose (ED) is the CIP mass collected on the adapter, throat and NGI stages relative to the total recovered CIP mass. The fine particle dose (FPD) is the CIP mass with an aerodynamic diameter < 5 μm deposited on the stages. The FPD relative to the total recovered mass is termed the fine particle fraction (FPF). The diameter under which the size of 50% of the particles by mass resides is the mass median aerodynamic diameter (MMAD). The FPD and MMAD were obtained by interpolation from the aerodynamic particle size distribution plot. The dispersions were performed in triplicate and data reported as mean ± SD.

2.6. Quantitative analysis of CIP: High-performance liquid chromatography (HPLC)

The high-performance liquid chromatography system (Model LC-20; Shimadzu, Japan) consisted of a DGU-20A degasser, CBM-20A controller, LC-20AT pump, SIL-20A HT auto-sampler, CTO-20A column oven, and ultraviolet (UV) detector. The applied parameters were: Detector at 277 nm wavelength for CIP quantification, Phenosphere-Next C-18 column (5 mm, 4.6 × 150 mm, Phenomenex, USA), a mixture of 0.5 % TEA in water, pH 3.0, and 100 % methanol (78:22 v/v) as the mobile phase at a flow rate of 0.9 mL min^{-1}, injection volume of 100 μL, and column oven temperature at 35 °C. The calibration curves of standards of CIP dissolved in deionized water were linear (R2 > 0.999) over the concentration range of approximately 0.25 – 25 μg mL^{-1}. Quality control samples were prepared at two levels of 1 and

10 µg mL^{-1}, and the accuracy and precision were within 5%. The CIP concentration is represented as CIP HCl.

2.7. Statistical analysis

Two-way analysis of variance (ANOVA) at a confidence level of 95% and Tukey's multiple comparisons test were employed on repeated observations to identify the statistical difference. Probability (p) values < 0.05 were measured as a statistically significant difference. For comparing the *in vitro* release profiles, difference factor (f1) and similarity factor (f2) were calculated as recommended by the FDA for modified release solid oral dosage forms (FDA, 1997). Release profiles with f2 values above 50 were considered similar while those with f1 values below 15 were not different (Riley et al., 2012). In this study, the similarity factor (f2) values were reported only when both their f2 values were above 50 and their f1 values were below 15.

3. Results

3.1. Physicochemical characteristics of CNL powders (after storage and subsequent reconstitution in saline)

3.1.1. Encapsulation efficiency (EE%)

Spray-dried liposomes retained about 90% of the ciprofloxacin within their core whether sucrose or lactose was used as a lyoprotectant (**Table 3**). Irrespective of the storage RH after a month, the EE values of the lactose CNL dropped significantly to 84% (*P*-values = 0.0002 - 0.04) which remained the same for up to 6 months of storage (*P*-values = 0.4 -1.0). The sucrose formulation stored at 20 %RH failed to maintain the initial EE value after 6 months of storage (*P*-value = 0.0001) while the one stored at 4%RH was stable (*P*-values = 0.5 -1.0).

Table 3. Encapsulation efficiency (%, w/w) of CNL powders (sucrose or lactose as lyoprotectant) before and after storage at 4 or 20 %RH. Mean ± SD, n=3.

Lyoprotectant	Storage RH (%)	Storage period (months)			
		0	1	3	6
Sucrose	4±1	90.1±1.2	90.0±2.3	89.1±0.2	88.1±0.1
	20±3		89.1±0.3	88.1±0.4	82.8±0.6
Lactose	4±1	89.2±1.1	84.5±2.3	82.0±1.7	84.6±2.2
	20±3		83.2±0.7	84.3±2.2	83.2±4.9

3.1.2. Particle size distribution and surface zeta potential of liposomes: Dynamic light scattering (DLS)

The size of the original liposomes (before SD) is about 96.8±2.1 nm for empty liposomes and 101.5±3.0 nm for liposomes encapsulating CIP as reported previously (Cipolla et al., 2016; Khanal et al., 2020; Khatib et al., 2021a). The freshly manufactured CNL powders, which contain either sucrose or lactose as lyoprotectant, consisted of liposomes with size about 125 nm (**Fig. 1**). Sucrose CNL powders stored at 4 %RH maintained a liposomal size close to the initial value of 125 nm (*P*-value = 0.6 - 1.0). However, those stored at 20 %RH showed a significant drop in their size (Δ = 10 nm) after 6 months of storage (*P*-value = 0.004). In contrast, lactose CNL powders stored at both RHs exhibited a size increase (Δ = 15 - 20 nm) after 1-month (*P*-value <0.0001), without any further change after 6 months storage. The liposome polydispersity indices during the 6 months of storage of the sucrose CNL powder remained the same at 4 %RH (**Fig. 2**) but increased substantially at 20 %RH (*P*-value <0.0001). Lactose CNL powders retained a monodispersed distribution of liposomes irrespective of the storage RH. Surface zeta potential value, which is about -9.93±0.09 mV for original liposomes (Khatib et al., 2021a), remained within a narrow range over the storage period at both RHs. The values were between -18.5 and -21.4 mV for sucrose CNL powders and between -16.0 and -17.9 mV for lactose CNL powders.

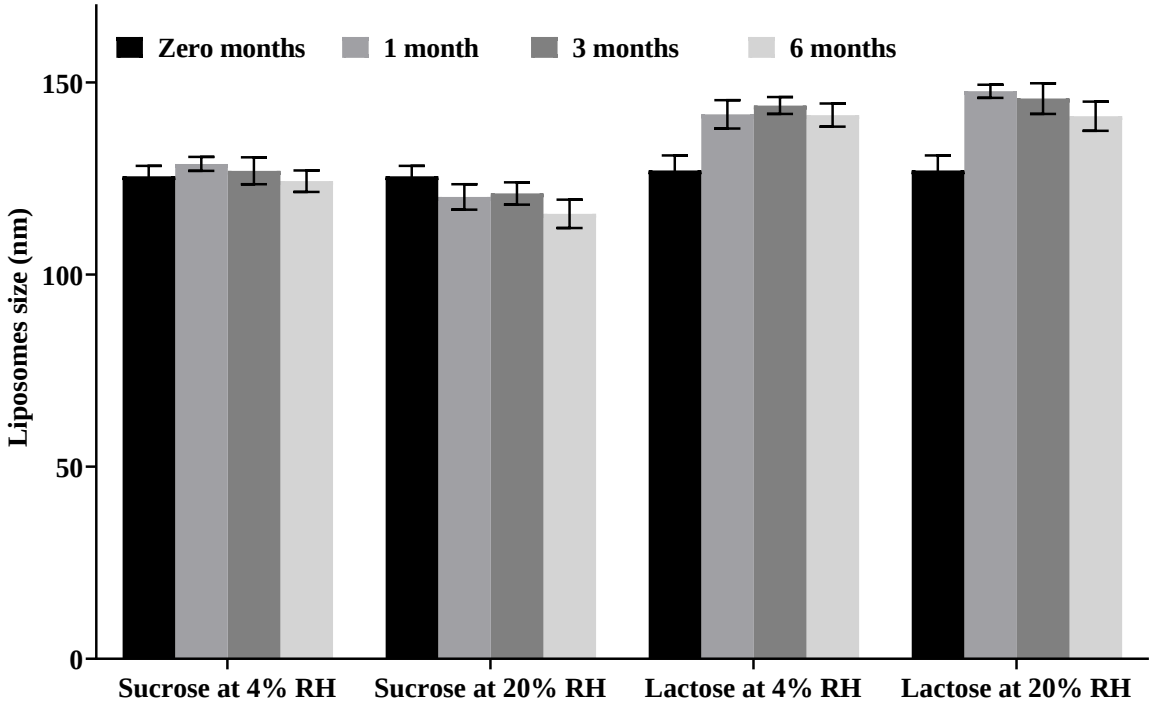

Fig. 1. The liposome particle size for reconstituted CNL powders (sucrose or lactose as lyoprotectant) before and after storage at 4 or 20 %RH. Mean with SD, n=3.

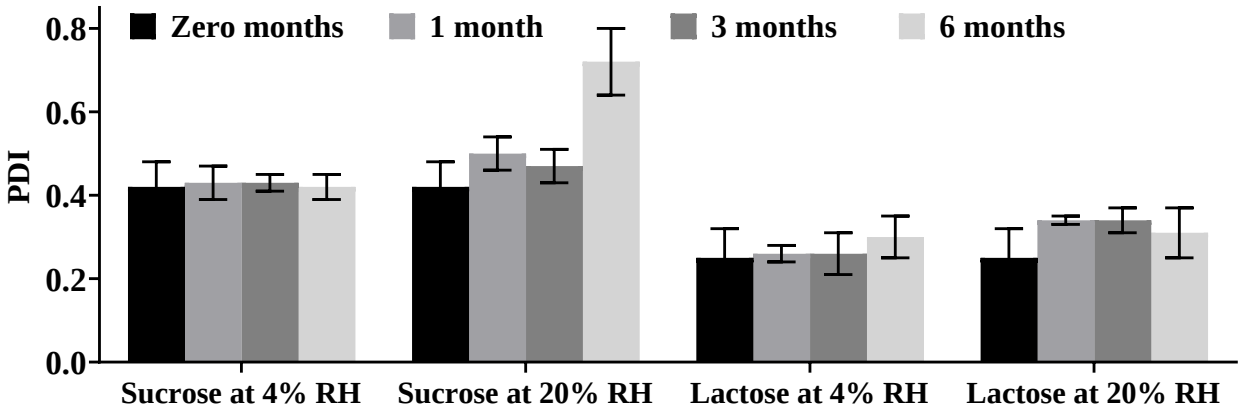

Fig. 2. The polydispersity index (PDI) of the liposome size of reconstituted CNL powders (sucrose or lactose as lyoprotectant) before and after storage at 4 or 20 %RH. Mean with SD, n=3.

3.1.3. In vitro assay of CIP release from liposomes

Modifying the release profile of encapsulated CIP inside liposomes was the main objective of producing CNL. Previous studies reported that CNL dry powder successfully exhibited a slower release of CIP than the original aqueous LC (Control) (Khanal et al., 2020; Khatib et al., 2019a). The current study was conducted to explore the reproducibility of the CIP release profiles of the stored CNL powders over time. **Figs. 3** is representing the percentage of CIP released from CNL powders at different storage time points in comparison to the control. **Fig. 4** is showing a quantitative estimation of the sought-after similarity ($f2 > 50$) between the CNL powder release profiles obtained at different storage time points and the dissimilarity ($f2 < 50$) between them and the control. Stable controlled release profiles were observed for sucrose-containing CNL powder over 6 months of storage at 4 %RH (**Fig. 3**). The same behaviour was obtained when stored for 3 months at 20 %RH, followed by a lack of similarity ($f2 < 50$) in the release profile after 6 months of storage as compared to those release profiles observed at the initial time points (**Fig. 4**). At both storage RHs, lactose CNL powders exhibited a gradual transition in their release behaviour. This transformation became significant at the 6-months release profile. The burst release of the drug due to the unencapsulated CIP was about 13±1% for the sucrose CNL powder stored at 4 %RH. For the powder stored at 20 %RH for 3 months, the burst release was about 14±2% then increased to 22±1% at the 6 months. Before storage, the lactose CNL powder released 17±2% of CIP at time zero of the release study with a slight increase to 20±2% after storage. The rate of change in both cases was in line with the observed EE values.

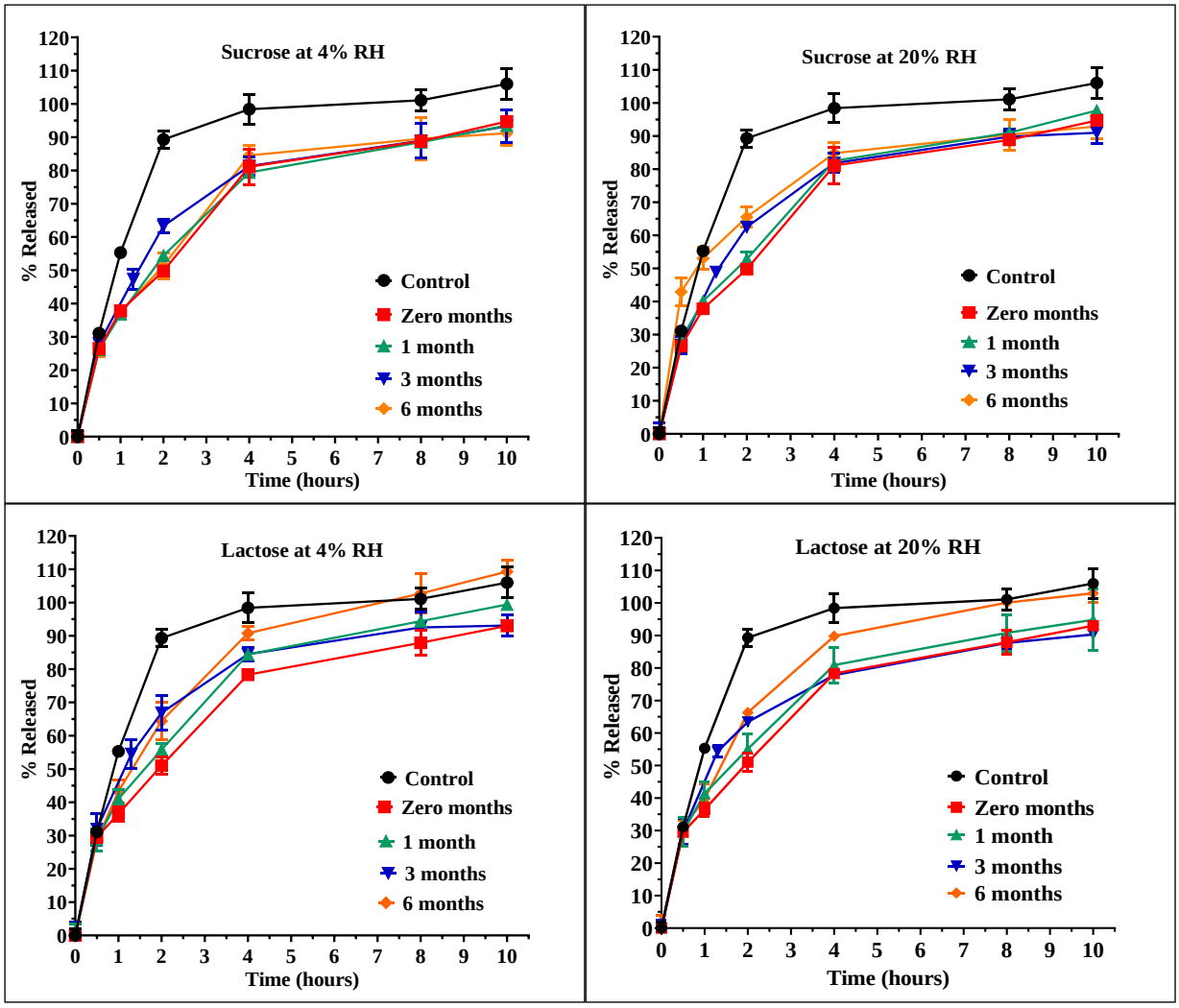

Fig. 3. *In vitro* release profiles of CNL powders containing sucrose or lactose as lyoprotectant in comparison to the control (aqueous LC) before and after storage at 4 or 20 %RH. Mean with SD, n = 3.

3.2. Physicochemical characteristics of stored CNL powder (solid form)

3.2.1. Particle size and morphology: Scanning electron microscopy (SEM)

Generally, all CNL powders appeared as agglomerates comprising spherical particles below 2 µm (**Fig. 5**). The surface of most sucrose CNL particles was smooth while lactose CNL particles were dimpled and corrugated. Elongated thread-like structures protruding from the particle surfaces occurred in sucrose powders after one and six months storage at 20 %RH. They were numerous after six months of storage which explains the significant drop that occurred in the *in vitro* aerosol performance (**Section 3.2.4**). Lactose powders started to develop similar structures after six months of storage at 20 %RH. However, a change in the dispersibility was not observed (**Section 3.2.4**).

3.2.2. Solid-state: Powder X-ray Diffraction (PXRD)

The X-ray diffraction of both CNL powders (sucrose and lactose) before storage produced similar patterns (**Fig. 6**) and the data were consistent with that reported previously (Khanal et al., 2020). Briefly, the PXRD patterns revealed a partially crystalline powder with diffraction peaks at 2θ around 21° and 26° which were attributed to the spray-dried liposomes and CIP, respectively. Sucrose and lactose in the powder were amorphous as no relevant peaks were observed.

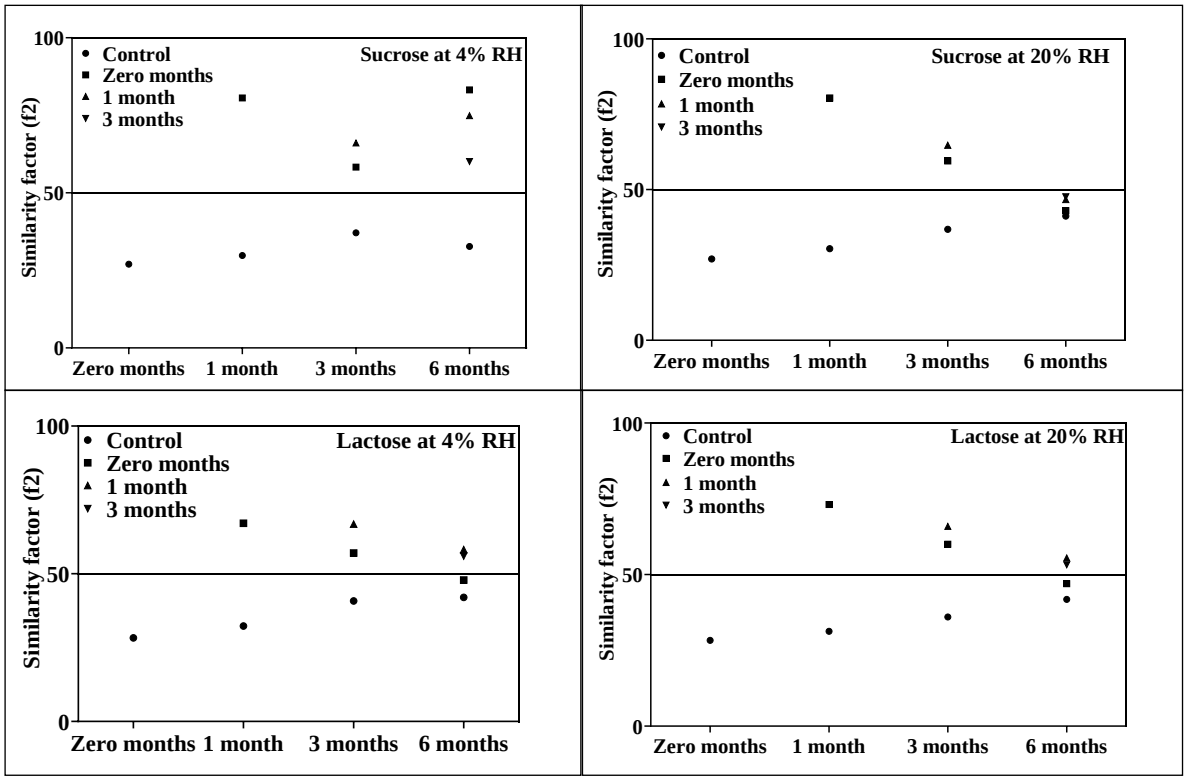

Fig. 4. Similarity factor analysis (f2) for CNL powders containing sucrose or lactose as lyoprotectant before and after storage at 4 or 20 %RH versus either control (aqueous LC) or the same powder at different storage time points.

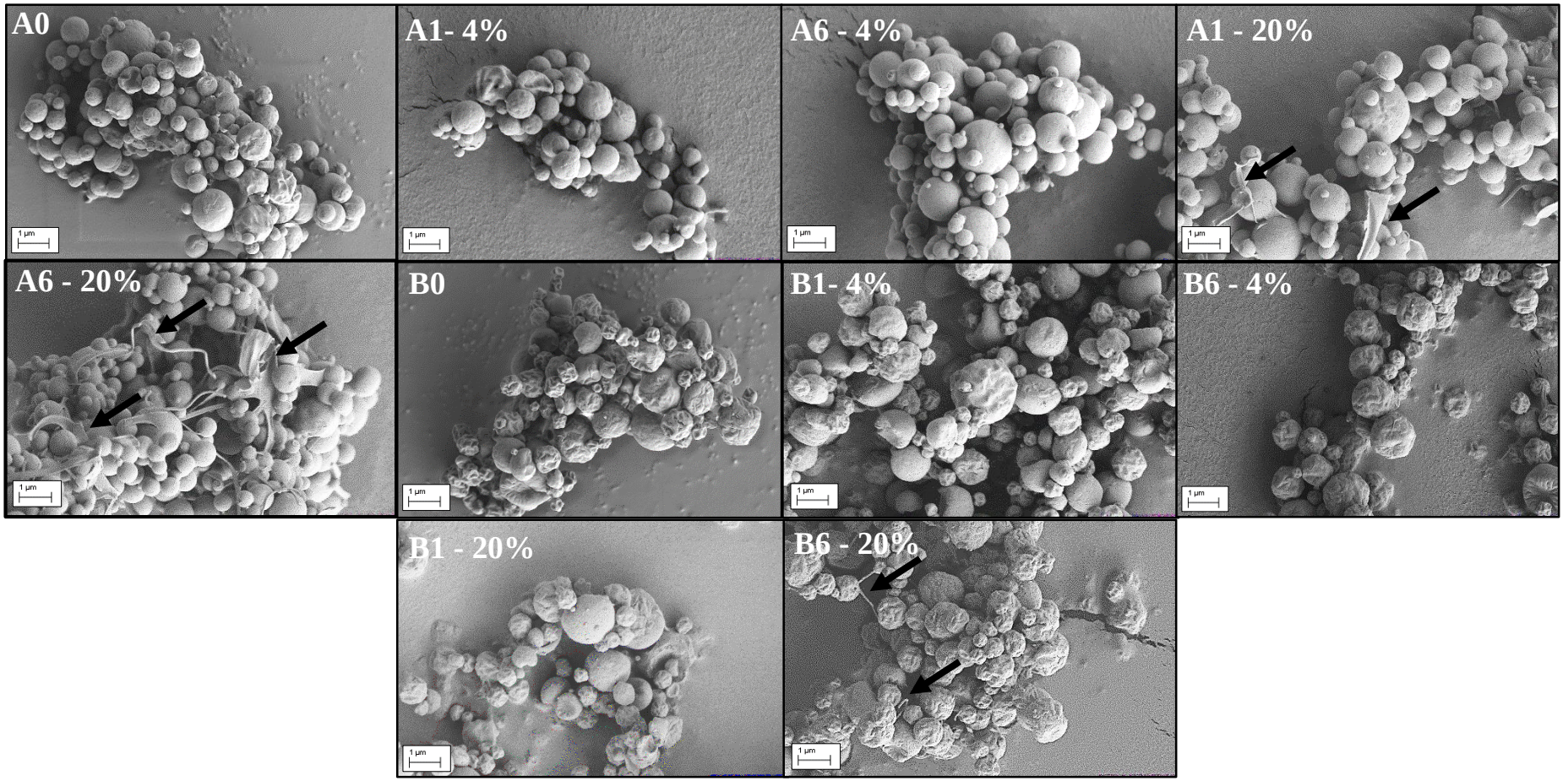

Fig. 5. SEM micrographs of CNL powders before (0) and after storage for one (1) and six (6) months at 4 or 20 %RH: sucrose (A0, A1-4%, A1-20%, A6-4% and A6-20%) and lactose (B0, B1-4%, B1-20%, B6-4% and B6-20%) (Black arrows refer to thread-like structures).

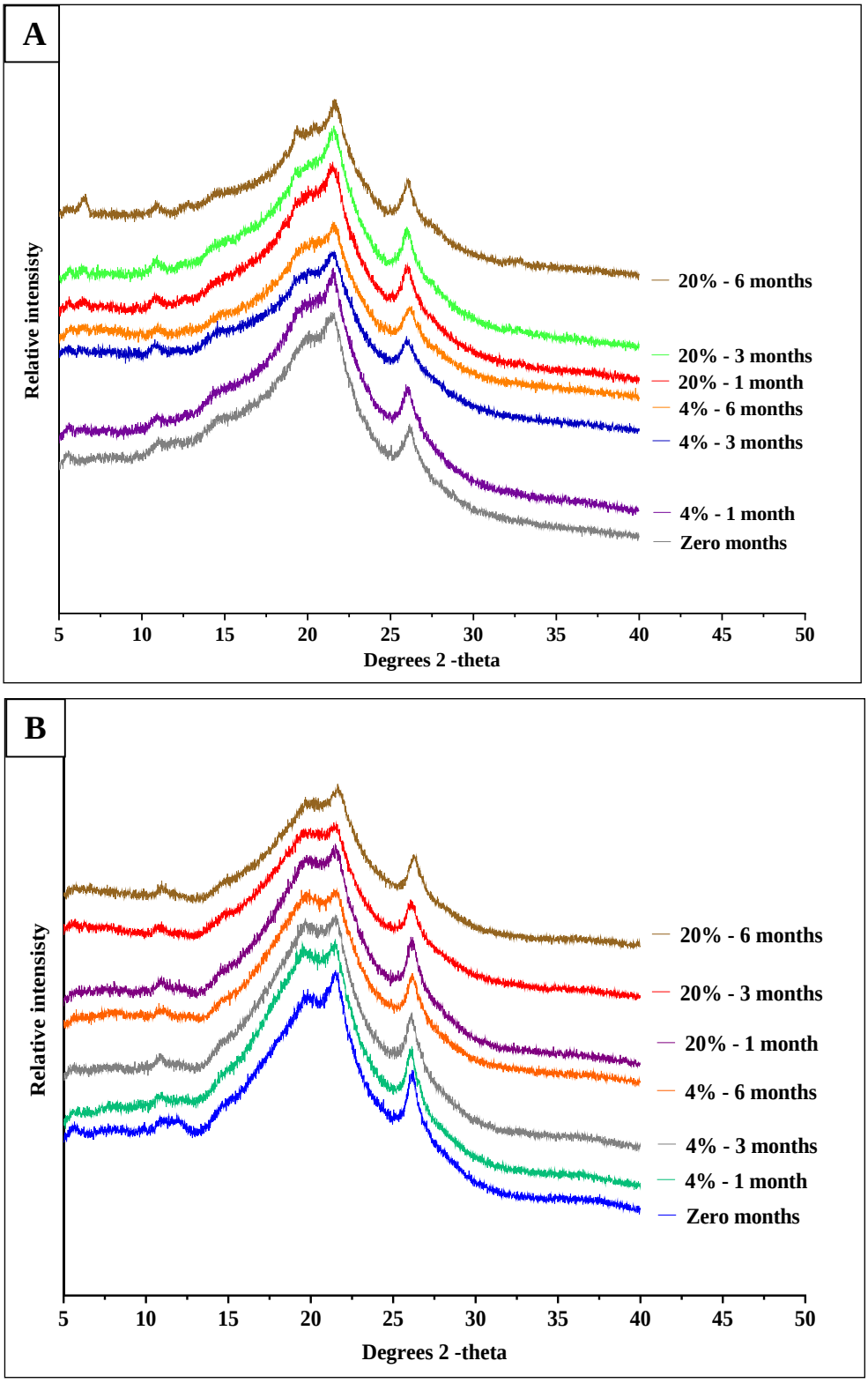

Fig. 6. X-ray powder diffraction patterns of sucrose (A) and lactose (B) CNL powders stored at 4 or 20 %RH for 6 months.

While the PXRD of lactose CNL powders remained unchanged over the entire storage period, the PXRD pattern of sucrose CNL powders showed differences when stored at 20 %RH for 6 months. A new peak appeared at 2θ around 6.7°. Additionally, the intensity of the peak around

19° slightly increased. These changes may be related to the crystallization of amorphous sucrose as reported in the investigation of the crystallization of sucrose glass (Kawakami et al., 2006b).

3.2.3. Thermal analysis:

A- Moisture content (TGA)

CNL powders containing either sucrose or lactose contained a low moisture content of < 3% (w/w) before and after storage (**Table 4**). However, a value of 2.78% (w/w) for sucrose CNL powders induced physicochemical instability over the six months storage period at 20 %RH.

Table 4. Moisture content (% w/w) of sucrose and lactose CNL powders stored at 4 or 20 %RH for 6 months.

Lyoprotectant	Storage RH (%)	Storage period (months)			
		0	1	3	6
Sucrose	4±1	2.54±0.34	2.42±0.64	2.78±0.03	2.03±0.48
	20±3		2.14±0.17	2.77±0.61	2.78±0.14
Lactose	4±1	2.63±0.36	2.01±0.37	2.14±0.14	1.70±0.23
	20±3		2.70±0.38	2.83±0.09	2.78±0.46

B- Thermal properties (DSC)

Before storage, sucrose CNL powder revealed amorphous sucrose with a T_g around 63 °C in the DSC thermogram (**Fig. 7**). The endothermic peak around 150 °C was attributed to the dehydration of nanocrystalline CIP HCl monohydrate (Shetty et al., 2018). The endothermic peak at ~170 to 180 °C represented sucrose melting (Jawad et al., 2018). After storage, the powders displayed similar thermograms except for the 6-month sample stored at 20 %RH which showed a lowering of the T_g toward ~50 °C and the appearance of an additional small peak at ~163 °C. These changes are indicative of the beginning of sucrose recrystallization. Kawakami et al. (2006a) reported that an imperfection in the crystal lattice of re-crystallized sucrose led to the appearance of two broad peaks at the melting temperature instead of one sharp peak. The DSC thermogram of lactose CNL powder immediately after manufacture exhibited a T_g around 63 °C. A broad endothermic peak was observed around 140 to 160 °C which is a combination of two dehydration events of nanocrystalline CIP HCl monohydrate and crystalline α-lactose monohydrate (Angberg, 1995). The DSC thermograms of the powder were not altered after storage at 4 or 20 %RH over the six months.

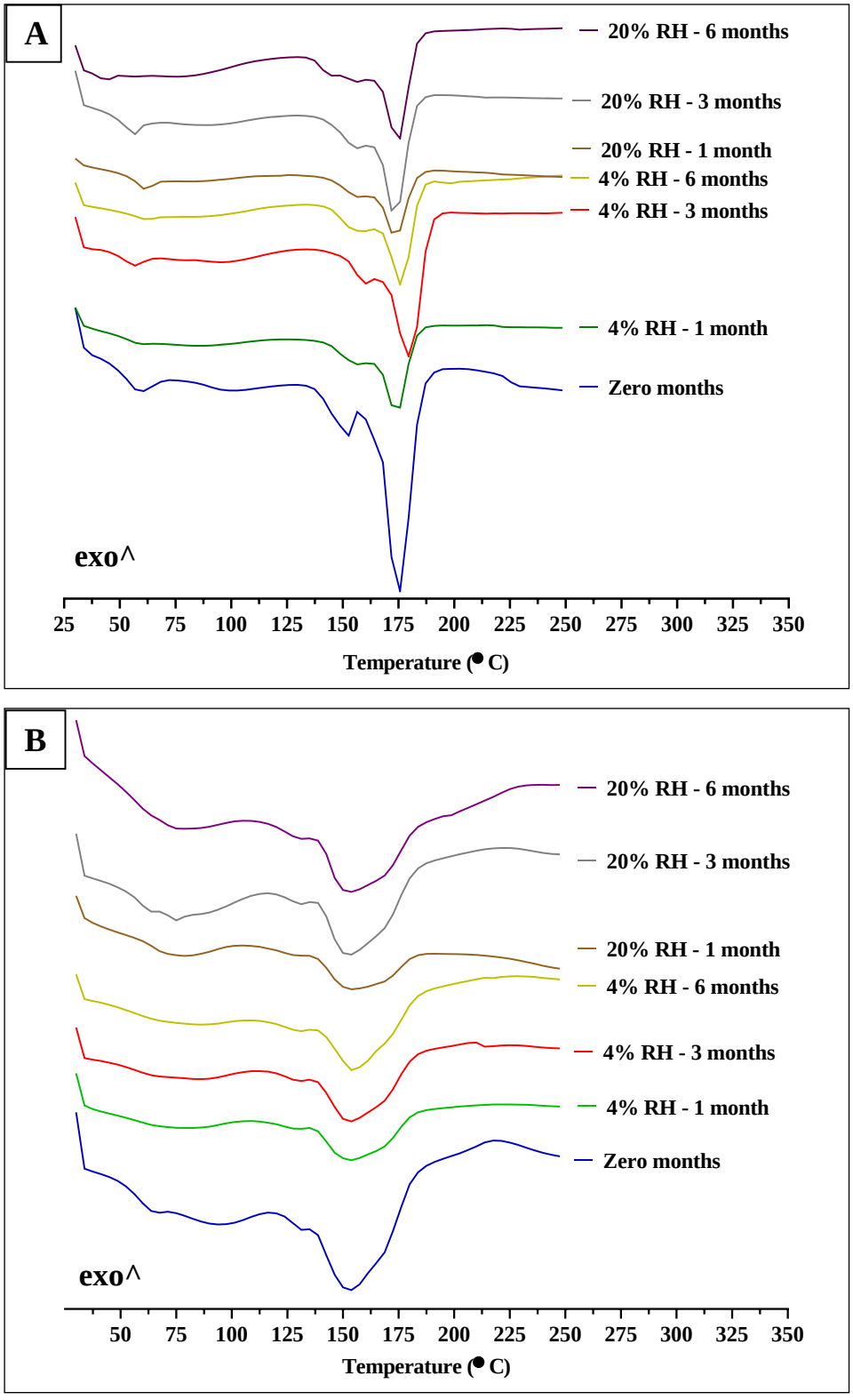

Fig. 7. DSC thermograms of sucrose (A) and lactose (B) CNL powders stored at 4 or 20 %RH for 6 months.

3.2.4. In vitro aerosol performance

The fine particle fraction (FPF) and per cent emitted dose (ED) were 61.7% and 85.0%, respectively, for sucrose CNL powder after manufacture and prior to storage (**Fig. 8**). Lactose

CNL powder possessed better aerosol performance (FPF = 76.2% and ED 84.3%) than the sucrose powders. Both powder types maintained consistent dispersibility throughout the six months of storage at 4 %RH (*P*-value > 0.05). The lactose CNL powder also displayed similar behaviour when stored at 20 %RH (*P*-value > 0.05). However, the aerosol performance of the sucrose CNL powder dropped significantly after six months of storage at 20 %RH. The FPF decreased to 47.3% (*P*-value <0.000001) while the ED increased to 92.0% (*P*-value = 0.005) likely due to solid bridging between particles as moisture adsorbed onto the surface of the particles (Shetty et al., 2020). The FPD of CIP declined from 2.94 to 1.87 mg (*P*-value = 0.0007) per capsule containing 4.5 mg CIP (**Table 5**). The MMAD increased from 2.48 to 4.11 µm (*P*-value = 0.000006).

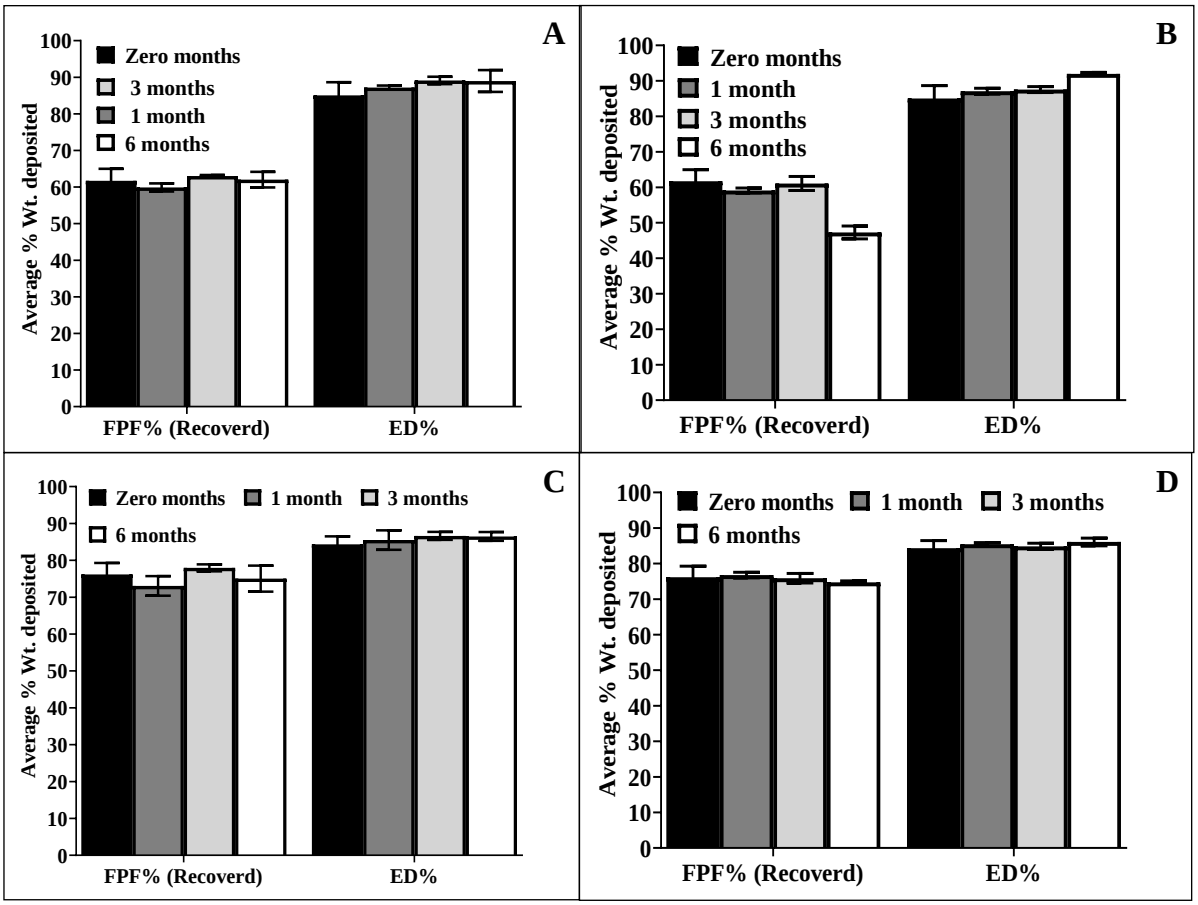

Fig. 8. Aerosol performance (FPF and ED) of CNL powders containing either sucrose (A and B) or lactose (C and D) stored for six months under 4 %RH (A and C) or 20 %RH (B and D). Mean with SD, n=3.

Table 5. Aerosol performance (FPD and MMAD) of CNL powders (sucrose and lactose) stored for six months at controlled RT and 4 or 20 %RH. Mean ± SD, n=3.

Storage RH (%)	Storage period (months)	Lyoprotectant			
		Sucrose		Lactose	
		FPD[a] (mg)	MMAD[b] (µm)	FPD[a] (mg)	MMAD[b] (µm)
	0	2.94±0.32	2.48±0.30	3.45±0.33	1.75±0.26
4±1	1	2.53±0.28	2.46±0.30	3.05±0.12	1.82±0.21
	3	2.62±0.01	2.73±0.06	3.29±0.13	1.80±0.06
	6	2.72±0.09	2.68±0.08	3.36±0.13	1.85±0.05
20±3	1	2.42±0.07	2.82±0.15	3.32±0.02	1.70±0.08
	3	2.51±0.06	2.76±0.04	3.23±0.06	1.80±0.02
	6	1.87±0.19	4.11±0.54	3.20±0.06	1.90±0.11

FPD[a]: Fine particle dose; MMAD[b]: Mass median aerodynamic diameter; RH: Relative Humidity.

4. Discussion

Dry powders that contain a high proportion of amorphous sugar typically suffer from instability upon storage at high relative humidity (Chang et al., 2020; Chen et al., 2016; Langrish and Wang, 2009; Naini et al., 1998; Shetty et al., 2018). Although the highest RH used in the present stability study was ~ 20%, a physical transformation occurred in the sucrose CNL powder after six months. As observed in the SEM images, thread-like structures emerged from the surfaces of sucrose CNL particles. Moisture adsorbed onto the particle surface increases molecular energetics and mobility to allow solid bridging between particles (Lechanteur and Evrard, 2020; Shetty et al., 2020). Consequently, while the mass of powder emptied from the capsule in the *in vitro* dispersion study increased, the mass of fine particles available in the dose decreased.

The PXRD data of sucrose CNL powders confirmed sucrose crystallization after six months of storage at 20 %RH. The appearance of a new peak and the increase in the intensity of the existing peak are two events reported previously for sucrose crystallization (Kawakami et al., 2006a). The T_g of sucrose CNL powder dropped thirteen degrees as detected by DSC thermograms, thus reducing the difference between the powder T_g and T_s. This evidence explains the liposomal instability and the decline in the aerosol performance of the sucrose CNL powder after six months of storage at 20 %RH. Even though sucrose and lactose are both unstable at high humidity (Naini et al., 1998; Ye et al., 2017), lactose exhibited better physical stability than sucrose. However, lactose was less capable of maintaining the liposomal

integrity. A decline in the EE value (~ 5% w/w) and increase in the liposomal size (~ 15 -20 nm) occurred after one-month storage irrespective of the storage RH. This can be attributed to liposomal fusion that happened in the solid-state during storage and led to drug leakage. Liposomal fusion results in an increase in liposomal size after powder reconstitution. Although lactose remained in the glassy state as no signs of recrystallization (no drop in T_g) were observed after one-month storage, liposomal fusion still could happen. A possible explanation is that the stability of dry liposomes depends not only on preserving a high T_g of the stabilizer but also on the stabilizer capability to maintain a depression in the T_m. The hydrogen bonding between the stabilizer and the phospholipids is directly responsible for T_m depression.

The overall stabilizing efficiency of sugars depends on more than one factor, and there is no single lyoprotectant suitable for all formulations. The stabilizing effects depend on the hydrogen bonding and/or vitrification capabilities of the lyoprotectant (Ingvarsson et al., 2011). Cipolla et. al. (2016) and Khatib et. al. (2021a) found sucrose to be superior to trehalose in protecting liposomes during either FT or SD. A specific hydrogen bond interaction between sucrose and the HSPC lipid was believed to provide this beneficial attribute. The apparent difference between the ability of these two sugars (i.e. sucrose and lactose) to preserve dry liposomes during storage may be related to essential differences in their method of interaction with the lipid bilayers (Crowe and Crowe, 1988). In this study, the change in the EE suggests that lactose-lipids bilayer interactions have changed over the one-month storage. Although a further drop in EE or an increase in particle size did not occur after one month of storage, the *in vitro* CIP release from lactose CNL was transitioning to a faster release profile over the six months storage. In the IVR study, liposomal integrity may decrease in the release media due to interactions with apoproteins and apolipoproteins, that simulate drug release in lung fluid. These proteins can bind to liposomal membranes leading to an increase in liposomal permeability to the dissolving CIP nanocrystals (Cipolla et al., 2014). Thus, CNL powders with partially effective lyoprotectants may exhibit a less consistent drug release behavior over time. Sucrose was effective as a lyoprotectant for liposomes during the stability study as long as the powder was stored at 4 %RH for six months. Otherwise, sucrose recrystallized which led to liposomal instability as represented by the ~ 7% drop in the EE, 10 nm decrease in liposomal size, and a significant increase in vesicle size polydispersity. Furthermore, the *in vitro* CIP release became similar to that of the non-crystalline liposomal dispersion ($f2 < 50$).

The sugar glassy state is essential for maintaining liposomal integrity as it prevents liposome fusion and drug leakage. However, if sugar crystallizes, the glassy state will be

disturbed (Franzé et al., 2018; Sun et al., 1996) causing instability of the liposomes. Although liposomal fusion, which increases the vesicle size after rehydration, was expected for recrystallized sucrose CNL, the liposomal size decreased about 10 nm from the initial size. This can be explained by probable drug leakage without liposomal fusion. A probable vesicle-vesicle adhesion happened rather than full fusion, the former causes strain on the membranes leading to drug leakage outside the liposome contact points. Some solute leakage with no liposome fusion was observed for egg PC liposomes in an aqueous solution as temperature increases (Anchordoguy et al., 1992; Kono et al., 1994). In another study, the observed 22% carboxyfluorescein leakage from liposomes protected by sucrose was not accompanied by an increase in the mean diameter of liposomes (Sun et al., 1996). Drug leakage will change the osmotic pressure differential across the liposomal membrane of the reconstituted liposomal vesicles. Thus, the entrapped water will diffuse out through the membrane bilayer which leads to a reduction in vesicles size in the absence of liposomal fusion, as observed with sucrose CNL stored at 20% RH for six months. However, the PDI of liposomal size distribution has increased from 0.4 to 0.7 which confirms the vesicular rearrangement into more than one size category, either large size due to the full fusion or small size because of drug leakage with only vesicle-vesicle adhesion.

To date, the only liposomal formulations encapsulating nanocrystalline CIP investigated for storage stability was an aqueous dispersion of CNL produced by FT (Cipolla et al., 2016). The study showed that frozen CNL was stable after three months of storage, and for optimum results, the drug should be thawed just before patient administration (Cipolla et al., 2015a). Besides enhancing the product stability at RT, the ultimate goal of dry CNL powders is to increase patient adherence to treatment with a small portable inhaler device for powder inhalation (Cipolla et al., 2013; Ingvarsson et al., 2011; Parumasivam et al., 2017; Willis et al., 2012). The nanocrystalline CIP formed during the spray drying step is believed to present in the dry powder form (solid-state). This hypothesis has been based on the SEM, DSC, and X-ray data obtained from our previous publications (Khatib et al., 2021b; Khatib et al., 2019a; Khatib et al., 2020). First, the images obtained by the SEM showed needle-like structures for liposomal samples spray-dried using the least amount of protectant (sucrose). These structures were representing the growth of CIP nanocrystals outside the liposomal vesicles due to the poor protection because of the suboptimal sugar amount (Khanal et al., 2020; Khatib et al., 2020). Second, the DSC thermogram of CNL powder revealed a CIP monohydrate peak, this is a typical peak in the DSC thermogram of the crystalline CIP. Finally,

the powder x-ray diffraction patterns of CNL powders either formed solely by spray drying or by freeze-thaw (to generate CIP nanocrystals) followed by spray drying (FT-SD) were similar (Khanal et al., 2020; Khatib et al., 2019a), and both showed the CIP monohydrate peak. However, these data only provided indirect evidence for our hypothesis. All discussions of CIP nanocrystals were based on data that involved the powder reconstitution step in liquid before testing by Cryo-TEM or measuring EE and IVR. CIP nanocrystals appeared in cryo-TEM images as elongated cylindrical structures within the core of liposomal vesicles (Khatib et al., 2021b; Khatib et al., 2019a; Khatib et al., 2020). EE was an indirect indicator of the presence of CIP inside the liposomal vesicles. Moreover, the IVR assay revealed that samples with low protectant amounts, which had a low EE and many empty liposomes in the Cryo-TEM images, had an immediate release of CIP (Khatib et al., 2020). In contrast, samples of proper protectants types and amounts had a controlled and delayed CIP release compared to the control (non-nanocrystalline CIP inside liposomes). Slow dissolution and release of crystalline structures entrapped inside a closed environment (liposomes) were evidenced with other crystallized drugs inside liposomes (Cipolla et al., 2016; Li et al., 2018). Taken together, these data did provide strong indirect evidence to show the location of CIP nanocrystals being inside the liposomes. However, these are not direct evidence as such data are currently unavailable due to the lack of analytical techniques that can visualize nanocrystals within liposomes at the nanoscale in the solid-state.

In this stability study, liposomal powder of nanocrystalline CIP protected by sucrose can be stored without refrigeration (controlled RT) and transported without the need for the cold chain for up to six months provided that the RH is kept low at 4%.

5. Conclusion

Dry powder inhaler of CNL with sucrose lyoprotectant remained physiochemically stable when stored under a dry condition (4% RH) at controlled RT. When stored at 20% RH, sucrose began to recrystallize at six months of storage, and consequently, liposome EE decreased along with CNL dry powder aerosol performance. Although lactose as lyoprotectant was an efficient protectant for liposomes during SD, it failed to maintain liposomal stability for six months of storage. Within one month, the EE declined while the liposome particle size increased. Moreover, after six months of storage, the CIP release from CNL powder was different and less controlled than the CIP release of the freshly prepared powder.

Overall, this research suggests that an inhalable CNL powder formulation with sucrose as a stabilizing excipient when stored under a relatively low humidity environment of 4 %RH and controlled RT storage possesses at least a six-month shelf-life.

Declaration of interest

This work is related to a provisional patent filed by 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 Hak-Kim Chan and Isra Khatib.

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Declaration of interests

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

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

This work is related to a provisional patent filed by 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 Hak-Kim Chan and Isra Khatib.

Authorship Statement

Manuscript title: Storage stability of inhalable, controlled-release powder formulations of ciprofloxacin nanocrystal-containing liposomes

Authorship contributions

Isra Khatib: Conceptualization, Data Curation, Formal Analysis, Investigation, Software, Methodology, Writing - Original Draft.

Wei-Ren Ke: Investigation, Data Curation, Writing - Review & Editing.

David Cipolla: Conceptualization, Resources, Writing - Review & Editing.

Kim Chan: Conceptualization, Funding Acquisition, Resources, Project Administration, Supervision, Writing - Review & Editing.