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Nebulisation of rehydrated freeze-dried beclomethasone dipropionate liposomes

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Abstract

Beclomethasone dipropionate (BDP) liposomes were prepared from various lipids, dilauroyl phosphatidylcholine (DLPC), dimyristoyl phosphatidylcholine (DMPC), dipalmitoyl phosphatidylcholine (DPPC), and hydrogenated soybean phosphatidylcholine (Epikuron 200 SH). A lipid with a low transition temperature (T_m) (DLPC) incorporated a higher amount of BDP than lipid with a high T_m. The nebulisation of rehydrated freeze-dried BDP liposomes was carried out using a Pari LC Plus nebuliser and the generated aerosol characterised by an Andersen Cascade Impactor operated at 28.3 l/min. The rehydrated BDP–DLPC liposomes showed a higher output (78.3%) and a higher fine particle fraction (FPF) (75.0%) and smaller mass median aerodynamic diameter (MMAD) (3.31 μm) than the other rehydrated liposome preparations. Liposomes containing lipid with a high T_m (DPPC and Epikuron) underwent aggregation during nebulisation. This was shown by the large increase in size of the DPPC liposomes from 15.78 to 47.51 μm and the Epikuron liposomes from 5.84 to 46.70 μm. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Beclomethasone dipropionate; Nebulisation; Rehydrated freeze-dried liposomes

1. Introduction

Liposomes are potential drug carriers for pulmonary drug delivery because they can be prepared from phospholipids, which are endogenous to the respiratory tract as a component of pulmonary surfactant (Mihalko et al., 1988). Liposomes composed of naturally occurring phospholipids at an appropriate dose should not pose a toxicological risk to this organ. The unique structural properties of liposomes allow drugs with widely varying lipophilicities to be encapsulated in liposomes either in the aqueous compartment, in the phospholipid bilayer, or at the bilayer interface. Liposomes are an example of a carrier system that can be used to deliver drugs to a designated body cavity where the liposomes may release their content at a sustained or controlled rate while reducing drug toxicity and side-effects

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(Szoka and Papahadjopoulos, 1980). Among the various categories of drug that should benefit from liposomal entrapment are immunosuppressive anti-inflammmatory agents, particularly corticosteroids, because even when used locally, these agents may produce serious systemic side effects.

Beclomethasone dipropionate (BDP), an insoluble glucocorticoid, is often delivered in the aerosol form for treatment of asthma and other inflammatory lung disease using pressurised metered dose inhalers (pMDIs) or dry powder inhalers (DPIs). Nebulisation of this steroid was reported to be less effective than a DPI or pMDI in children with moderately severe asthma (Webb et al., 1986). Occasionally, direct administration of glucocorticoid to the lung may cause localised side-effects of candidiasis and dyspnea (Brogden et al., 1975, 1984; Toogood, 1993). The utilisation of liposomes for aerosol delivery of glucocorticoid via a nebuliser has advantages over aerosol delivery of microcrystalline glucocorticoid suspension. Liposomes offer a solubilisation matrix for the insoluble drug and sustained pulmonary release to maintain therapeutic drug concentrations. Nebulisation has been reported to be a suitable method for delivery of liposomes to the lung (Farr et al., 1985; Barker et al., 1994). However, the long-term stability of a liposome suspension is one of the main concerns in the development of liposomes. During storage, the liposome suspension may undergo physical and chemical changes that lead to vesicle alteration, chemical degradation and leakage of entrapped drug.

In an attempt to improve the stability of liposomes, freeze-dried BDP liposomes of various lipids (DLPC, DMPC, DPPC, Epikuron) were prepared. The preparations were rehydrated and nebulised using a Pari LC Plus nebuliser, and the size distribution of aerosolised particles was measured using an Andersen Cascade Impactor.

2. Materials

DLPC, DMPC and DPPC were purchased from Avanti Polar Lipids, Albaster (USA). Hydrogenated soybean phosphatidylcholine (Epikuron 200 SH) was obtained from Lukas Meyer Inc. (Germany), while beclomethasone dipropionate was a gift from Glaxo Wellcome (UK). All other chemicals were of reagent grade and were obtained commercially.

3. Methods

3.1. Preparation of liposomes

The required amount of lipids (DLPC, DMPC, DPPC and Epikuron 200 SH) and BDP were dissolved in *t*-butanol. The solutions were frozen in liquid nitrogen and lyophilised at ambient temperature using a freeze dryer (Edwards Super Modulyo, Gloucestershire, UK). Distilled water prewarmed to a temperature exceeding the lipid transition temperature (T_m) (DLPC and DMPC at 37°C, DPPC at 56°C and Epikuron at 65°C) was added to the dried mixtures to deliver a final concentration of 10 mg/ml. The mixtures were then shaken mechanically for 1 h in a water bath at a temperature above the lipid T_m to produce multilamellar vesicles (MLVs). The liposome suspensions obtained were maintained for another hour at the temperature above the lipid T_m to anneal the liposomes structure. Small multilamellar vesicles (SMLVs) were produced by extrusion through two stacked polycarbonate 25 mm filters of 0.2 μm pore size (Costar). The extrusion process was performed above the lipid T_m using an extrusion unit (Lipex Bioemembranes Inc.). The liposomes in the suspension were separated from the free BDP, and the amount of BDP encapsulated in the liposomes was determined by HPLC.

The SMLV liposomes prepared as above were frozen in liquid nitrogen and freeze-dried at ambient temperature overnight using the freeze dryer.

3.2. Separation of liposomes from free BDP

Liposome suspension (0.5 ml) was mixed with 1 ml of 30% w/v Ficoll. Then, 3 ml of 10% w/v Ficoll were gently layered on the top of the liposome suspension. The upper Ficoll layer was covered with 1 ml of distilled water and centrifuged at 35 000 rpm for 30 min at 20°C (Sorvall T-865, Du Pont, USA). The liposome pellets were col-

lected using a Pasteur pipette at the interface between the water and the 10% w/v Ficoll layer. The non-encapsulated BDP remained in the lowest Ficoll layer. The amount of BDP encapsulated in liposomes was determined by HPLC.

3.3. Interaction of BDP with lipids

The interaction of BDP with lipids (DMPC, DPPC, Epikuron) was studied using a differential scanning calorimeter (DSC) (Perkin-Elmer DSC-7). MLV liposome suspensions were weighed and hermetically sealed in aluminum pans (Perkin-Elmer). Following calibration with pure indium (Perkin-Elmer), the transition temperature (T_m) of the MLV liposome suspensions was measured. The liposomal suspensions were scanned over the range of 10–50°C at a rate of 5°C/min.

3.4. Determination of liposome size

The size of the rehydrated freeze-dried liposomes before and after nebulisation was measured using a Malvern 2600 HSD particle sizer (Malvern Instruments, UK). Measurement was carried out using a 63.0 mm focal length lens, which is capable of measuring vesicles between 1.2 and 118.4 μm. The rehydrated liposomes were diluted with filtered water, and the sample was not sonicated prior to particle-size measurement.

3.5. Nebulisation of rehydrated liposomes

The particle-size distribution was determined using an Andersen Cascade Impactor (ACI) (Mark ll, Andersen Samplers, Atlanta, GA) calibrated at a flow rate of 28.3 l/min. The impaction surfaces of the ACI were coated with 1% polyethylene glycol 300 in acetone to prohibit reentrainment of particles. This coating was achieved by spraying the solution at a distance of 20 cm for 2×10 s. A period of 10 min was allowed in between the sprays for the solution to evaporate. Eight milliliters of rehydrated freeze-dried liposomes were placed in a Pari LC Plus nebuliser, and compressed air from the Pari Master pump was supplied to the nebuliser. A vacuum pump was operated at a flow rate of 28.3 l/min for 15 min. Nebulisation was at room temperature (20°C) and a humidity of 60%. The aerosolised liposomes were collected, and the amount of BDP deposited was analysed by HPLC.

3.6. Determination of BDP concentration

The amount of BDP was determined by HPLC analysis using a Highchrome ODS (4.6 × 250 mm) column at room temperature. The mobile phase used was 60:40 acetonitrile:water at a flow rate of 1.5 ml/min. Peak detection was performed at 239 nm using an LCD Milton Roy detector with quantification on an SP 4270 integrator.

3.7. Data analysis

The total amount of drug recovered in the ACI and the throat represents the emitted dose. Particles < 5.8 μm represent the fine particle fraction (FPF) calculated as a percentage relative to the total BDP emitted dose. Student's t-test or single factor analysis of variance (ANOVA) followed by a Tukey–Kramer multiple comparison test were employed on the data wherever appropriate. A P-value of < 0.05 was regarded as an indication of statistical significance.

4. Results and discussion

Table 1 shows that relatively small amounts of BDP were encapsulated in liposomes. This result agrees with the data reported by many previous authors, who found a low entrapment of

Table 1
Comparison of encapsulation of BDP in liposomes separated by the density gradient method[a]

Lipid	Encapsulation (mg/mg%)	
	MLV	SMLV
DLPC	3.69 (0.10)	2.03 (0.08)
DMPC	3.31 (0.06)	1.37 (0.02)
DPPC	1.59 (0.03)	0.66 (0.01)
Epikuron	1.43 (0.06)	0.22 0.07)

[a] Mean ± S.D., $n = 3$.

lipophilic compounds such as testosterone in DPPC (Montenegro et al., 1996), steroids in DPPC (Shaw et al., 1976), dexamethasone palmitate in egg phosphatidylcholine (EPC) (Benameur et al., 1993), cortisol palmitate in DPPC (Fildes and Oliver, 1978) and cortisone esters in DPPC (Arrowsmith et al., 1983). These authors attributed the low entrapment of steroid drugs in the liposomes to the geometric structure of steroid molecules, which prevent the steroid drugs from strongly interacting with the lipid bilayers of the liposomes. The present investigation shows that the order from the greatest to the least BDP encapsulation in the four lipids studied was DLPC, DMPC, DPPC and Epikuron. These results suggested that the ability of the bilayer structure of the liposomes to accommodate BDP is inversely proportional to the T_m of the phospholipid. The T_m values of DLPC, DMPC, DPPC and Epikuron are -1.8 (Szoka and Papahadjopoulos, 1980), 23, 41 and 52°C (Darwis, 2000), respectively. These findings were probably caused by high-T_m lipids producing more solid and rigid liposomes, resulting in their inability to encapsulate BDP. This also suggested that the bilayers of the phospholipid with longer acyl chains have less ability to accommodate the BDP. A similar finding has been reported by Wu et al. (1978), in the study of gramicidin S encapsulated in DMPC and DPPC. The authors reported that 95% of gramicidin S was encapsulated in DMPC, compared to only 45% encapsulated in DPPC. Szoka and Papahadjopoulos (1980) suggested that in addition to hydrophobic drugs associating with the bilayer of liposomes, it was also possible for them to form two types of complexes: the lipid–drug complex and the drug–drug complex, which precipitates. The authors also pointed out the difficulty in separating these complexes. The encapsulation of BDP in SMLV liposomes was significantly lower than MLV liposomes (Table 1). The loss of BDP during extrusion through the membranes could be a result of not all of the BDP residing in the bilayers. Some may form complexes with the lipid head groups on the surface of the liposomes. Hence, during extrusion through the membrane pores, some of the complexes may have broken up and the drug liberated from the surface of the liposomes. A similar finding was reported by Stamp and Juliano (1979) for the less lipophilic drugs vinblastin and actinomycin D, encapsulated in egg PC. The authors observed that 50–60% of the drugs remained in the liposomes after 10 min of sonication. They explained that this portion of the drugs might be tightly bound to the liposomal membrane, thus being able to resist extensive sonication. In contrast, Taniguchi et al. (1987) reported that highly lipophilic dexamethasone esters were more than 90% encapsulated in egg PC liposomes and remained intact after prolonged (20 min) sonication. The authors used ultrafiltration to separate liposomes from the free steroid esters, although this method, as pointed out by Szoka and Papahadjopoulos (1980), was not suitable for purifying liposomes. Therefore, the high entrapment of steroid esters obtained could be due to liposomes still being contaminated with non-encapsulated drug.

Interaction of BDP with lipid bilayers was observed using DSC and showed that the T_m values of DMPC, DPPC and Epikuron were shifted to a lower value when BDP was encapsulated in the liposomes (Fig. 1). Lipophilic drugs have been previously shown to change the pre-transition temperature and to shift the T_m to a lower value (Fildes and Oliver, 1978; Szoka and Papahadjopoulos, 1980; Arrowsmith et al., 1983).

Fig. 2 shows the distribution of aerosolised BDP liposomes in the ACI and nebuliser. The greatest concentration of aerosolised particles was found at stage 3 of the ACI. There was a significant difference in deposition of BDP liposome aerosols collected at stage 3. Among the preparations studied, BDP–DLPC was the highest (19.9%) followed by BDP–Epikuron (16.8%), BDP–DMPC (10.3%) and finally BDP–DPPC (5.3%). The aerosol output from the nebuliser of BDP–DLPC liposomes (78.3%, T_m — 1.8°C) was significantly higher than BDP–DMPC, BDP–DPPC and BDP–Epikuron liposomes with T_m values higher than ambient temperature. Among the liposome preparations, the aerosol output of BDP–DPPC liposomes was the lowest. This result is in agreement with the finding reported by Waldrep et al. (1993), who found a higher output of cyclosporine A liposomes of low-T_m lipids

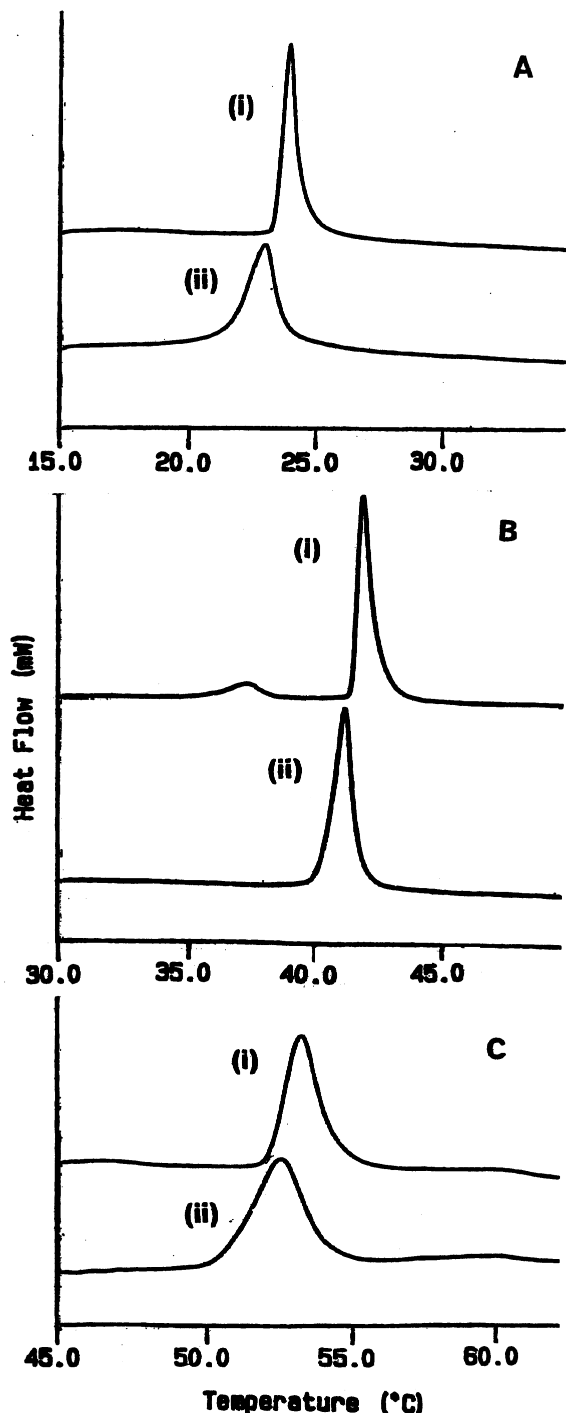

Fig. 1. DSC spectra of (A) DMPC, (B) DPPC and (C) Epikuron liposomes (i) without BDP and (ii) with encapsulated BDP.

[(DLPC, dioleoyl phosphatidylcholine (DOPC) and palmitoleoyl phosphatidylcholine (POPC)] from a Puritan Bennett 1600 twin jet nebuliser compared to liposomes of higher-T_m lipids (DMPC and DPPC). In another study, Waldrep et al. (1994), reported nebulisation of glucocorticoid liposomes comprising different lipids (DPPC, DMPC, DLPC, POPC, DOPC and EPC). The authors again reported a low output of DMPC and DPPC liposomes from a Puritan Bennett 1600 twin jet nebuliser. They suggested that the inefficient nebulisation of the DMPC and DPPC liposomes was due to the rigidity of the lipid bilayers. A lipid will behave more like a liquid than a solid above its T_m. During nebulisation, the temperature in the nebuliser chamber could drop to 16°C (Waldrep et al., 1993, 1994; Khanna et al., 1997), which is well below the T_m of the lipids, DMPC, DPPC and Epikuron (23, 41 and 52°C, respectively). The efficient formation of liposome aerosols is related to the T_m of the lipid used (Waldrep et al., 1993, 1994), which determines the solidity and fluidity of the liposomes (Szoka and Papahadjopoulos, 1980). Hence, lipids with a high T_m produce a low output of aerosols. This may be due to the solid and rigid liposomes of high T_m being inefficiently extruded through the nebuliser jet orifice.

The FPFs (size < 5.8 μm) of the emitted dose were significantly higher for BDP–DLPC liposomes (75.0%) than other liposome preparations, BDP–DMPC (57.6%), BDP–DPPC (64.0%) and BDP–Epikuron (63.96%) (Fig. 3). Although the FPF for BDP–DMPC liposomes was the lowest among the three liposome preparations (BDP–DMPC, BDP–DPPC and BDP–Epikuron), there were no significant differences in FPF of these preparations because a large variability in the BDP–DMPC liposome data was observed. The deposition of the aerosols on the throat and preseparator (size > 10 μm) and on stage 0 and stage 1 ($10 > \text{size} > 5.8$ μm) was significantly lower for BDP–DLPC than for the other three liposome preparations. However, there was no significant difference in the deposition of the aerosols on the throat and preseparator and on stage 0 and stage

1 for the BDP–DMPC, BDP–DPPC and BDP–Epikuron liposomes.

Table 2 shows the mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) of aerosolised liposomes. The MMAD of BDP–DLPC liposomes (3.31 μm) was significantly smaller than the BDP–DMPC (3.80 μm), BDP–DPPC (3.79 μm) and BDP–Epikuron liposomes (4.18 μm). Clearly, the MMAD of BDP–Epikuron was the largest among the liposome preparations studied. However, there was no significant difference in the MMAD of BDP–DMPC and BDP–DPPC liposomes. The distribution of the aerosolised particles of all liposome preparations was demonstrated to be polydispersed in nature as the value of GSD was greater than 1.2.

A cumulative percentage undersize versus effective cut-off diameter (ECD) plot is shown in Fig. 4. DLPC liposomes demonstrated a significantly higher percentage of under size (size < 4.7, < 5.8 and < 9 μm) than the other three liposome preparations. Among the three liposome preparations (DMPC, DPPC and Epikuron), DMPC liposomes showed the lowest cumulative percentage under size (size < 5.8 and < 9 μm). However, there was no significant difference found in cumulative percentage under size for ECD 4.7 μm for DMPC, DPPC and Epikuron liposomes. The cumulative percentage undersize of Epikuron for ECD 3.3–0.4 μm was significantly lower than DLPC, DMPC and DPPC liposome preparations. Fig. 5 depicts the mass fraction of BDP versus ECD of liposome preparations. It shows a similar, broad distribution for all liposome preparations with maximum deposition being achieved on the plate of ECD 3.3 μm. It was observed that the mass fraction of BDP–DMPC liposomes at this size was significantly lower than for the other liposomes studied.

The liposome suspension was extruded through a 0.2 μm polycarbonate pore membrane before freeze-drying. However, the particle size had increased greatly after lyophilisation and rehydration of the liposomes, probably reflecting the absence of a cryoprotectant in the freeze-drying protocol. The increase in particle size could be due to aggregation and/or fusion of liposomes during freeze-drying and rehydration. Vesicle aggregation and size changes may be less important

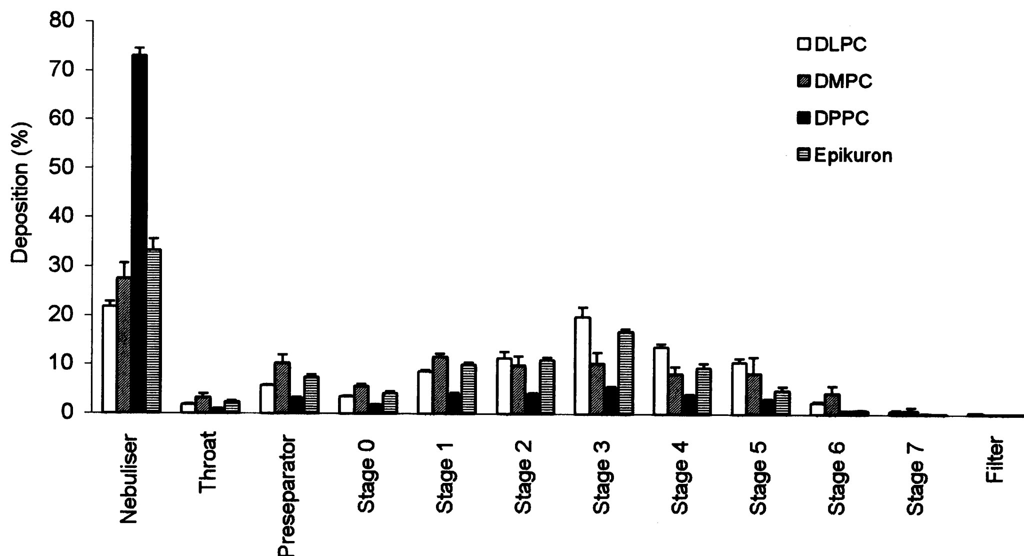

Fig. 2. Distribution of DLPC, DMPC, DPPC and Epikuron rehydrated liposomes in the nebuliser and ACI following nebulisation at a flow rate of 28.3 l/min for 15 min. Mean ± S.D., $n = 5$.

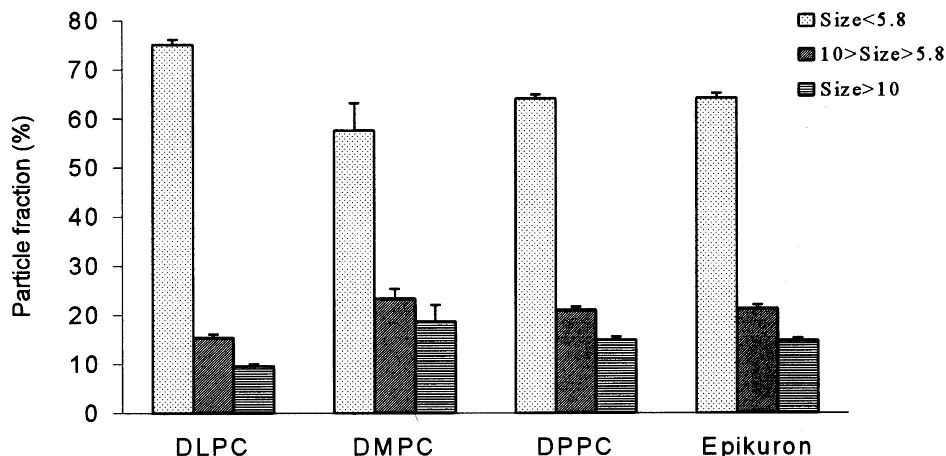

Fig. 3. Fractions of the emitted dose of particle size < 5.8 μm (FPF), 10 > size > 5.8 μm (stage 0–1) and size > 10 μm (throat and preseparator) of DLPC, DMPC, DPPC and Epikuron rehydrated liposomes following nebulisation at 28.3 l/min for 15 min. Mean ± S.D., $n = 5$.

for bilayer associated steroids than for entrapped solutes. The size of rehydrated liposome vesicles before and after nebulisation was measured by laser diffraction (Table 3). The size of the rehydrated BDP–DLPC liposomes retained in the reservoir was reduced after nebulisation from 10.30 to 3.87 μm and BDP–DMPC liposomes from 10.21 to 4.50 μm. Similar results have been reported by Farr et al., (1985) in the nebulisation of ^{99m}Tc labelled-DMPC and DPPC MLV liposomes and Taylor et al. (1990) in nebulisation of sodium cromoglycate-EPC/cholesterol MLV liposomes. They suggested that the reduction in liposome vesicle size could be due to breakdown of the vesicle aggregates during nebulisation. In contrast, this study showed that the size of BDP–DPPC and BDP–Epikuron liposomes retained in the reservoir was increased significantly after nebulisation. The size of BDP–DPPC and BDP–Epikuron liposomes was increased from 15.78 to 47.51 μm and from 5.84 to 46.70 μm, respectively. Comparable studies in the literature often assume that the size of the vesicles remaining in the nebuliser reservoir reflects the size of the vesicles delivered. However, these findings clearly indicate that this is not necessarily the case. The increase in vesicle size could be due to aggregation of liposomes in the reservoir during nebulisation, as the temperature in the nebuliser reservoir was below the T_m of the lipids and resulted in a low output of liposome aerosols. Waldrep et al. (1994) also reported aggregation of glucocorticoids-DPPC MLV liposomes during nebulisation. Although the BDP–DPPC and BDP–Epikuron liposomes appeared to aggregate in the nebuliser reservoir, they were effectively delivered to the lower stages of the ACI. The output of the BDP–Epikuron liposome aerosols was significantly higher than the BDP–DPPC liposomes, even though BDP–Epikuron had a higher T_m than BDP–DPPC liposomes. This could be due to the

Table 2
MMAD and GSD of aerosols deposited in the ACI following nebulisation of DLPC, DMPC DPPC and Epikuron rehydrated liposomes at a flow rate of 28.3 l/min for 15 min[a]

Lipid	MMAD (μm)	GSD
DLPC	3.31 (0.10)	2.08 (0.07)
DMPC	3.80 (0.20)	2.19 (0.09)
DPPC	3.79 (0.06)	2.09 (0.03)
Epikuron	4.18 (0.10)	1.93 (0.02)

[a] Mean ± S.D., $n = 5$.

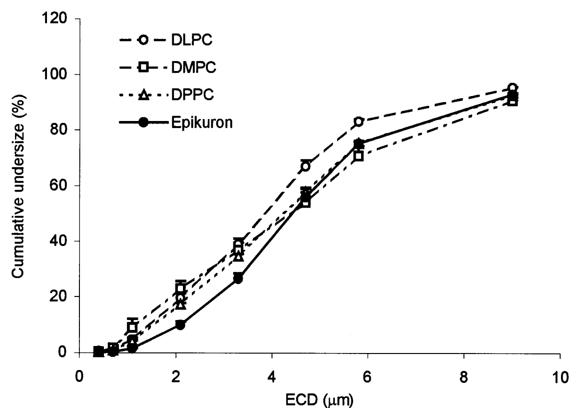

Fig. 4. Cumulative percentage undersize versus ECD of aerosolised DLPC, DMPC, DPPC and Epikuron rehydrated liposomes following nebulisation at a flow rate of 28.3 l/min for 15 min. Mean ± S.D., $n = 5$.

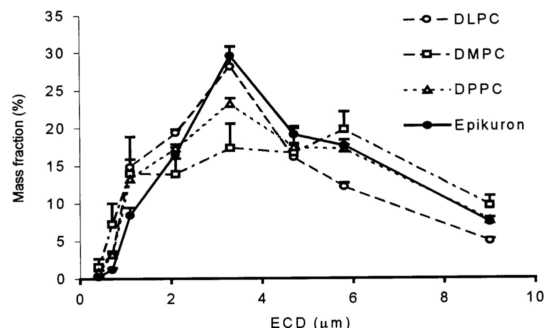

Fig. 5. Mass fraction versus ECD of aerosolised DLPC, DMPC, DPPC and Epikuron rehydrated liposomes following nebulisation at a flow rate of 28.3 l/min for 15 min. Mean ± S.D., $n = 5$.

size of BDP–Epikuron liposomes before nebulisation being significantly smaller than BDP–DPPC liposomes. Therefore, the BDP–Epikuron liposomes could be nebulised efficiently, even though the temperature in the nebuliser chamber was well below the T_m of the BDP–Epikuron liposomes.

5. Conclusions

The interaction of BDP with the bilayer of liposomes depended on the acyl chain composition of the lipid forming the liposomes. Lipids with shorter acyl chains and lower T_m values encapsulated a larger amount of BDP compared to lipids with a higher T_m. Among the four lipids studied, DLPC was found to incorporate the greatest amount of BDP. Nebulisation of BDP liposomes prepared from different lipids (DLPC, DMPC, DPPC and Epikuron) showed that the output of liposomes from the nebuliser depended on the lipid used. Liposomes formed from lipids with a low T_m such as DLPC nebulised efficiently and showed a high output of aerosol from the nebuliser reservoir. Conversely, liposomes formed from lipids with a high T_m (DPPC and Epikuron) showed a low output of aerosols, presumably due to aggregation during nebulisation. The size of these liposomes before nebulisation may influence the aerosol output, as observed for the BDP–Epikuron, which had a small particle size and a high aerosol output compared with BDP–DPPC. The results also indicated that BDP–DLPC liposomes produced the best aerosol output, MMAD and FPF, among the liposome preparations studied.

Table 3
Volume mean diameter of rehydrated liposomes of DLPC, DMPC, DPPC and Epikuron vesicles before and after nebulisation[a]

Lipid	Before nebulisation		After nebulisation	
	Median diameter (μm)	Span	Median diameter (μm)	Span
DLPC	10.30 (1.35)	1.78 (0.17)	3.87 (0.20)	0.85 (0.19)
DMPC	10.21 (1.40)	1.90 (0.52)	4.50 (0.17)	1.40 (0.26)
DPPC	15.78 (1.62)	2.19 (0.35)	47.51 (8.32)	1.76 (0.25)
Epikuron	5.84 (0.20)	1.25 (0.10)	46.70 (2.41)	1.83 (0.10)

[a] Mean ± S.D., $n = 5$. Span = (D-90 − D-10)/D-50.

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