

## Article

# Spray-Dried Proliposome Microparticles for High-Performance Aerosol Delivery Using a Monodose Powder Inhaler

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1 **Spray-Dried Proliposome Microparticles for High Performance Aerosol**  
2 **Delivery using a Monodose Powder Inhaler**

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42

43 **Abstract**

44 Proliposome formulations containing salbutamol sulphate (SS) were developed using spray drying,  
45 and the effects of carrier type (lactose monohydrate (LMH) or mannitol) and lipid to carrier ratio  
46 were evaluated. The lipid phase comprised soy phosphatidylcholine (SPC) and cholesterol (1:1), and  
47 the ratios of lipid to carrier were 1:2, 1:4, 1:6, 1:8 or 1:10 w/w. X-ray powder diffraction (XRPD)  
48 revealed an interaction between the components of the proliposome particles, and scanning electron  
49 microscopy (SEM) showed that mannitol-based proliposomes were uniformly sized and spherical,  
50 whilst LMH-based proliposomes were irregular and relatively large. Using a two-stage impinger  
51 (TSI), fine particle fraction (FPF) values of the proliposomes were higher for mannitol-based  
52 formulations, reaching 52.6%, which was attributed to the better flow properties when mannitol was  
53 used as carrier. Following hydration of proliposomes, transmission electron microscopy (TEM)  
54 demonstrated that vesicles generated from mannitol-based formulations were oligolamellar, while  
55 LMH-based proliposomes generated “worm-like” structures and vesicle clusters. Vesicle size  
56 decreased upon increasing carrier to lipid ratio, and the zeta potential values were negative. Drug  
57 entrapment efficiency (EE) was higher for liposomes generated from LMH-based proliposomes,  
58 reaching 37.76% when 1:2 lipid to carrier ratio was used. The in vitro drug release profile was similar  
59 for both carriers when 1:6 lipid to carrier ratio was used. This study showed that spray drying can  
60 produce inhalable proliposome microparticles that can generate liposomes upon contact with an  
61 aqueous phase, and the FPF of proliposomes and the EE offered by liposomes were formulation-  
62 dependent.

63 **Key words:** Aerosol; Morphology; Particle size; Powder; Pulmonary

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70 **1. Introduction**

71 Inhalation of therapeutic materials in liposome formulations has been studied as a strategy for  
72 controlled drug release in the lungs. There is evidence that liposomes can retain the drug in the  
73 pulmonary tissues for prolonged periods, reducing the need for frequent dosing and minimizing the  
74 potential of systemic adverse effects (1-5). Many studies have investigated the pulmonary delivery  
75 of relatively large volumes of liposome dispersions using medical nebulizers (1,6,7). There are at  
76 least two nebulizable liposome formulations currently in clinical trials for potential approval in the  
77 treatment of pulmonary infections, for example, Arikace® (liposomal Amikacin) for the treatment of  
78 pseudomonas aeruginosa in cystic fibrosis patients (7,8), and Pulmaquin® (liposomal ciprofloxacin)  
79 for the treatment of non-cystic fibrosis lung infections (9).

80 Although nebulization is the most studied means of delivering liposomes by inhalation, there are a  
81 number of disadvantages associated with using nebulizers for this purpose. First, liposome instability  
82 caused by shearing during nebulization and concomitant losses of the originally entrapped drug is a  
83 major challenge, necessitating extensive work to engineer the optimal liposome size and bilayer  
84 composition, and select nebulizers with appropriate designs and operating parameters (7,10). Second,  
85 the performance of the aerosol (e.g. output, droplet size, 'FPF', etc.) generated from these nebulizers  
86 is greatly influenced by the physicochemical properties of formulation (11,12), which means that  
87 nebulizer performance for one liposome dispersion may not be the same for another formulation (13).  
88 Third, the large volumes delivered via nebulizers may contribute to toxicity not only by the drug but  
89 also by the accompanying excipients. For example, in pulmonary infections, inhalation of relatively  
90 large antibiotic doses is needed to eradicate the infection, necessitating the use of large phospholipid  
91 quantities to entrap the antibiotic and sustain its release. In spite of the established biocompatibility  
92 and biodegradability of liposomes, dose-limiting toxicity of phospholipids in animals has been  
93 reported (14).

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94 Dry powder inhalers (DPIs) offer the advantages of delivering small doses of drug and excipients,  
95 and avoidance of shearing-induced liposome instability during delivery. Compared to the number of  
96 studies published for the delivery of liquid liposome dispersions via nebulization, a limited number  
97 of reports have attempted to explore the potential of liposomes and phospholipid formulations for  
98 pulmonary applications delivered in the dry powder form. It has been postulated by several  
99 investigators that dried liposomes would exploit the aqueous environment of the lung to be hydrated  
100 *in situ* within the respiratory tract (5). Freeze-drying of liposomes in the presence of a cryoprotectant  
101 followed by micronization has been studied for the generation of inhalable dry powder liposomes;  
102 however, milling may exert a deleterious effect on vesicle stability, causing leakage of the drug upon  
103 rehydration (15,16). As an alternative to freeze-drying (lyophilization), spray-drying of liposomes  
104 dispersed in carbohydrate solutions has been investigated, with high powder 'respirability' being  
105 reported in a range of studies (17-21).

106 As an alternative to traditional liposome powders, particulate proliposome formulations have been  
107 developed for inhalation. Proliposomes are carbohydrate carriers coated with phospholipid to  
108 generate liposomes upon addition of aqueous phase (22,23). Proliposomes, in the context of this  
109 study, are phospholipid and drug blended with diluent carbohydrate carriers, aiming to generate  
110 liposomes upon contact with the pulmonary physiological milieu (5). In one approach, phospholipid  
111 and drug were mixed with lactose followed by air-jet milling. The resultant proliposome blend  
112 generated multilamellar vesicles (MLVs) upon dispersion in aqueous phase, with entrapment  
113 efficiencies (24) and fine particle fractions (FPF) (25) being dependent on formulation. Spray drying  
114 of alcoholic phospholipid solutions may also generate proliposome particles (26-28) with FPFs in the  
115 range of 20-30% using antibiotics such as pyrazinamide (28). Thus, the potential of inhalable  
116 proliposome powders has been explored for delivering antimicrobial drugs. Although the antibacterial  
117 activity of hydrated proliposome has been established *in vitro* (27), the need for large doses for the  
118 eradication of lung infections *in vivo* raises doubts about the suitability of DPIs in delivering  
119 therapeutic amounts of antimicrobial agents. Unlike pulmonary infections, the doses needed to treat

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120 asthma are very small, hence powdered formulations (e.g. in the form of proliposomes) can  
121 potentially be used as antiasthma delivery systems.

122 In this study, we have introduced a potentially applicable approach for pulmonary delivery using  
123 spray-dried proliposomes loaded with salbutamol sulphate (SS). Proliposomes consisted of  
124 carbohydrate carriers (lactose monohydrate or mannitol) and lipids (soya phosphatidylcholine and  
125 cholesterol; 1:1) were formulated using a range of lipid to carriers ratios. The resultant proliposome  
126 powders were thoroughly characterized, and using a two-stage impinger (TSI), the deposition of the  
127 drug was evaluated following proliposome delivery from a Monodose inhaler device. Furthermore,  
128 the ability of the proliposomes to generate liposomes following hydration was studied and drug  
129 entrapment was determined, to evaluate the potential of the formulations in providing a reservoir, *in*  
130 *situ*, for sustaining the drug release. The findings of this study using proliposomes were evaluated in  
131 light of the progress achieved in the field of pulmonary delivery of dry powder formulations.

132

## 133 **2. Materials and methods**

134

### 135 **2.1 Materials**

136 Lactose monohydrate (LMH) was purchased from VWR, UK, and D-mannitol and cholesterol (CH)  
137 were bought from Sigma Aldrich, UK. Solvents used in HPLC experiments including Water and  
138 methanol were HPLC-grade and HPLC-grade 99.9%, respectively and were supplied by Fisher  
139 Scientific, UK. Absolute ethanol and ethanol (96%) were also purchased from Fisher Scientific, UK.  
140 Sodium 1-hexane sulfonate monohydrate (99%), acetic acid glacial (99%) and salbutamol sulphate  
141 (SS; 99%) were purchased from Alfa-Aesar, UK. The phospholipid used, namely soya  
142 phosphatidylcholine (SPC; Lipoid S-100) was kindly gifted by Lipoid, Switzerland.

143 **2.2 Methods**

144

145 **2.2.1 Spray drying of mannitol and LMH**

146 Carbohydrate carriers (LMH or mannitol) were spray-dried by dissolving the carrier particles in  
147 distilled water (1% w/v), followed by spraying the carbohydrate solution through the spray-drier's  
148 nozzle (diameter = 0.7 mm) using a B-290 spray drier (Büchi, Switzerland). The inlet temperature  
149 was set at 130°C, spraying flow rate was 600 L/h, feed rate was 17%, and the outlet temperature was  
150  $70 \pm 2^\circ\text{C}$ . The resultant spray-dried microparticles were used as core carriers to prepare particulate-  
151 based proliposomes. This step of spray drying aimed for enhancing the dispersion of the carbohydrate  
152 carriers in ethanol during the preparation of proliposomes, as described in the subsequent section.

153 **2.2.2 Manufacture of particulate-based proliposome particles via spray drying**

154 The constituents used to prepare proliposomes are demonstrated in Table 1. Spray-dried mannitol or  
155 LMH microparticles were employed as core carriers for manufacturing proliposomes. This was  
156 achieved by weighing a total of 100 mg lipid consisting of SPC and CH (1:1 mole/mole), followed  
157 by addition of 100 mL ethanol (96% grade), and SS (10 mg). The alcoholic mixture was sonicated  
158 for 1 min to aid complete dissolution of lipids in ethanol. The spray-dried mannitol or LMH in various  
159 ratios were dispersed in the ethanolic solution followed by sonication for 15 min to break up any  
160 agglomerates of the carbohydrate particles before performing spray drying using the same Büchi B-  
161 290 Mini Spray Dryer equipment, but this time connected with the Büchi's inert loop system (Büchi,  
162 Switzerland). The homogeneity of the resultant alcoholic mixture was maintained by continuous  
163 stirring while feeding the mixture into the spray drier. The spray drying conditions were optimized  
164 by adjusting the inlet temperature at 120°C, spray flow rate was 600 L/h, feed rate was 11% and the  
165 outlet temperature was  $73 \pm 3^\circ\text{C}$ . The resultant powder constituting lipid/carbohydrate was collected  
166 and referred to as 'proliposomes', which were stored in a desiccator for later use in the same day.

167

168 **2.2.3 Hydration of spray-dried powder**

169 Samples of spray-dried powders were hydrated by adding deionized water followed by vortex mixing  
170 for 2 min in order to test for the possible generation of liposomes. The liposome dispersion was  
171 allowed to “anneal” for around 1 h at room temperature before performing further characterization.  
172 Allowing the liposomes to anneal at temperatures above that of the lipid phase transition ( $T_m$ ) may  
173 promote the stability of liposomes by overcoming structural defects of the bilayers [29].

174

175 **2.2.4 Product yield estimation**

176 The product yield ( $PY$ ) of spray-dried powders was determined using the weight of the final spray-  
177 dried powder ( $W^o$ ) divided by the initial weight of carrier, lipid and drug employed ( $WT$ ). The product  
178 yield, drug recovery and content drug uniformity were determined according to the following  
179 equations (30):

180 
$$PY (\%) = \frac{W^o}{WT} \times 100 \quad Eq. 1$$

181

182 
$$Drug\ recovery (\%) = \frac{Pw}{WT} \times 100 \quad Eq. 2$$

183 Where  $Pw$  is the practical weight of the drug after spray-drying was performed, as quantified using  
184 high performance liquid chromatography (HPLC).

185 
$$Content\ drug\ uniformity (\%) = \frac{Drug\ recovery (\%)}{PY (\%)} \times 100 \quad Eq. 3$$



186

187 **2.2.5 Scanning electron microscopy (SEM)**

188 Particle size and morphology of spray-dried formulations were studied using scanning electron  
189 microscopy (SEM, Quanta-200, FEI at 20 kV). Spray-dried particles were spread onto an aluminium  
190 stub and coated with a film of gold using the JFC-1200 Fine Coater (JEOL, Tokyo, Japan).

191

192 **2.2.6 X-ray powder diffraction (XRPD) studies**

193 X-ray diffraction (XRPD) studies of powder were performed by utilizing the Equinox 2000 (Inel,  
194 France) using a diffracted-beam monochromator with Cu radiation. The intensity of diffractions was  
195 recorded at an angle of 2-theta, at a diffraction scan duration of 20 min. The experiments were  
196 conducted by keeping the voltage at 32 kV and the current generator at 28 mA.

197

198 **2.2.7 Studies of vesicle morphology using transmission electron microscopy (TEM)**

199 A drop of hydrated spray-dried powder was carefully positioned on carbon-coated copper grids (400  
200 mesh; TAAB Laboratories Equipment Ltd., UK), followed by negative staining (using 1%  
201 phosphotungstic acid; PTA). The samples were imaged using the Philips CM 120 Bio-Twin electron  
202 microscope (Philips Optics BV, the Netherlands).

203

204 **2.2.8 Particle size measurements of hydrated samples**

205 Size and size distribution of liposomes generated upon hydration of spray-dried proliposomes were  
206 analysed by laser diffraction using the Mastersizer 2000 (Malvern Instruments Ltd., UK). The median  
207 size, also referred to as volume median diameter (VMD; 50% undersize), and Span were measured  
208 to represent the size and size distribution (polydispersity) of liposomes, respectively. Span value

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209 represent the difference between 90% undersize and 10% undersize divided by the VMD. Span is a  
210 unit-less term introduced by the manufacturer of the Malvern Mastersizer 2000 instrument.

211

#### 212 **2.2.9 Zeta potential measurements**

213 The zeta potential of vesicles was determined via the Zetasizer Nanoseries instrument (Malvern  
214 Instruments Ltd., UK) by choosing the relevant software option of the instrument. Proliposomes were  
215 hydrated with deionized water with shaking. The resultant liposomes (70 µL) were loaded via a  
216 Gilson pipette into the Malvern's zeta potential cells, after setting the temperature at 25°C and  
217 allowing 2 min for sample equilibration in order to obtain consistent zeta potential measurements.

218

#### 219 **2.2.10 Drug content and entrapment efficiency (EE) studies**

220 Spray-dried powder (10 mg) was dispersed with deionized water (1 mL) to generate liposomes. The  
221 dispersion was transferred into a volumetric flask (10 mL), and methanol (1 mL) was added to  
222 dissolve the lipid followed by making up the volume with water. HPLC was employed to determine  
223 the drug content in the flask (i.e. total drug loaded into the 10 mg powder sample) by adapting a  
224 method we published established (13). The mobile phase was made by making a buffer solution  
225 constituting a mixture of sodium hexane sulfonate in water (5 mM) and methanol (75:25 v/v), to  
226 which glacial acetic acid was added to have 1% of the total volume. The high-performance liquid  
227 chromatography (HPLC; Agilent 1200 - UV detector system; Hewlett-Packard Co., USA) was set up  
228 with a Symmetry C-18 column (150 mm, 4.6 mm, 5µm; Waters, UK). The samples (injection volume  
229 of each is 20 µL) were analysed at 276 nm. The flow rate of mobile phase was set up at 1 mL / min  
230 at 40 °C. To determine the entrapment efficiency (EE), spray-dried powder (10 mg) was hydrated  
231 using deionized water (50 µL) followed by vortex mixing for 2 min and dilution with deionized water  
232 (950 µL). The liposomes were left for 1 h at room temperature to anneal, followed by further dilution  
233 to 8 mL with deionized water. The liposomes were then centrifuged at 55,000 rpm (277,000 x g) for

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234 35 min at 6°C (Beckman LM-80 ultracentrifuge; Beckman Coulter Instruments, USA). The  
235 supernatant was aspirated for subsequent analysis of SS (the untrapped fraction of drug). The EE  
236 was determined using the following equation:

237

$$238 \quad EE(\%) = \frac{\text{Total drug loading} - \text{Untrapped drug}}{\text{Total drug loading}} \times 100 \quad Eq.4$$

#### 239 **2.2.11. In vitro drug release study**

240 The release of SS from liposomes generated from the proliposome powders was investigated using  
241 the dialysis method. Spray-dried powder (71 mg containing 1 mg SS) was dispersed with deionized  
242 water (0.5 mL) followed by vortex mixing for 1 min and dilution with deionized water (1.5 mL) to  
243 generate liposomes. The dispersion was placed in a dialysis tube (MWCO 3500) and tightly sealed.  
244 For free drug, 1 mg SS was dissolved in 1 ml of ethanol: water: tween 80 (20:79.9:0.1%). Then, the  
245 dialysis tube was immersed in 50 mL (total volume) release medium (deionized water) containing  
246 0.1% (v/v) Tween 80) and incubated with stirring in for 24 h at 37°C. Samples (0.3 mL) were taken  
247 at time intervals from the release medium for 24 h, and replaced by a similar volume of fresh medium.  
248 The concentration of SS was determined by HPLC using the methods described above.

249

#### 250 **2.2.12 Assessment of aerosol performance *in vitro***

251 Using the Two-Stage Impinger (TSI), also called the Twin Impinger or the Single Stage Glass  
252 Impinger (Copley Scientific Ltd, Nottingham, UK), the performance of spray-dried proliposome  
253 aerosols and deposition profile were investigated (31) using a Miat Monodose powder inhaler (Miat,  
254 Italy). TSI is designed with two stages: the upper stage (S1) and the lower stage (S2), attempting to  
255 represent the upper airways and lower airways, respectively. The flow rate through the TSI was set  
256 up at 60 L/min. The cut-off aerodynamic diameter between the two stages at 60 L/min is 6.4 µm,  
257 hence particles smaller than this aerodynamic diameter will most likely deposit in the lower impinger  
258 and will constitute the 'fine particle fraction' ('FPF') (31). Proliposome powder (25 mg) was filled

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259 into hydroxypropyl methylcellulose (HPMC; size 3) capsules. Each capsule was loaded into the Miat  
260 device which was then connected to the impinger that contained deionized water as collection  
261 medium (7 mL in the upper stage (S1) and 30 mL in the lower stage (S2). In each experiment, the  
262 capsule content was pulled by applying negative pressure (60 L/min) through the actuated inhaler  
263 device over 5 s. Then, the impinger was dismantled and each stage, the inhaler device and capsule  
264 were separately rinsed with deionized water for subsequent drug quantification using HPLC. The  
265 total amount of drug in the inhaler device, S1 and S2 constitutes the recovered dose (RD) of the drug.  
266 The amount of drug deposited in S1 and S2 of the impinger constitutes the emitted dose (ED)  
267 calculated as the percentage proportion of the RD (Eq. 5). The percentage proportion of the drug that  
268 is deposited in S2 of the impinger was calculated as the “FPF” (Eq. 6).

$$269 \quad ED = \frac{S1 + S2}{RD} \times 100 \quad Eq. 5$$

$$270 \quad FPF = \frac{S2}{RD} \times 100 \quad Eq. 6$$

271

### 272 **2.2.13 Proliposome flowability studies**

273 Aiming to understand the behaviour of selected formulations, the bulk density of the spray-dried  
274 proliposome powder was measured by using the ERWEKA tapped density meter (ERWEKA®p  
275 GmbH, D-63150 Heusenstamm, Germany). A defined mass of powder was poured into a calibrated  
276 measuring cylinder and the volume occupied by the powder was recorded. The tapped density of  
277 spray dried powder was determined by volume measurement of the tapped mass until no further  
278 changes in the powder volume were observed. Hausner ratio and Carr’s index, also called Carr’s  
279 compressibility index, for each spray dried powder were derived according to the following  
280 equations:

$$281 \quad BD = \frac{W}{V} \times 100 \quad Eq. 7$$

$$282 \quad TD = \frac{W}{Vt} \quad Eq. 8$$

$$\text{Hausner Ratio} = \frac{V}{V_t} \quad \text{Eq. 9}$$

$$\text{Carr's Index} = \left[1 - \left(\frac{BD}{TD}\right)\right] \times 100 \quad \text{Eq. 10}$$

Where BD and TD are bulk density and tapped density, respectively, and V and V<sub>t</sub> are actual volume and tapped volume, respectively.

#### 2.2.14 Statistical analysis

All experiments were conducted three times using three different proliposome batches. Statistical significance was studied using one-way analysis of variance (ANOVA) and student's *t*-tests, for comparing more than two sets and two groups of results, respectively. P values < 0.05 indicate that difference between compared groups is statistically significant.

### 3. Results and discussion

#### 3.1 Product yield and drug content uniformity of spray-dried proliposomes

Table 2 shows the product yield (PY), drug recovery and drug content uniformity of powder formulations. Since spray-drying conditions were the same for all formulations, any difference in PY was attributed to formulation composition, namely, carrier type and lipid to carrier ratio. Spray-drying parameters such as atomizer design, flow rate and temperature of the drying air, solid content of the feed solution can all influence PY of spray-dried powder (32).

For both carriers, a direct relationship was observed between PY and carrier concentration (Table 2). PY of F1 was lower than F2 (*p*<0.05), and PY of F2 was lower than F3, F4 and F5 (*p*<0.05) (Table 2). However, when PY values of F3, F4 and F5 were compared, the difference was not significant statistically (*P*>0.05). Moreover, PY of F6 was significantly (*p*<0.05) lower than PY of F7 which, in turn, was significantly (*p*<0.05) lower than PY values observed with F8, F9 and F10. Only a trend of higher PY was also found for F9 and F10 when compared to F8 (*p*>0.05) (Table 2). The decrease in

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307 PY of spray-dried formulations is attributed to possible adherence of the sprayed droplets and dry  
308 particles to the inner walls of the drying compartment, or because of poor collection of fine powder  
309 by the cyclone separator [33]. Thus, the high lipid content of F1, F2, F6 and F7 can be responsible  
310 for the low PY of these formulations, causing adherence of lipid to the inner walls of the drying  
311 compartment.

312 The PY of LMH formulations (F6, F7, F8, F9 and F10) was generally higher than PY of proliposomes  
313 based on mannitol carrier (F1, F2, F3, F4 and F5), indicating that it is not only lipid content that can  
314 affect PY but also carrier type (Table 2). Other reports have demonstrated that glass phase transition  
315 ( $T_g$ ) of carbohydrates has a prime effect on the behaviour of formulations during spray drying (34-  
316 37). When the temperature during spray drying is higher than  $T_g$  of the sugar employed, adherence  
317 of formulation components on the walls of the drying chamber may increase, resulting in lower  
318 powder yields (38). The  $T_g$  of lactose is 101°C (39,40) which is higher than the outlet temperature  
319 used in the present study, whilst  $T_g$  of mannitol is lower than the outlet temperature used (41-44),  
320 making mannitol-based proliposomes more adhesive with concomitant lower PY than LMH-based  
321 formulations (Table 2).

322 Table 2 also shows the recovery values of salbutamol sulphate (SS). The recovery of drug increased  
323 with increasing the carrier ratio in the proliposomes, regardless of carrier type. The enhanced drug  
324 recovery is paralleled with the higher PY obtained when higher carrier ratios (i.e. lower lipid  
325 concentrations) were used. Thus, low drug recovery for F1, F2, F6 and F7 formulations is attributed  
326 to the incorporation of high lipid contents. Table 2 also shows drug content uniformity in the spray-  
327 dried proliposomes, which was in the range of 90 - 109%, indicating uniform distribution of SS in  
328 the powder. Drug content uniformity using LMH carrier was higher than formulations based on  
329 mannitol carrier (Table 2). In an attempt to provide the reasons behind these differences, particle  
330 morphology of proliposomes was investigated as illustrated in the subsequent section.

331

332 **3.2 Morphology of spray-dried proliposome particles**

333 Particle morphology of proliposomes presented in Table 1 was studied using SEM (Figure 1).  
334 Mannitol-based particles looked spherical regardless of lipid to carrier ratio (Figure 1), coming in  
335 agreement with previous investigations employing this carrier (26). Particles of F1 and F2 were  
336 spherical and had small sizes, and apparently smooth surfaces, and tended to form large agglomerates  
337 (Figure 1a, b). The agglomeration of these two formulations can be attributed to their high lipid  
338 content, and may justify their low PY values (Table 2), making their potential for ‘deep lung’  
339 deposition questionable. By contrast, F3 particles were small, porous and spherical, with less  
340 propensity to form agglomerates (Figure 1c). Porosity of particles can enhance their aerosol  
341 performance (45). F4 and F5 were small and spherical with apparently smooth surfaces and evidence  
342 of particle agglomeration (Figure 1d, e), possibly due to high surface energy of the particles, which  
343 commonly increases cohesiveness and compromises flowability (46-48).

344 By contrast, LMH-based proliposome microparticles were irregular, rough and not similar in size  
345 (Figure 2). LMH is practically insoluble in ethanol used as the solvent in the present investigation.  
346 Upon atomization during spray drying, it appears that ethanol did not form uniform droplets; hence,  
347 the resultant proliposome particles had an irregular shape and wide size distribution. Studies have  
348 correlated particle surface morphology with aerosol performance (49). Smooth particles have high  
349 flowability and are potentially applicable for aerosolization (50). The irregular shape and rough  
350 surface of lactose microparticles can promote the interaction between carrier and drug (49).

351 Particles that have spherical shape may have high chance for deposition in the peripheral airways,  
352 especially when the aerodynamic size is in the range of 1-5  $\mu\text{m}$  (51-54). Thus, LMH-based  
353 proliposomes might have lower suitability for delivery from DPI devices than mannitol-based  
354 formulations, since LMH-based particles are larger and more irregular in shape (Figure 2). According  
355 SEM, the potentially most appropriate proliposome formulation for use as DPIs would be F3 (i.e.  
356 using mannitol carrier with 1:6 w/w lipid to carrier ratio) (Figure 1c).

357

### 358 **3.3 Crystallinity of spray-dried formulations**

359 XR diffraction profiles of SS are shown in Figure 3. The intensity of drug peaks before and after  
360 spray drying indicates that the crystalline characteristics of SS were preserved. The intensity peak  
361 after spray drying increased slightly, because ethanol can increase powder crystallinity (55-58).  
362 Mannitol had high crystallinity before spray drying (Figure 3c); however, the peak intensity decreased  
363 by spray drying (Figure 3d), indicating reduced crystallinity of this sugar (59). High amorphous  
364 content of solids can facilitate dispersion of powder in aqueous media (60-62), which is advantageous  
365 in dry powder formulations.

366 Spray-dried mannitol is crystalline (Figure 3d). The X-ray diffraction profile of the drug was not  
367 detected in the proliposome formulations (Figure 4; Figure 6), because of the low drug concentration  
368 when compared to the other formulation components (i.e. mannitol and lipid). Moreover, the drug  
369 might have been coated by SPC that is known to be amorphous, resulting in poor detection of  
370 crystalline drug traces. Similarly, DSC curve of the formulation F3, for example, did not show a  
371 thermogram for the drug. However, the pure drug shows an endothermic melting peak with the onset  
372 of about 200°C.

373 The X-ray diffraction patterns of mannitol proliposomes are presented in Figure 4. The crystalline  
374 characteristics of mannitol were dominant in all formulations because of the high content of sugar  
375 compared to the other components of the formulations (i.e. drug and lipid). X-ray diffraction profile,  
376 as evident from the intensity of the main peak of mannitol-based proliposomes was formulation-  
377 dependent, when F1-F5 formulations were considered. The intensity of the main peak increased  
378 slightly by increasing the ratio of mannitol, owing to the high crystallinity of this type of sugar. The  
379 intensity peak of mannitol-based proliposomes (F1-F5) was lower than mannitol alone, indicating  
380 other formulations components (mainly the lipid) have decreased powder crystallinity.



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381 Figure 5 shows the X-ray diffraction profiles of LMH before and after spray-drying in aqueous or  
382 alcoholic solutions. As shown in Figure 5a, LMH demonstrated a crystalline profile before spray-  
383 drying (Figure 5a), and converted into amorphous because of spray-drying in aqueous solution  
384 (Figure 5b), agreeing with previous reports employing this type of sugar (63-67). By contrast, LMH  
385 preserved its crystallinity after spray drying from its ethanolic solution, possibly because of the lower  
386 solubility of this sugar in ethanol when compared to its aqueous solubility. However, the intensity  
387 peak of LMH was diminished by spray drying from ethanol compared to before spray drying (Figure  
388 5c). LMH is crystalline in all formulations due to the high sugar content, regardless of formulation  
389 (Figure 6); however, slight differences in the intensity of the main peak was observed when the  
390 formulations F6-F10 were compared. Thus, crystallinity increased slightly with increasing LMH ratio  
391 in the formulation.

392 The DSC thermographs for F3 and F8 conformed the preserved crystallinity of both mannitol and  
393 LMH, respectively, after spray-drying from ethanolic solution. However, no peaks appeared for the  
394 drug in both formulations thermograms (data not shown).

#### **3.4 Drug entrapment in liposomes generated upon hydration of spray-dried proliposomes**

396 Drug entrapment efficiency (EE) in liposomes was determined after hydration of the proliposome  
397 powder (Table 3). For mannitol-based proliposomes, entrapment differed slightly for different  
398 formulations (Table 3). This can be attributed to different proportions of lipid recovered after spray  
399 drying, or difference in morphology of proliposome microparticles with accordance to using different  
400 formulations. Rough carrier particle surfaces may facilitate carrier-drug interactions due to having  
401 high surface area, whilst smooth surfaces may result in loose interactions between the drug and carrier  
402 (49,68,69). Thus, the apparently rough surfaces of LMH-based formulations could be responsible for  
403 enhanced drug-carrier interactions, facilitating drug encapsulation by liposomes upon hydration. This  
404 explains the higher drug entrapment in vesicles generated upon hydration of LMH-based  
405 proliposomes compared to mannitol-based formulations (Table 3). Furthermore, F6 and F7 (i.e. lower  
406 LMH content; higher lipid proportion) had greater drug entrapment efficiencies than F8-F10 (i.e.

407 higher LMH content; lower lipid content) formulations. Thus, the relatively low lipid concentrations  
408 in F8, F9 and F10 caused the generation of dilute liposome dispersions; thus, lower drug proportions  
409 were encapsulated by the vesicles (Table 3). LMH-based proliposomes with 1:2 lipid to carrier ratio  
410 (i.e. F6 formulation) gave the greatest drug entrapment efficiency, because of the high lipid content  
411 in this formulation. These findings demonstrated a correlation between PY, particle morphology and  
412 drug entrapment efficiency. Other investigators have hypothesized possible hydration of proliposome  
413 powders *in situ* within the lung after inhalation by exploiting the aqueous physiological environment  
414 of the lung (24-28). *In vivo* investigations are merited in the future to explore the validity of this  
415 hypothesis. Our ongoing studies involving the use of simulated lung fluids to explore the potential of  
416 dehydrated liposome and proliposome formulations when the hydration environment is made from  
417 aqueous systems other than simple solutions are supportive to the aforementioned hypothesis (results  
418 unpublished).

419

### 420 **3.5 Size analysis of hydrated proliposomes**

421 The volume median diameter (VMD), also referred to as median size, of liposomes after  
422 reconstitution of the powders in deionized water was in the range of 3.38 - 6.01  $\mu\text{m}$  and 3.23 - 5.96  
423  $\mu\text{m}$  for mannitol-based vesicles and LMH-based liposomes, respectively (Table 3). Liposome size is  
424 an influential factor on drug entrapment, retention time of the vesicle components in the lung, and  
425 drug release profile (70). F1 and F6 had the largest VMD measurements and highest drug entrapment  
426 efficiencies, whilst F4 and F10 had the smallest VMD values and lowest drug entrapment  
427 measurements (Table 3). The high lipid content in F1 and F6 could be responsible for generating the  
428 largest vesicles that demonstrated the highest drug entrapment efficiencies. Drug release and  
429 absorption of liposome-encapsulated drug are influenced by liposome size and lipid phase  
430 composition. For instance, the localized time of terbutaline in the pulmonary system was prolonged  
431 by enriching the liposome formulations with CH or by using phospholipids with saturated alkyl chains  
432 (71). Large liposomes and multilamellarity can promote drug entrapment and prolong drug release in

433 the lung (72). The Span measurements were approximately 2 or less except for F4, F8 and F9 (Table  
434 3), indicating different polydispersity for different formulations. The high Span values (i.e.  
435 polydispersity) could be due to aggregation of liposomes.

436

### 437 **3.6 Zeta potential measurements**

438 Particles with a net surface charge (negative or positive) may repel each other, causing lower tendency  
439 for aggregation during storage, which improves physical stability of formulation stability (73).  
440 Furthermore, surface charge on particles, especially negative charge, may influence the interaction of  
441 particles with biological membranes (74-76). Liposomes in all formulations had very slightly negative  
442 zeta potential values (Table 3), indicating that lipid to carrier ratio, and carrier type did not affect the  
443 surface charge of vesicles. These findings support the potential of our liposome formulations, as  
444 particles with negative charge may demonstrate enhanced cellular uptake (74-76).

445

### 446 **3.7 Studies of vesicle morphology using transmission electron microscopy (TEM)**

447 Liposomes generated upon hydration of mannitol-based proliposomes were a mixture of large  
448 unilamellar (LUVs) and oligolamellar vesicles (OLVs), while structures generated from LMH-based  
449 powders were rich of vesicle clusters and “worm-like” shapes (Figure 7). These findings are in  
450 agreement with one of our previous investigations, for liposomes generated from LMH-based  
451 proliposomes manufactured using a modified rotary evaporator (77). Based on this TEM study,  
452 liposome morphology was unaffected by other formulation factors such as lipid to carrier ratio or  
453 lipid composition. The slow dissolution of carrier, may slow the hydration of lipid, resulting in  
454 retarded deaggregation of vesicles and formation of elongated bilayer structures (77). By contrast,  
455 mannitol-based proliposomes may have better dispersion properties in water, which might be due to  
456 the small size, smooth surfaces and spherical shape of mannitol-based particles, as shown earlier by  
457 SEM (Figure 1), causing formation of spherical LUVs and OLVs (Figure 7). Thus, the different in

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458 hydration patterns of phospholipid because of using proliposomes with different morphologies  
459 resulted in generation of vesicles with different morphologies (Figure 7). Interestingly, previous  
460 studies employing traditional proliposomes manufactured using modified rotary evaporators revealed  
461 instant generation of liposomes upon hydration of proliposomes under static conditions (i.e. without  
462 shaking) via the ‘budding off’ mechanism (22, 23, 77). Further studies should investigate the role of  
463 carrier type on the behaviour of liposomes generated from proliposomes.

464

### 465 **3.8 Powder aerosolization performance *in vitro***

466 Proliposome with spherical shapes would be expected to have better flowability, and when combined  
467 with having small particle size (i.e. in the ‘respirable’ range), they become likely to deposit in the  
468 lower airways. However, SEM used to evaluate particle morphology does not give information about  
469 aerodynamic size. The deposition site of inhaled particle in the pulmonary system is influenced by  
470 particle shape and aerodynamic size (78). For this reason, inertial impaction studies using the two-  
471 stage impinger (TSI) were conducted. Using the MIAT inhaler device, deposition of proliposome  
472 particles in the stages of the TSI was studied, in order to determine the recovered dose (RD), emitted  
473 dose (ED) and ‘FPF’ (Figure 8).

474 Figure 8 shows that the performance of proliposome aerosols was dependent on carrier type and lipid  
475 to carrier ratio (Figure 8). The RD for all formulations approached 100% (95.62 – 99.79%), with  
476 higher values for LMH-based proliposomes than mannitol-based formulations ( $p < 0.05$ ). However, the  
477 delivery of coarse LMH-based proliposome particles from the capsule was better (i.e. ED was higher)  
478 than ED of mannitol-based proliposomes.

479 The ED was high for all formulations (77.46 – 94.59%). However, LMH-based proliposomes had  
480 higher deposition in the upper stage (S1) of the impinger (i.e. lower ‘FPF’). These findings are in  
481 agreement with the earlier SEM studies, since all LMH-based proliposome formulations (F6-F10)  
482 (shown to have large sizes and irregular shapes; Figure 2) had extremely poor ‘FPF’ (0 - 3.99%)

483 (Figure 8). By contrast, mannitol-based microparticles were smaller and more spherical (Figure 1).  
484 Hence, they offered much greater 'FPF' (2.79 - 52.14%) compared to LMH-based powder. Particles  
485 having size of 1-5 $\mu$ m are likely to reach the peripheral airways following inhalation (51). F1 had the  
486 lowest FPF amongst mannitol formulations, which can be ascribed to particle agglomeration due to  
487 the high lipid content of this formulation. For F2 and F5, the 'FPF' values were 33.57 and 33.63%,  
488 respectively. These values were lower than those determined for F3 and F4, which can be attributed  
489 to the agglomeration occurring in F2 and F5 formulations because of the presence of small particles.  
490 By contrast, the lower agglomeration tendency of F3 and F4 might be responsible for the enhanced  
491 deposition of particles into the lower stage of the TSI (Figure 8). The subsequent section elaborates  
492 on studying the characteristics of proliposomes and the generated liposomes, using two distinguished  
493 formulations with superior aerosol performance.

494

### 495 **3.9. Additional powder characterization and drug release studies**

496 Further powder characterization studies and drug release investigations were conducted on best  
497 performing proliposome formulations, mainly relying on the aerosol performance findings of the  
498 powders (Figure 8). Proliposome powders exhibited their best performance in terms of 'FPF' when  
499 the lipid to carrier ratio was 1:6; thus F3 (mannitol-based proliposomes) and F8 (LMH-based  
500 proliposomes) were further investigated in terms of powder flowability and moisture content, and the  
501 release profile of SS from the subsequently generated liposomes. Upon reflection on the earlier  
502 findings in this study, it was further observed that F3 and F8 formulations exhibited desirable  
503 characteristics in terms of drug recovery, PY and drug content uniformity (Table 2). Moreover, as  
504 observed earlier with SEM studies, F3 proliposomes were spherical and apparently smooth with low  
505 agglomeration propensity (Figure 1c), justifying the superior aerosol performance of this formulation  
506 (Figure 8) and supporting the rationale behind conducting flowability and drug release studies on this  
507 particular proliposome composition.

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508 Accordingly, flowability studies were conducted on both F3 (as representative for mannitol-based  
509 proliposomes) and F8 (as representative for LMH-based formulations). Spray-dried powders of  
510 mannitol and LMH were used for comparison with the proliposome formulations to investigate the  
511 effect of lipid on powder flowability (Table 4). Flowability was assessed using Carr's compressibility  
512 index and Hausner ratio according to the reference published in 1965 (79) and summarized in Table  
513 4. Lipid-free carriers (i.e. spray-dried powders of mannitol or LMH) exhibited 'Fair' flowability  
514 according to both Hausner ratio and Carr's index (Table 4; Table 5). When these carriers were used  
515 to manufacture spray-dried proliposomes (i.e. F3 as a representative for mannitol-based formulations,  
516 and F8 as a representative for LMH-based formulations), the flow characteristics were markedly  
517 compromised, so that F3 exhibited 'Poor' flowability, whilst F8 was regarded to have 'Very very  
518 poor' flowability according to both Hausner ratio and Carr's index (Table 4; Table 5). Although the  
519 flowability findings do not look encouraging for both proliposome formulations (F3 or F8), the  
520 emitted dose (ED) of SS from the capsules to the impinger was considerably high, exceeding 80%  
521 (Figure 8), indicating that the 'inspiratory' flow rate through the impinger was sufficiently powerful  
522 to pull the proliposome powders from the inhaler device. However, the relatively better flow  
523 characteristics of F3 (Table 5) in addition to the spherical morphology, the relatively small physical  
524 size, and apparent smooth surfaces of this formulation (Figure 1c) were the prime reasons for the  
525 superior 'FPF' value observed for F3 (Figure 8). By contrast, F8 demonstrated very poor 'FPF'  
526 (Figure 8), possibly due to the relatively large size, irregular shape and rough surfaces (Figure 2c)  
527 and the extremely poor flow properties of the particles (Table 5). The flow rate through the impinger  
528 was appropriate to aspirate a large dose of F8 particles, but most of the drug dose was deposited in  
529 the upper impinger, resulting in extremely poor 'FPF' values (Figure 8). Particle surface morphology  
530 can influence aerosol performance (49) and smooth particles may exhibit better aerosolization  
531 characteristics (50). It is worth to mention that the residual moisture contents for both formulations  
532 F3 and F8 were less than 0.8% as determined by [thermogravimetric analysis \(TGA\)](#) at 110°C (data

533 not shown). TGA is a well-established analytical tool for the determination of residual moisture  
534 content in powdered formulations and solid excipients (80, 81).

535 When the in vitro drug release profiles of F3 and F8 were compared, the difference was insignificant  
536 for most intervals studied ( $p > 0.05$ ); however, a trend for faster drug release was observed with the  
537 F3 formulation (Figure 9). After 24 h, the cumulative release for SS was 79% and 72% for F3 and F8  
538 formulations, respectively, demonstrating advantageous sustained release for both formulations in  
539 comparison to the free SS which demonstrated 93% cumulative release after as short as 8 h and full  
540 release after 24 h (Figure 9). The drug release findings of F3 and F8 indicate that carrier type  
541 (mannitol or LMH) had no significant influence on the drug release profile.

542  
543 In our opinion, if nebulizable liposome formulations like Arikace® are considered the first generation  
544 of inhalable liposome medicines, liposomal and proliposomal DPIs might constitute the second-  
545 generation formulations. The proliposome formulations introduced in this study offer the rationale of  
546 delivering small therapeutic doses of SS with relatively small doses of phospholipid and sugar; this  
547 may reduce the risk of ‘overwhelming’ the lung with large amounts of exogenous lipids. Future  
548 investigations should expand to explore the role of materials used to manufacture the capsules that  
549 accommodate the powder prior to loading into the inhaler device (82), and the role of inhaler device  
550 design (83, 84), aiming to maximize formulation output and FPF. Importantly, aerosol flow rate  
551 from DPI devices may affect the deposition profile for conventional powders (85); thus, studying  
552 the role of flow rate through an impinger and its influence on the emitted dose and FPF is part of our  
553 ongoing investigations using phospholipid-based powders. Finally yet importantly, in vivo studies  
554 using experimental animals are needed in the future to further explore the potential of DPI  
555 proliposomes for inhalation to treat pulmonary diseases (e.g. asthma).

556

557 **4. Conclusions**

558 In this study, novel spray-dried proliposome formulations for delivery as dry powders were  
559 investigated using LMH or mannitol as carriers and SPC and CH as lipid composition. The ratio of  
560 carrier to lipid has influenced the product yield, particle morphology, and powder crystallinity and  
561 deposition pattern in the TSI. The characteristics of proliposome microparticles have accordingly  
562 influenced the vesicles generated, in terms of size, surface charge and drug entrapment. The  
563 production yield of spray-dried LMH formulations was higher than the yield values shown for  
564 mannitol-based proliposomes. X-ray diffraction patterns demonstrated the crystallinity of  
565 proliposomes after spray drying from ethanolic suspensions, indicating interaction between the  
566 proliposome constituents. SEM, impinger investigations, and powder flowability studies showed  
567 mannitol to be a more appropriate carrier for manufacturing DPI proliposome formulations because  
568 its particles were spherical, smooth and small after spray drying, and offered higher 'FPF' using the  
569 TSI (exceeding 50%). By contrast, LMH-based proliposomes were irregular in shape, had rough  
570 surfaces, larger sizes and poorer flowability, compromising its suitability for as DPI formulations.  
571 However, the vesicles generated upon hydration of proliposomes using LMH carrier offered higher  
572 drug entrapment efficiencies. The higher drug entrapment in liposomes generated from LMH-based  
573 formulations can be ascribed to the larger size of vesicles generated using this carrier. The zeta  
574 potential values were slightly negative, regardless of formulation composition. Moreover, TEM  
575 showed that mannitol-based proliposomes generated spherical vesicles, while bilayer structures  
576 generated upon hydration of LMH-based proliposomes were "worm-like" clusters. Following on the  
577 advanced development stages achieved by nebulizable liposome dispersions for inhalation, we expect  
578 phospholipid-based dry powders delivered via DPI devices, similar to the formulations developed in  
579 this study, to constitute the second generation of inhalable liposomes.

580



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585

586 **6. Conflict of interests**

587 The authors of this manuscript declare no conflict of interests.

588

589 **7. References**

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**Table 1: Composition of the proliposome formulations manufactured using spray drying**

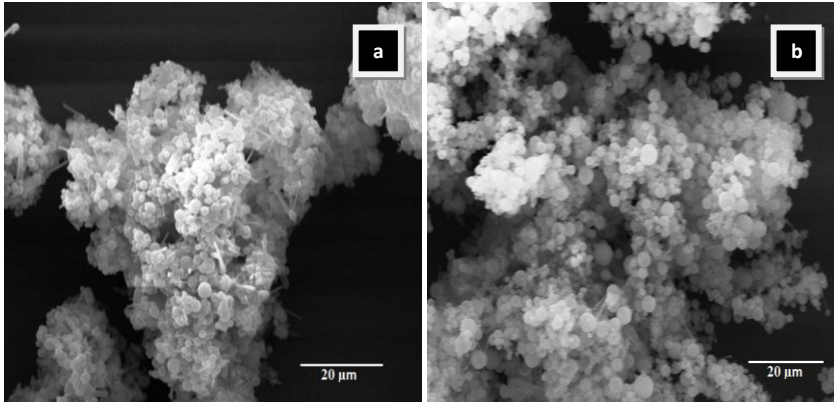
<u>Formulation</u>	<u>Lipid : Carrier</u> <u>(w/w)</u>	<u>Lipids (SPC:CH; 1:1)</u> <u>(mg)</u>	<u>Mannitol</u> <u>(mg)</u>	<u>LMH</u> <u>(mg)</u>	<u>SS</u> <u>(mg)</u>
<u>F1</u>	<u>1:2</u>	<u>100</u>	<u>200</u>	<u>-</u>	<u>10</u>
<u>F2</u>	<u>1:4</u>	<u>100</u>	<u>400</u>	<u>-</u>	<u>10</u>
<u>F3</u>	<u>1:6</u>	<u>100</u>	<u>600</u>	<u>-</u>	<u>10</u>
<u>F4</u>	<u>1:8</u>	<u>100</u>	<u>800</u>	<u>-</u>	<u>10</u>
<u>F5</u>	<u>1:10</u>	<u>100</u>	<u>1000</u>	<u>-</u>	<u>10</u>
<u>F6</u>	<u>1:2</u>	<u>100</u>	<u>-</u>	<u>200</u>	<u>10</u>
<u>F7</u>	<u>1:4</u>	<u>100</u>	<u>-</u>	<u>400</u>	<u>10</u>
<u>F8</u>	<u>1:6</u>	<u>100</u>	<u>-</u>	<u>600</u>	<u>10</u>
<u>F9</u>	<u>1:8</u>	<u>100</u>	<u>-</u>	<u>800</u>	<u>10</u>
<u>F10</u>	<u>1:10</u>	<u>100</u>	<u>-</u>	<u>1000</u>	<u>10</u>

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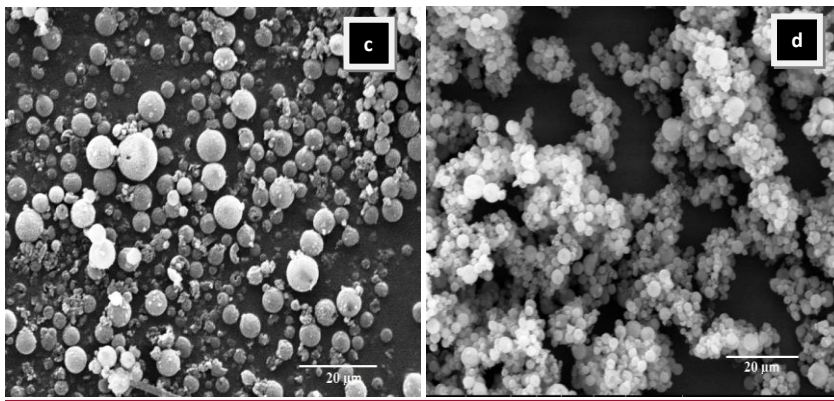
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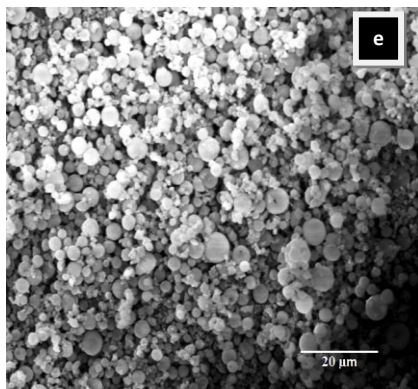
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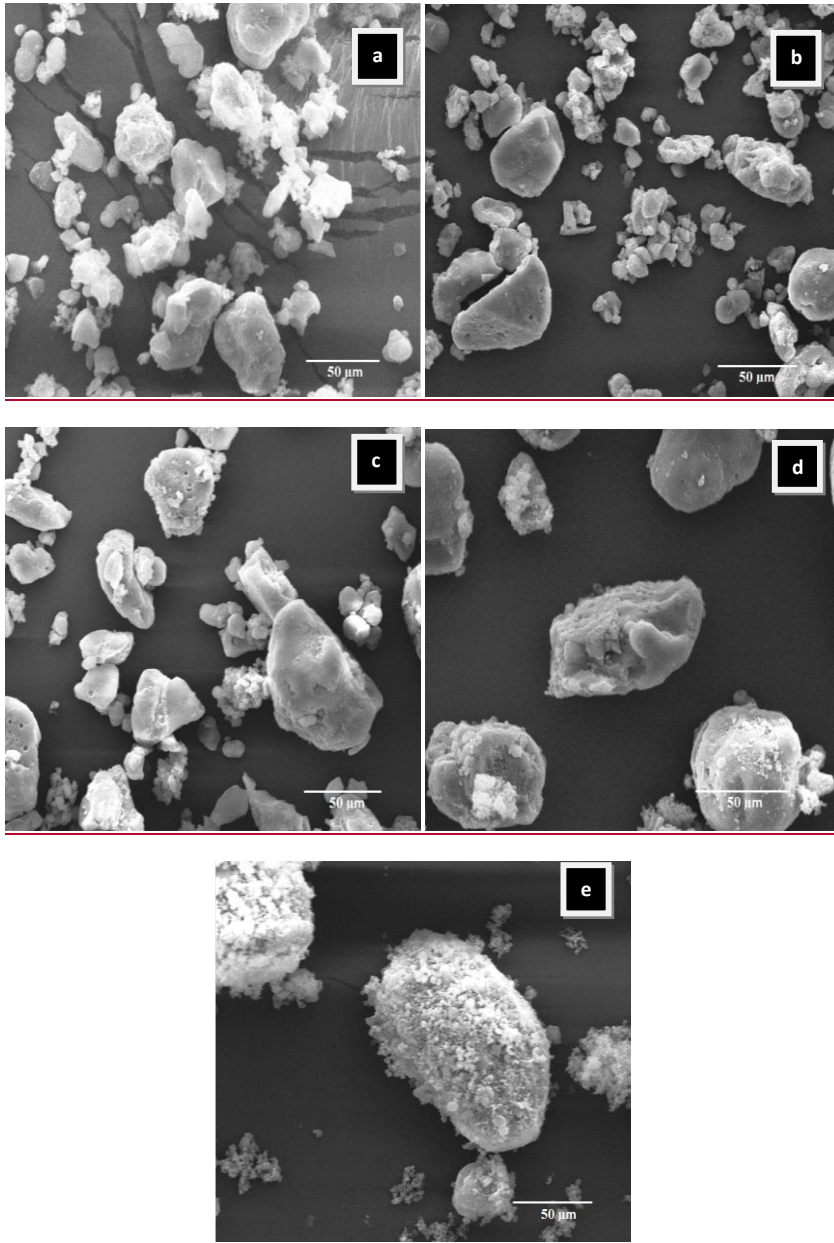


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805 **Figure 1: SEM images of mannitol-based proliposomes: (a) F1, (b) F2, (c) F3, (d) F4 and (e) F5**

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*Spray-dried proliposomes for aerosol delivery*



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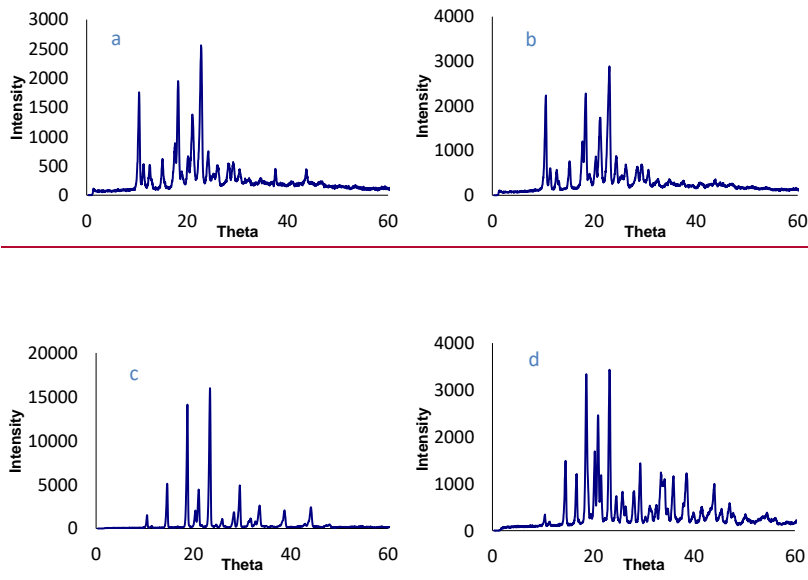
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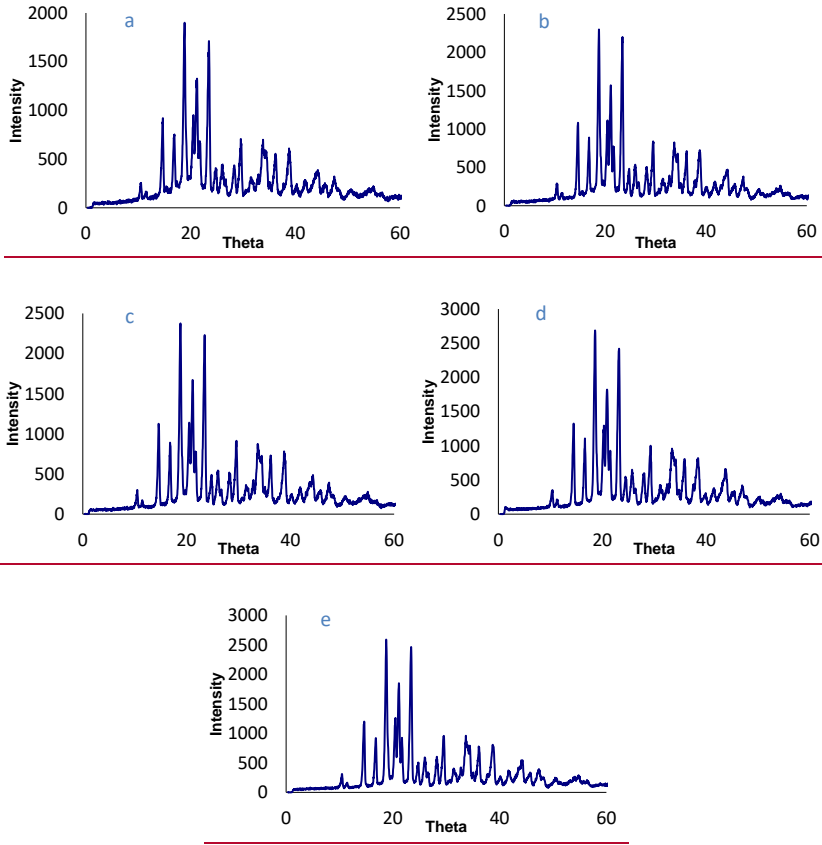
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**Figure 2: SEM images of LMH-based proliposome formulations: (a) F6, (b) F7, (c) F8, (d) F9 and (e) F10**



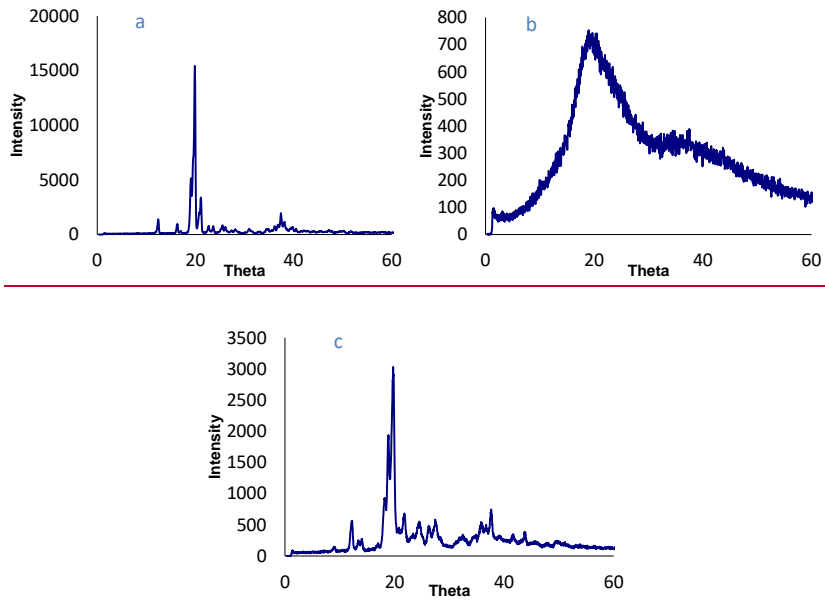
**Figure 3: X-ray powder diffraction of SS (a), mannitol (c) prior to spray-drying, and SS (b), mannitol (d) after spray-drying in ethanolic suspension**

Spray-dried proliposomes for aerosol delivery



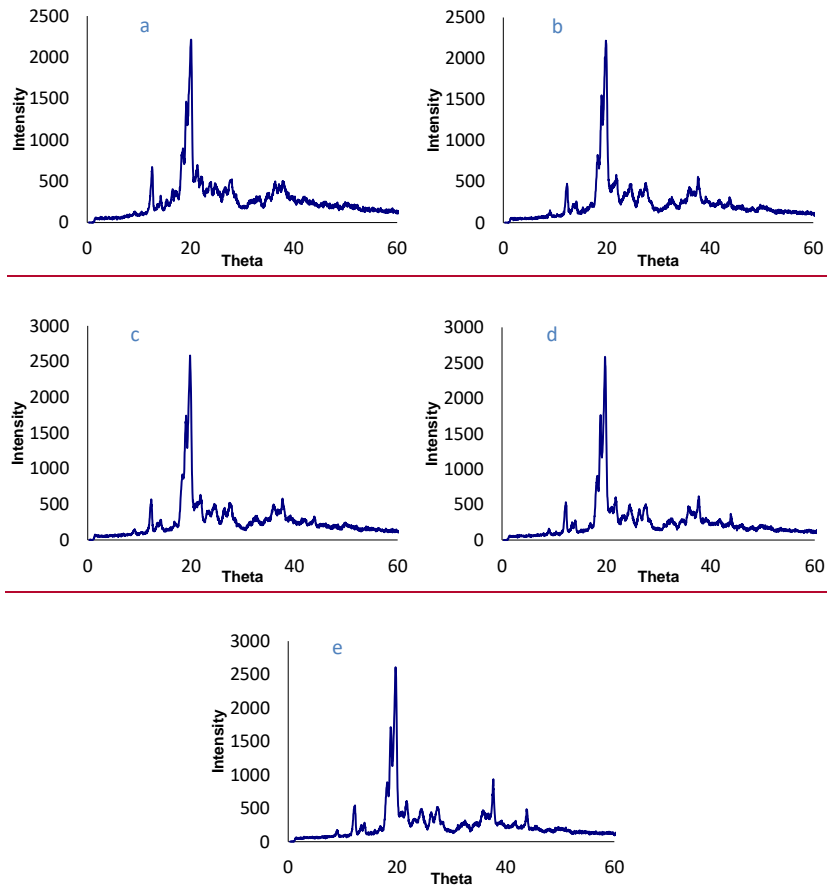
**Figure 4: X-ray powder diffraction profiles of mannitol-based proliposomes: (a) F1, (b) F2, (c) F3, (d) F4 and (e) F5**

Spray-dried proliposomes for aerosol delivery

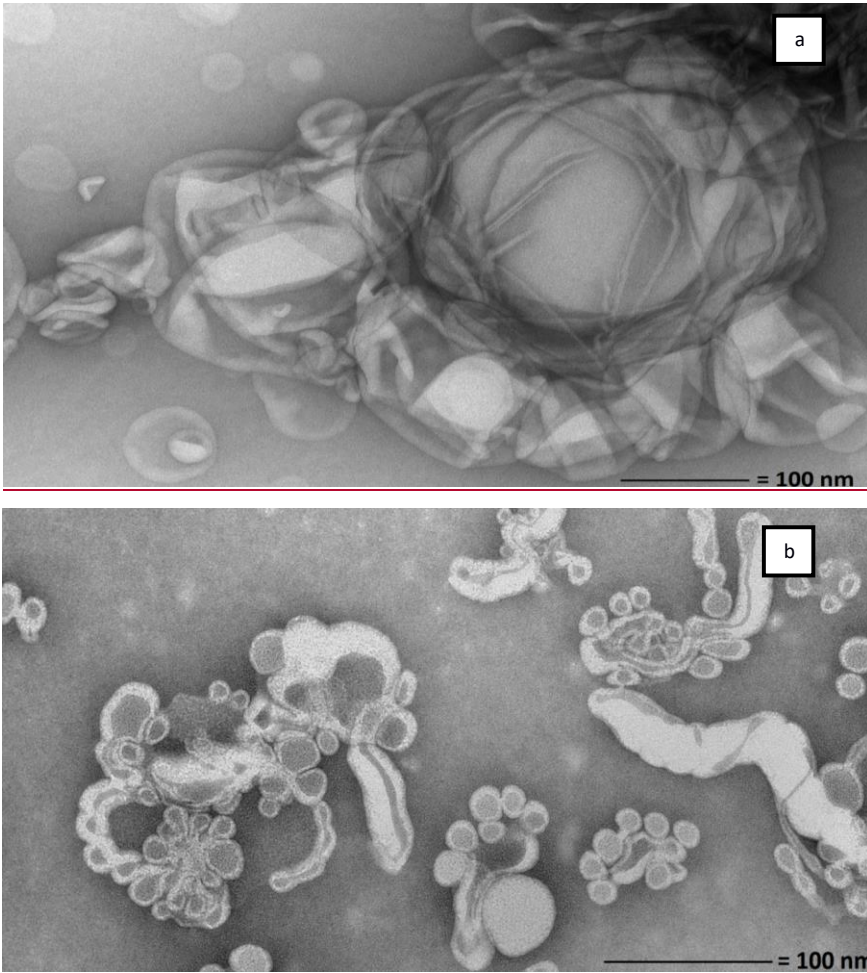


**Figure 5: X-ray powder diffraction profiles of LMH: (a) prior to spray-drying, (b) after spray-drying from its aqueous solution and (c) after spray-drying from its ethanolic suspension**

Spray-dried proliposomes for aerosol delivery



**Figure 6: X-ray powder diffraction profiles of LMH-based proliposomes: (a) F6, (b) F7, (c) F8, (d) F9, and (e) F10**



**Figure 7: TEM of (a) OLV liposomes generated upon manual hydration of mannitol-based proliposomes and (b) elongated worm-like bilayer liposomes and liposome clusters generated from LMH-based proliposomes using 1:6 w/w lipid to carrier**

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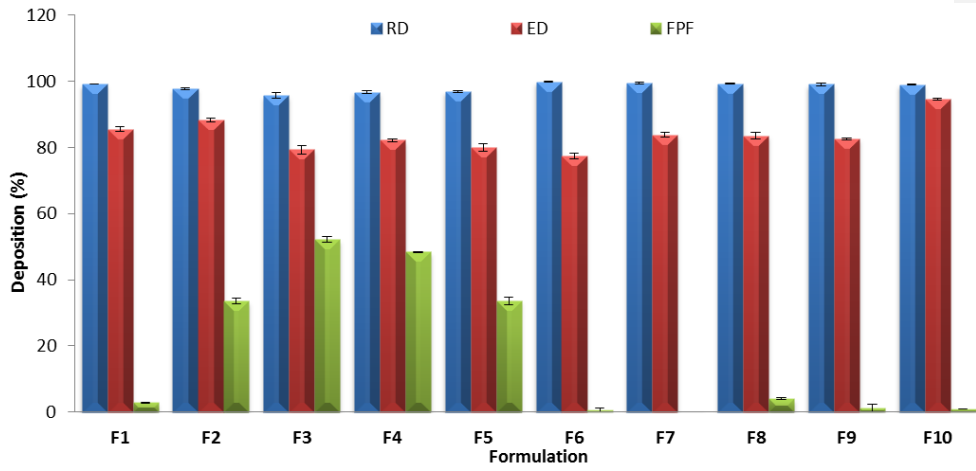
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Spray-dried proliposomes for aerosol delivery



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875 **Figure 8: Recovered dose (RD; %), emitted dose (ED; %) and 'fine particle fraction' ('FPF'; %) of**  
876 **mannitol-based and LMH-based proliposomes (n = 3 ± SD)**

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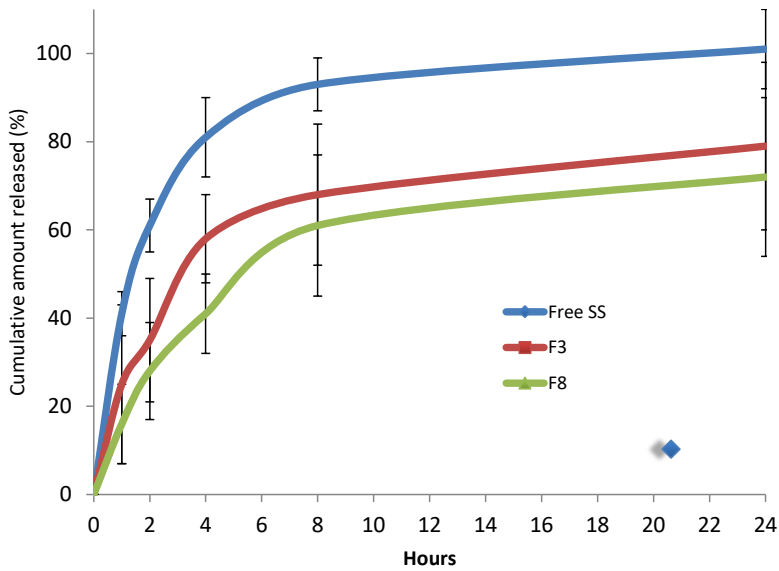
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Spray-dried proliposomes for aerosol delivery



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**Figure 9: Drug release profile from liposomes generated from mannitol-based proliposomes (F3) and LMH-proliposomes (F8) in comparison to free SS (n = 3 ± SD)**