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Physical stability and aerosol properties of liposomes delivered using an air-jet nebulizer and a novel micropump device with large mesh apertures

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Abstract

The aerosol properties of liposomes and their physical stability to aerosolization were evaluated using an air-jet nebulizer (Pari LC Plus) and a customized large aperture vibrating-mesh nebulizer (Aeroneb Pro-8 μm). Soya phosphatidylcholine: cholesterol (1:1 mole ratio) multilamellar liposomes (MLVs) entrapping salbutamol sulfate were nebulized directly, or after being reduced in size by extrusion through 1 or 0.4 μm polycarbonate membrane filters. MLVs were very unstable to jet nebulization and stability was not markedly enhanced when vesicles were extruded before nebulization, such that drug losses from delivered liposomes using the Pari nebulizer were up to 88% (i.e. only 12% retained in liposomes). The Aeroneb Pro-8 μm nebulizer was less disruptive to liposomes, completed nebulization in a much shorter time, and produced greater mass output rate than the Pari nebulizer. However, aerosol droplets were larger, total drug and mass outputs were lower and aerosolization performance was dependent on formulation. Vibrating-mesh nebulization was less disruptive to liposomes extruded through the 1 μm membranes compared with the non-extruded MLVs, so that the retained entrapment of the drug in the nebulized vesicles was 56% and 37%, respectively. However, extrusion of liposomes to 0.4 μm resulted in reduced stability of liposomes to vibrating-mesh nebulization (retained entrapment = 41%) which was attributed to the reduced liposome lamellarity and subsequent reduced resistance to nebulization-induced shearing. This study has shown that vibrating-mesh nebulization using the customized large aperture mesh nebulizer (Aeroneb Pro-8 μm) had a less disruptive effect on liposomes and produced a higher output rate compared with the Pari LC Plus air-jet nebulizer. On the other hand, the air-jet nebulizer produced higher total mass and drug outputs and smaller aerosol droplets.

Keywords: Aerosol; Liposome; Nebulization; Nebulizer; Salbutamol sulfate; Stability

1. Introduction

Air-jet nebulizers have been widely investigated in the delivery of antiasthma liposome formulations to the respiratory system of man (Taylor et al., 1989; Waldrep et al., 1997; Saari et al., 1998, 1999, 2002). Medical nebulizers are commonly classified into two types, which are air-jet (pneumatic) and ultrasonic nebulizers. Recently, a third type, namely vibrating-mesh nebulizers have been commercially available (Dhand, 2002).

Air-jet nebulizers employ high velocity gas passing through a venturi nozzle to convert liquids into a mist. Liquid is drawn from the nebulizer reservoir up a feed tube and emerges as fine filaments that collapse into aerosol droplets (O'Callaghan and Barry, 1997). Ultrasonic nebulizers employ a piezoelectric crystal vibrating at high frequency to produce a fountain at the liquid–air interface, which results in the generation of large droplets from the apex, whilst a fog of small droplets are produced from the lower regions. For both of these nebulizer types, baffles recycle large droplets whilst smaller ones are released for inhalation (O'Callaghan and Barry, 1997).

Vibrating-mesh nebulizers employ vibrating plates with multiple apertures through which liquid is extruded to generate aerosols (Dhand, 2002). In one approach (e.g. Omron MicroAir NE-U22 nebulizer) a vibrating piezoelectric crystal attached to a transducer horn transmits vibrations to a perforated plate placed on top of it. The plate comprises up to 6000 tapered apertures, approximately 3 μm in diameter through which liquid is passed to generate the aerosol. Alternatively, a micropump technology (e.g. Aeroneb Pro and Aeroneb Go nebulizers) employs an aerosol generator, comprising a domed aperture plate with up to 1000 holes and a vibrational element that contracts and expands on application of an electrical current, resulting in upward and

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downward movements of the mesh by a few micrometers in order to generate the aerosol (Dhand, 2002).

Air-jet nebulization has a detrimental effect on the physical stability of liposomes, resulting in loss of the entrapped hydrophilic material (Taylor et al., 1990b; Niven et al., 1991). Similarly, ultrasonic nebulization can damage liposomal structures (Leung et al., 1996). However, the ability of vibrating-mesh nebulizers to deliver liposomes has been recently shown (Elhissi and Taylor, 2005; Elhissi et al., 2006) and the destabilizing effect they exerted on liposomes generated from proliposomes tended to be less than that produced by a jet nebulizer (Elhissi et al., 2006).

In this study, the liposome-entrapped hydrophilic bronchodilator salbutamol sulfate was employed to study the effect of liposome size on the physical stability of liposomes to aerosolization using a standard air-jet nebulizer (Pari LC Plus) and a novel vibrating-mesh nebulizer with large mesh apertures (Aeroneb Pro-8 μm) customized by the manufacturer for this study. The influence of nebulizer and liposome size on the aerosolization performance (e.g. droplet size, mass output, etc.) was also studied.

2. Materials and methods

2.1. Materials

Chloroform, sodium chloride (NaCl) and glacial acetic acid were all AnalaR grade and purchased from BDH, UK. Cholesterol (>99%) and Triton X-100 were purchased from Sigma–Aldrich, UK. Sodium 1-hexane sulfonate hydrate (>99%) was supplied by Alfa Aesar, UK. Salbutamol sulfate (99%) was supplied by Avocado Research Chemicals Ltd., UK. Soya phosphatidylcholine (SPC; Lipoid S-100) was a gift from Lipoid, Germany. Phosphotungstic acid (analytical reagent grade) was purchased from TAAB Laboratories Equipment Ltd., UK. Water and methanol used for high performance liquid chromatography (HPLC) were HPLC grade and purchased from Fisher Scientific Ltd., UK. Nuclepore Polycarbonate Track-Etch Membrane filters with a pore size of 3, 1, 0.6 or 0.4 μm were purchased from Whatman, USA. Pari LC Plus air-jet nebulizers and Pari TurboBoy *N* compressor were kindly supplied by Pari, GmbH, Germany. The Aeroneb Pro-8 μm nebulizer is a customized large aperture vibrating-mesh device and was manufactured for this study by Aerogen, Inc., USA (now Nektar, USA).

2.2. Methods

2.2.1. Preparation of liposomes

Phospholipid (70 mg) comprising soya phosphatidylcholine (SPC) and cholesterol (1:1 mole ratio) was dissolved in chloroform (60 mg/ml) in a pear-shaped flask. The flask was attached to a rotary evaporator (Büchi Rotavapor R-114, Büchi, Switzerland) under reduced pressure using a vacuum pump (Büchi Vac V-500, Büchi, Switzerland). The rotation speed of the rotary evaporator was set at maximum and water bath was set at 35 °C. After 1 h, the negative pressure was released and the flask detached. The thin lipid film formed was flushed with nitrogen for 2 min to remove chloroform residues, if any. Salbutamol sulfate was dissolved in NaCl (0.9% w/v) to produce a solution of 35 mg/ml. Drug solution (2 ml) was added to the thin lipid film at ambient temperature with vigorous hand shaking for 10 min. This was followed by flask immersion in the water bath for 15 min followed by another 10 min hand shaking. Drug-free NaCl (0.9%) solution was added to give liposomes having a phospholipid concentration of 10 mg/ml. The resultant liposomes were reduced in size by extrusion, using a Liposofast-50 extruder (Avestin, Canada) through a series of polycarbonate membrane filters having a pore size of 3, 1, 0.6 and 0.4 μm, to yield liposomes of the desired size.

2.2.2. Size analysis of liposomes

The size distribution of liposomes unextruded or extruded through the 1 μm membrane filters was analyzed using a Malvern Mastersizer S laser diffraction size analyzer (Malvern Instruments Ltd., UK). A magnetically stirred cell dispersion unit (Malvern Instruments Ltd., UK) was employed with medium speed stirring in order to keep the liposomes dispersed during size measurement. The active beam length was set at 14.3 mm and polydisperse mode of analysis was chosen. These set up conditions permitted accurate measurement of particles having a size range between 0.5 and 900 μm. The median size and size distribution were measured by the instrument as the volume median diameter (VMD) and Span, respectively. Span is a unit-less term introduced by Malvern Instruments Ltd., UK to express the size distribution of the sampled particles, and is calculated as: Span = (90% undersize − 10% undersize)/VMD.

The size distribution of liposomes extruded through the 0.4 μm membrane filters was analyzed using a Zetasizer (Malvern Instruments Ltd., UK). This instrument gives an accurate measurement for particles having a size less than 1 μm. The size and size distribution were measured by the instrument as Z_{Ave} and polydispersity index (PI), respectively.

2.2.3. Transmission electron microscopy (TEM) analysis

Samples from liposome dispersions were deposited on carbon-coated copper grids (400 mesh) and negatively stained with phosphotungstic acid (1%, w/v). Liposome morphology was viewed using a Philips CM 120 BioTwin transmission electron microscope (Philips Electron Optics BV, The Netherlands).

2.2.4. Laser diffraction size analysis of aerosol droplets

The size distribution of aerosol droplets generated from nebulizers was analyzed using a Malvern 2600c laser diffraction size analyzer with 63 mm lens (Malvern Instruments Ltd., UK). Liposomes (5 ml, 10 mg/ml) were added to the reservoir of the nebulizer. The nebulizer mouthpiece was clamped 2.5 cm from the center of the laser beam, and adjusted so that the aerosol cloud traversed the beam at a distance of 2.5 cm from the lens. A vacuum line was employed to draw the aerosol cloud through the laser beam. VMD and Span were reported at the mid-point of nebulization to “dryness”. The VMD measured using laser diffraction represents the median size of a spherical particle which has the same volume as the particle in question. Thus,

VMD is different in principle from mass median aerodynamic diameter (MMAD) which is dependent on physical size and particle density. Laser diffraction is well established for size measurement of aerosol droplets generated using nebulizers. Moreover, VMD may be equivalent to MMAD for non-volatile aerosols and has shown a good correlation with pulmonary deposition findings (Clark, 1995).

2.2.5. Determination of size and morphology of liposomes delivered to a twin impinger (TI)

The twin impinger (TI) (Hallworth and Westmoreland, 1987), also known as the Single Stage Glass Impinger, was employed with a flow rate of 60 l/min to collect the nebulized aerosol. The TI comprises two stages. The upper stage represents the upper airways, and the lower stage represents the lower respiratory airways with a cut-off aerodynamic diameter of 6.4 μm at 60 l/min (Hallworth and Westmoreland, 1987). Liposomes (5 ml, 10 mg/ml) were placed in a Pari LC Plus or an Aeroneb Pro-8 μm nebulizer with the device mouthpiece directed into the throat of the TI. Nebulizers were operated to "dryness" which was 30 s after aerosols completely ceased. Samples were taken from the nebulizer reservoir and upper and lower stages of the TI for particle size analysis using laser diffraction or photon correlation spectroscopy, or for liposome morphology study using TEM.

2.2.6. Separation of entrapped and unentrapped salbutamol sulfate

Liposomes (10 mg/ml), unextruded or extruded, were centrifuged for 50 min at 41,000 × *g* and 4 °C using a 3K30 bench centrifuge (Sigma Laboratory Centrifuges, Germany). The supernatant was collected and liposome pellets in the centrifuge tubes were redispersed in 10 ml NaCl (0.9%) solution to remove drug adsorbed onto liposomes. Centrifugation was repeated for 50 min and the supernatant was again collected and added to the first supernatant to comprise the unentrapped fraction of the drug. The liposome pellets were solubilized using Triton X-100 (1%, w/v) and the released drug was the liposome-entrapped fraction. The entrapment efficiency (EE) of salbutamol sulfate in liposomes was calculated after quantification of entrapped and unentrapped drug using HPLC. EE (%) = (amount of drug entrapped in liposomes/overall amount of the drug in formulation) × 100%.

2.2.7. Determination of retained entrapment after nebulization

Unentrapped salbutamol sulfate was removed from liposome formulations as described previously and liposome pellets were redispersed in NaCl (0.9%) solution to give liposomes having a lipid concentration of 10 mg/ml and theoretically entrapping the entire amount of the drug present in the formulation. Liposomes (5 ml) were placed in the nebulizer and nebulization commenced and continued to "dryness". At the end of nebulization, the nebulizer reservoir, and upper and lower stages of the TI were washed separately with NaCl (0.9%) and made up to 50, 50 and 75 ml, respectively. The separation (centrifugation) procedure described earlier was repeated but the supernatants were collected this time for quantification of the unentrapped salbutamol sulfate using HPLC. The liposome pellets were solubilized using Triton X-100 (1%, w/v), and the drug released was analyzed by HPLC as the liposome-entrapped fraction. Entrapment efficiency was expressed here as the "retained entrapment" since the entrapment at the starting point (i.e. immediately before nebulization) was 100%.

2.2.8. Determination of aerosol mass output, drug output and mass output rate

Liposomes (5 ml, 10 mg/ml) were placed in a pre-weighed nebulizer and aerosolized to "dryness" into the mouthpiece of the TI. Aerosol mass output (%) was determined by calculating the weight difference of the nebulizer before and after nebulization. The time required to reach "dryness" status was recorded and mass output rate was accordingly calculated as mg/min. Drug output was calculated as the percentage fraction of drug (entrapped + unentrapped) released from the nebulizer when nebulization took place to "dryness".

2.2.9. HPLC analysis of salbutamol sulfate

Sodium 1-hexane sulfonate hydrate (5 mM) in water was mixed with methanol (75:25, v/v) to comprise the mobile phase. Glacial acetic acid was added to constitute 1% of the total mobile phase volume. The HPLC instrument (HP 1050 with UV detector; Agilent, USA) was set up with a SunFire^{TM} C18 column (4.6 mm × 150 mm, 5 μm; Waters Corporation, Ireland). The flow rate of the mobile phase was set at 1 ml/min and 40 °C. A 100 μl of the sample was automatically injected and detected at 276 nm. A calibration curve of ascending drug concentrations was made and drug in samples was accordingly quantified.

2.2.10. Statistical analysis

Statistical analysis was performed using the analysis of variance (ANOVA) and Student's *t*-tests. A value of $P < 0.05$ denotes a statistically significant difference. All experiments were undertaken in triplicate.

3. Results and discussion

3.1. Size and morphology of liposomes before nebulization

Extrusion was employed as a method of producing liposomes having different sizes (Table 1). Liposomes had a size larger than the pores through which they were extruded (Table 1). Similar findings for extruded liposomes have been previously reported (Olson et al., 1979; Szoka et al., 1980), which is attributed to the elastic deformation of liposomes (Lesieur et al., 1991). Compared with unextruded MLVs, the span value of liposomes extruded through the 1 μm polycarbonate membranes was significantly decreased ($P < 0.05$), indicating a considerable reduction in vesicle polydispersity on extrusion (Table 1).

Extrusion of liposomes resulted in considerable loss of the originally entrapped salbutamol sulfate, which was manifested by the decrease in the EE (Table 1). Szoka et al. (1980)

Table 1
Size and size distribution of unextruded MLVs and liposomes extruded through 1 or 0.4 μm polycarbonate membrane filters, and the resultant entrapment efficiency (EE) of salbutamol sulfate ($n = 3 \pm$ S.D.)

Liposome formulation	Size	Size distribution	EE (%)
Unextruded MLVs	VMD: 5.19 ± 0.49 μm	Span: 1.72 ± 0.16	13.17 ± 0.92
Extruded (1 μm)	VMD: 1.34 ± 0.13 μm	Span: 1.13 ± 0.14	7.78 ± 0.53
Extruded (0.4 μm)	Z_{Ave}: 408.8 ± 9.34 nm	PI: 0.49 ± 0.03	3.34 ± 0.14

demonstrated a marked reduction in the entrapped aqueous volume when reverse phase evaporation liposomes were extruded through 0.1 μm polycarbonate membranes. In another study, Taylor et al. (1990a) showed that extrusion of MLVs markedly reduced the entrapment of the hydrophilic drug sodium cromoglicate. However, the EE of salbutamol sulfate in this study is superior to the literature reported entrapment of hydrophilic agents in MLVs. This is attributed to the application of a two-step hydration protocol in this study.

TEM showed that MLVs extruded through 1 μm polycarbonate membranes retained their multilamellar morphology (Fig. 1a). However, extrusion through the 0.4 μm membranes produced oligolamellar liposomes (Fig. 1b), indicating that lamellarity of liposomes was reduced by extrusion through this membrane.

3.2. Size and morphology of liposomes after nebulization

Nebulization produced a significant ($P < 0.05$) reduction in the measured VMD of MLVs collected from the TI stages (Table 2) when compared with MLVs before nebulization (Table 1). This suggests that liposomes were disrupted during nebulization regardless of the nebulizer type. Air-jet nebulization may result in liposome size reduction (disruption) and subsequent loss of the entrapped hydrophilic material, with greater losses from larger liposomes (Taylor et al., 1990b; Niven et al., 1991). For the unextruded MLVs, size analysis of vesicles sampled from the TI did not demonstrate clearly that one nebulizer was more disruptive to the MLVs than the other (Table 2).

Span values of the unextruded MLVs delivered to the TI were significantly ($P < 0.05$) larger and highly non-reproducible for the Pari LC Plus (jet) nebulizer compared with the Aeroneb Pro-8 μm (vibrating-mesh) device (Table 2). Span value is dependent on 90% undersize, 10% undersize and VMD of the particles. For vesicles delivered by the jet nebulizer to the lower stage of the TI, the 90% undersize was 146 μm. The nebulization of particles having such a huge size to the lower stage of the TI is not possible as the cut-off between stages is 6.4 μm (Hallworth and Westmoreland, 1987). It is likely then, that liposomes have aggregated in the impinger after they were delivered from the jet nebulizer. Liposome aggregation in the lower stage of the TI has been previously reported when an ultrasonic nebulizer was employed (Leung et al., 1996).

For liposomes extruded through 1 μm polycarbonate membranes (Table 1), nebulization to the TI resulted in vesicle aggregation as the Span values were high for both nebulizers, and this was accompanied by large measured VMDs for the jet nebulizer (Table 2). For the lower stage of the TI, the 90% undersize was 111 and 45 μm for the Pari and Aeroneb Pro nebulizers, respectively, with size distribution being unimodal and bimodal, respectively (figures not shown). This means that the aggregation behaviour was affected by the mechanism of nebulization. It is possible that liposome bilayers were disrupted during nebulization and this resulted in a subsequent aggregation or fusion of the destabilized vesicles in the TI as shown in Fig. 2. The higher 90% undersize of liposomes delivered from the jet nebulizer might be attributed to solvent evaporation during atomization, resulting in the generation of aerosols having a higher concentration of

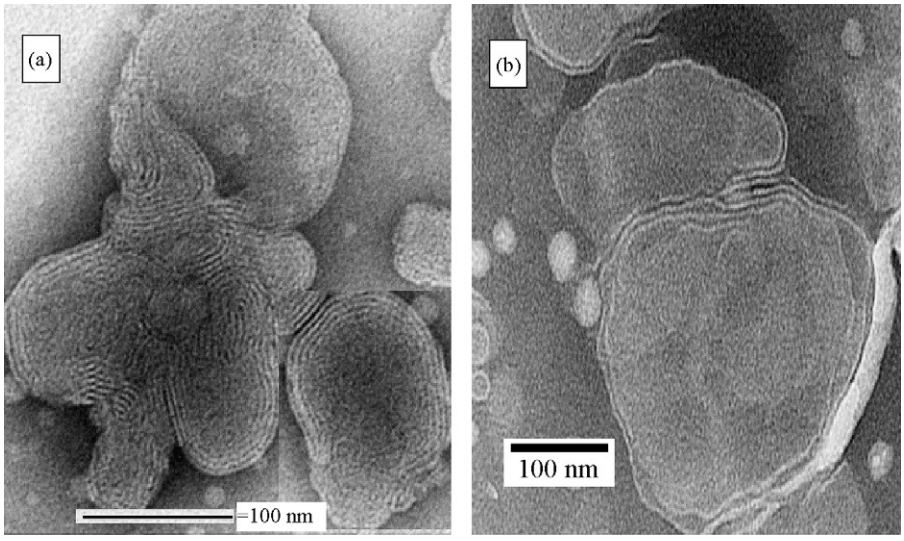

Fig. 1. TEM of liposomes extruded through 1 μm (a) and 0.4 μm (b) polycarbonate membrane filters.

Table 2
Size and size distribution of liposome formulations as collected from different compartments after nebulization using a Pari LC Plus (air-jet) and an Aeroneb Pro-8 μm (vibrating-mesh) nebulizers ($n = 3 \pm$ S.D.)

Nebulizer type	Nebulizer reservoir		Upper stage of the TI		Lower stage of the TI	
	VMD (μm)	Span	VMD (μm)	Span	VMD (μm)	Span
Unextruded MLVs						
Pari LC Plus (air-jet)	3.20 ± 0.09	1.45 ± 0.08	4.23 ± 0.12	2.06 ± 0.58	2.86 ± 0.29	48.54 ± 36.78
Aeroneb Pro (vibrating-mesh)	4.87 ± 0.27	1.43 ± 0.17	3.43 ± 0.31	1.18 ± 0.07	2.83 ± 0.32	1.25 ± 0.17
Liposomes extruded through 1 μm membrane filters						
Pari LC Plus (air-jet)	1.03 ± 0.12	1.22 ± 0.66	4.17 ± 0.79	8.99 ± 4.06	8.54 ± 0.16	12.27 ± 0.84
Aeroneb Pro (vibrating-mesh)	1.09 ± 0.03	1.64 ± 0.11	1.30 ± 0.21	5.55 ± 1.56	1.68 ± 0.47	25.77 ± 2.55

	Nebulizer reservoir		Upper stage of the TI		Lower stage of the TI	
	Z_{Ave} (nm)	PI	Z_{Ave} (nm)	PI	Z_{Ave} (nm)	PI
Liposomes extruded through 0.4 μm membrane filters						
Pari LC Plus (air-jet)	488.5 ± 181.89	0.75 ± 0.43	338.5 ± 54.72	0.56 ± 0.20	278.87 ± 10.47	0.40 ± 0.26
Aeroneb Pro (vibrating-mesh)	378.40 ± 23.43	0.31 ± 0.15	408.37 ± 31.04	0.39 ± 0.04	305.8 ± 17.87	0.44 ± 0.14

lipids and subsequent higher propensity to vesicle aggregation. Further work is required to evaluate the significance of such difference in aggregation behaviours *in vivo*.

For liposomes extruded through 0.4 μm membranes and delivered to the lower stage of the TI, the Z_{Ave} and PI decreased significantly ($P < 0.05$) (Table 2) compared to pre-nebulization (Table 1). There was no difference ($P > 0.05$) in the measured Z_{Ave} and PI between the nebulizers, suggesting that such liposomes demonstrated comparable degrees of instability for the vesicles delivered to the lower stage.

The size reduction of liposomes (0.4 μm-extruded) delivered to the upper stage was not statistically significant ($P > 0.05$) for either nebulizer (Table 2), suggesting a higher stability of vesicles delivered to the upper stage compared with those sampled from the lower stage of the TI. However, compared with liposomes before nebulization (Table 1), the PI of vesicles deposited in the upper stage increased ($P < 0.05$) for the jet nebulizer and decreased ($P < 0.05$) for the vibrating-mesh device, indicating a change in particle size distribution in a manner dependent on nebulizer type. This suggests that vesicles were destabilized by being broken up and/or aggregated or fused on nebulization to this stage of the TI, which has been confirmed earlier by TEM (Fig. 2).

3.3. Aerosol droplet size analysis

Fig. 3 shows the relationship between liposome size and size of the aerosol droplets generated from the nebulizers. For all formulations, the Pari nebulizer generated droplets having a smaller VMD than the Aeroneb Pro (Fig. 3a) whilst the Span was similar for the two nebulizers (Fig. 3b).

The VMD (Fig. 3a) and Span (Fig. 3b) of the aerosol generated from the Pari nebulizer were largely unaffected by the size of liposomes used for nebulization. Previously, for jet and ultra-

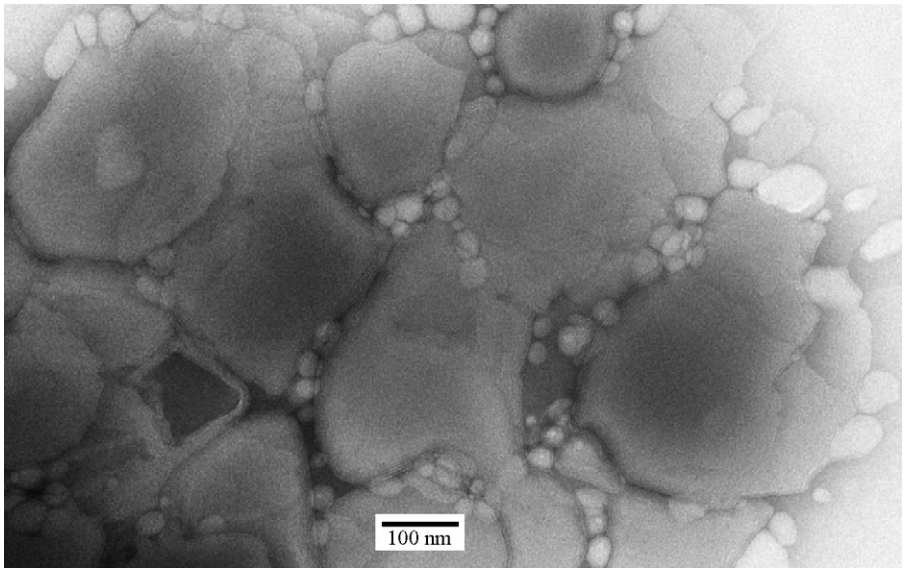

Fig. 2. TEM of liposomes extruded through 0.4 μm and delivered to the TI using the Aeroneb Pro-8 μm nebulizer.

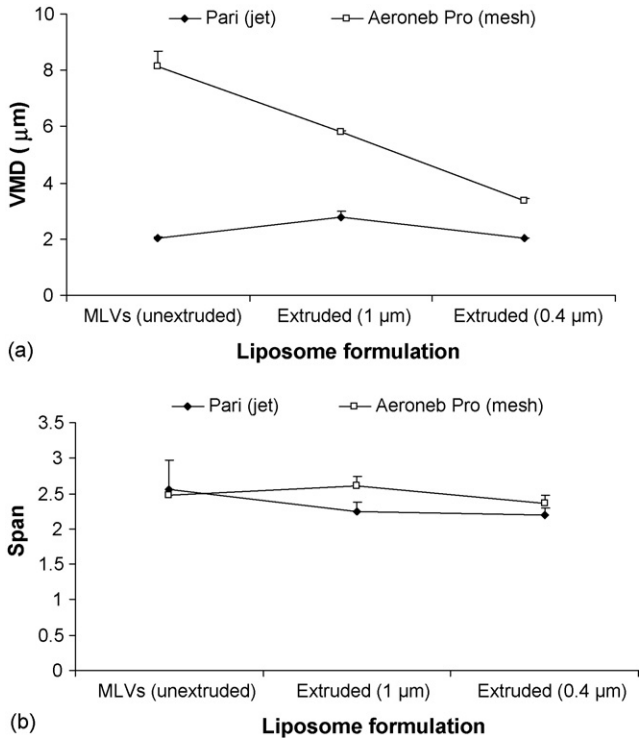

Fig. 3. Size (a) and size distribution (b) of the aerosols generated from liposome formulations using Pari LC Plus air-jet nebulizer and Aeroneb Pro-8 μm vibrating-mesh nebulizer ($n = 3 \pm$ S.D.).

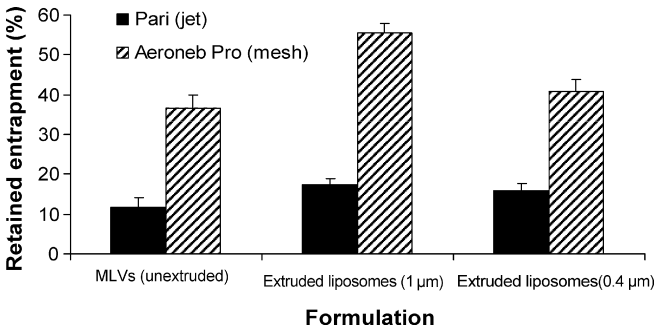

Fig. 4. The retained entrapment of salbutamol sulfate in the aerosolized liposomes using Pari LC Plus air-jet nebulizer and Aeroneb Pro-8 μm vibrating-mesh nebulizer ($n = 3 \pm$ S.D.).

sonic nebulizers, the size of aerosol droplets has been shown to be dependent on the nebulizer rather than liposome size (Bridges and Taylor, 1998). For the Aeroneb Pro nebulizer, the VMD of the aerosol was highly dependent on the size of liposomes used for nebulization ($P < 0.05$), as the VMD of the droplets decreased when the VMD of liposomes decreased (Fig. 3a). The Aeroneb Pro nebulizer produced aerosols with the smallest size when liposomes were extruded before nebulization through the 0.4 μm membranes (Fig. 3a). Using air-jet and ultrasonic nebulizers, aerosol droplet size and output of single phase liquids have been found to be dependent on physicochemical properties such as viscosity and surface tension (McCallion et al., 1995; Steckel and Eskandar, 2003). The performance of the Pari (jet) nebulizer was not greatly affected by the range of formulations employed in this study. However, ongoing studies in our laboratory show that vibrating-mesh nebulizers are highly dependent on formulation properties (Elhissi et al., 2005). Thus, the difference in the properties of aerosols produced by the Aeroneb Pro device might be due to small differences in the viscosity or surface tension between the liposome formulations employed.

3.4. Retained entrapment of salbutamol sulfate in liposomes after nebulization

Nebulization resulted in considerable losses of the originally entrapped salbutamol sulfate from vesicles delivered to the TI (Fig. 4). For the Pari (jet) nebulizer, the retained entrapment of the drug in the nebulized vesicles was low and slightly ($P < 0.05$) dependent on liposome size before nebulization (Fig. 4). Losses of the entrapped drug for the jet nebulizer were up to 88% (i.e. 12% retained) (Fig. 4). Niven et al. (1992) reported that jet nebulization may result in up to 88.2% losses of the entrapped hydrophilic marker carboxyfluorescein from liposomes extruded through 1 μm membranes. Recently, using a proliposome approach to liposome formulation, we have shown that vibrating-mesh nebulization is advantageous compared with jet nebulization in terms of vesicle stability to nebulization (Elhissi et al., 2006). The Aeroneb Pro-8 μm nebulizer employed in this study is a device customized by the manufacturer with large mesh apertures to reduce disruption of the bilayers when liposomes pass through the mesh pores. This device was found to be much less disruptive to the liposomes, so that up to 56% of the originally entrapped drug was retained in the delivered liposomes at the end of nebulization (Fig. 4). However, unlike the Pari nebulizer, the performance of the vibrating-mesh device was highly dependent on liposome formulation, demonstrating that vesicles extruded through 1 μm membranes were optimal in terms of liposome physical stability since the retained entrapment of the drug was at highest (Fig. 4).

For unextruded MLVs, the retained entrapment using the vibrating-mesh nebulizer was approximately 37% compared with only 12% for the air-jet nebulizer (Fig. 4). Extrusion through 1 μm membranes prior to nebulization increased the retained entrapment markedly ($P < 0.05$) for the customized vibrating-mesh nebulizer, and only slightly ($P < 0.05$) for the Pari (jet) nebulizer, being 56% and 17.5%, respectively (Fig. 4). However, further extrusion of liposomes through 0.4 μm membranes reduced the retained entrapment significantly ($P < 0.05$) for the vibrating-mesh nebulizer and produced no change ($P > 0.05$) for the air-jet device, being 41% and 16%, respectively. It was expected that extrusion of liposomes through 0.4 μm membranes would enhance their stability to nebulization. It is possible that the reduced lamellarity of vesicles extruded through 0.4 μm filters (Fig. 1b) has counteracted the desirable effect of using smaller liposomes for nebulization. It seems that the reduced lamellarity of these liposomes resulted in reduced resistance of vesicles to shearing within the nebulizers. It is also possible that the reduced size of droplets generated from this formulation (Fig. 3a) means that liposomes had to undergo more size reduction (i.e. disruption) to be incorporated in the aerosols produced by the Aeroneb Pro nebulizer.

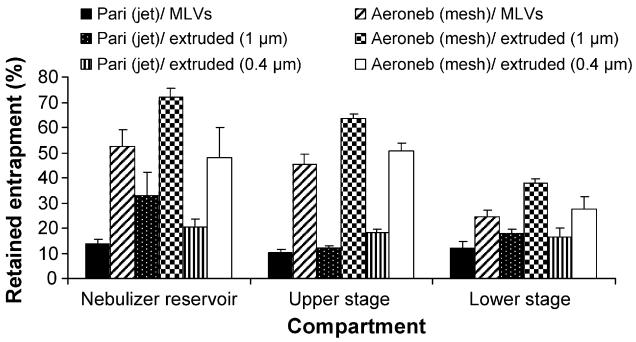

Fig. 5. The retained entrapment of salbutamol sulfate in liposomes delivered to the TI or remained in the "residual volume" of the nebulizers ($n = 3 \pm$ S.D.).

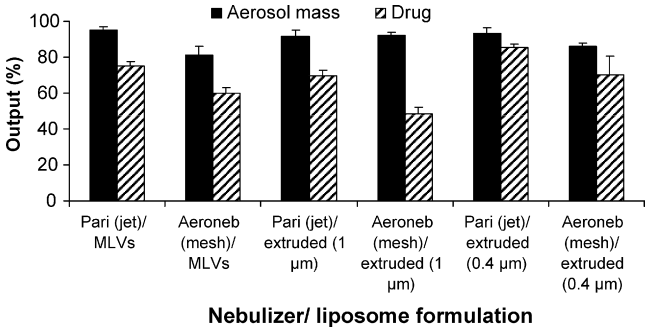

Fig. 6. Aerosol mass and drug output of liposome formulations delivered using Pari LC Plus air-jet nebulizer and Aeroneb Pro-8 μm vibrating-mesh nebulizer ($n = 3 \pm$ S.D.).

For all formulations, the Aeroneb Pro nebulizer delivered liposomes having higher retained entrapment ($P < 0.05$) than the jet nebulizer when both stages of the TI were considered (Fig. 5). Moreover, the retained entrapment of vesicles processed in the Pari nebulizer was similar for the nebulizer reservoir and both stages of the TI (Fig. 5). By contrast, liposomes in the residual volume of the Aeroneb Pro nebulizer had larger retained entrapment than the delivered vesicles (Fig. 5), suggesting that passage of liposomes through the mesh pores had a disruptive effect on the bilayers. In jet nebulizers, between 93% and 99% of the generated "primary" aerosol is caught and recycled by the baffles of the nebulizer and this is responsible for the prolonged nebulization (Nerbrink et al., 1994). Bridges and Taylor (1998) have shown a time-dependent reduction in the size of MLVs using a range of air-jet nebulizers. This suggests that the prolonged atomization within the Pari nebulizer was markedly detrimental to the physical integrity of liposomes and caused most losses of the entrapped salbutamol sulfate. By contrast, the passage of liposomes through the large mesh pores of the customized Aeroneb Pro was less disruptive to the bilayers and resulted in greater retention of the originally entrapped drug.

The lower stage of the TI collects the "fine particle fraction", i.e. the proportion of the emitted aerosol which is likely to be therapeutically available. For all formulations, liposomes delivered using the Aeroneb Pro (mesh) nebulizer had a higher retained entrapment in the upper stage compared with the lower stage of the TI (Fig. 5). This was not observed for the air-jet nebulizer (Fig. 5). Large liposomes entrap a greater proportion of water soluble phase than smaller liposomes (Table 1; Szoka et al., 1980; Taylor et al., 1990a). This probably means that the higher retained entrapment of salbutamol sulfate in the upper stage is due to the "size fractionation" of liposomes between the TI stages. In other words, larger vesicles/aggregates which have largely avoided disruption were incorporated in larger droplets which deposited in the upper stage, whilst smaller vesicles resulting from disruption during nebulization were incorporated in smaller droplets which deposited in the lower stage of the TI. Such "size fractionation" was observed previously for liposomes delivered from an ultrasonic nebulizer (Leung et al., 1996), air-jet nebulizers (Bridges and Taylor, 2001; Elhissi et al., 2006) and vibrating-mesh nebulizers (Elhissi et al., 2006). It is also possible that a fraction of droplets deposited in the lower stage were too small to incorporate liposomes and rather they contained free drug only, and that resulted in reduced entrapment in this stage.

3.5. Aerosol mass and drug output

Nebulization to "dryness" did not result in complete atomization of the nebulizer contents and hence mass and drug output were less than 100% (Fig. 6). Some fluid remains as the "residual" or "dead" volume in nebulizers at the end of nebulization (Clay et al., 1983). Aerosol mass output exceeded ($P < 0.05$) drug output for all formulations and both nebulizers (Fig. 6). This means that the concentration of drug/liposomes was increased in the nebulizers at the end of nebulization. In jet nebulizers, aerosol output comprises aerosol droplets and solvent vapour which saturates the outgoing air (Ferron et al., 1976; Dennis et al., 1990; Smye et al., 1992), thus an increased concentration of salbutamol sulfate in the Pari (jet) nebulizer was expected. However, vibrating-mesh nebulizers do not concentrate solutes during nebulization (Fink et al., 2001a) and have been shown to be as efficient as jet nebulizers in delivery of therapeutic suspensions (Fink et al., 2001b; Eskandar et al., 2003; Fink and Simmons, 2004; Caponetti et al., 2006). Consequently, the increase in the concentration of salbutamol sulfate within the Aeroneb Pro-8 μm nebulizer was unexpected. It seems likely that large liposomes were left in the residual volume of this nebulizer and that these were responsible for the retention of most of the drug remaining in the residual volume while the continuous aqueous phase was more efficiently nebulized. We have shown previously that phospholipids may accumulate during nebulization of liposomes in air-jet (Bridges and Taylor, 2000; Elhissi and Taylor, 2005; Elhissi et al., 2006), ultrasonic (Elhissi and Taylor, 2005) and vibrating-mesh (Elhissi and Taylor, 2005; Elhissi et al., 2006) nebulizers.

Overall, the Pari (jet) nebulizer generated aerosols with higher mass and drug output than the Aeroneb Pro (mesh) device (Fig. 6). However, the vibrating-mesh nebulizer completed the nebulization in a much shorter time than the air-jet nebulizer for all formulations (Fig. 7). Whilst approximately 10 min was sufficient to achieve "dryness" for the customized Aeroneb Pro, the Pari LC Plus required more than 25 min (Fig. 7). Consequently, much higher aerosol mass output rates were achieved using the vibrating-mesh nebulizer compared with the Pari (jet) nebulizer

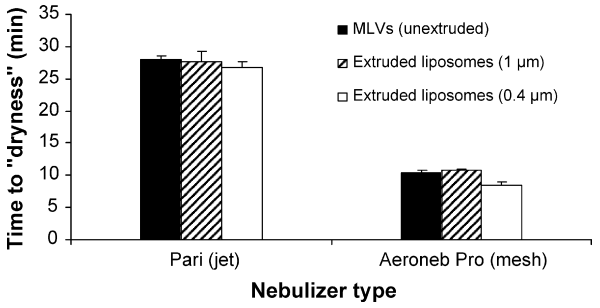

Fig. 7. Time required to achieve "dryness" for liposome formulations using Pari LC Plus air-jet nebulizer and Aeroneb Pro-8 μm vibrating-mesh nebulizer ($n = 3 \pm$ S.D.).

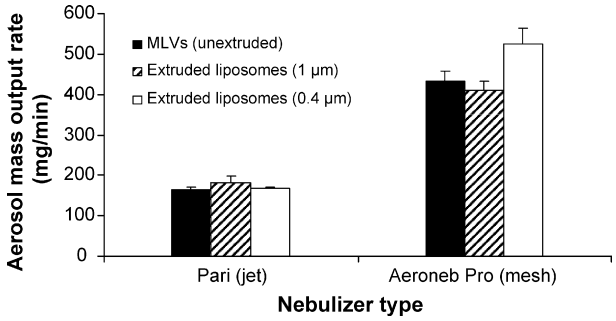

Fig. 8. Aerosol mass output rate for liposome formulations delivered using Pari LC Plus air-jet nebulizer and Aeroneb Pro-8 μm vibrating-mesh nebulizer ($n = 3 \pm$ S.D.).

(Fig. 8). For instance, using liposomes extruded through the 0.4 μm membranes, the mass output rate was 525 mg/min for the Aeroneb Pro nebulizer whilst only 167 mg/min for the Pari LC Plus nebulizer. The shorter nebulization time and higher output rate using the customized Aeroneb Pro may offer advantages over the Pari (jet) nebulizer in terms of patient compliance and nursing economics.

4. Conclusions

Liposome morphology and entrapment efficiency of the hydrophilic bronchodilator salbutamol sulfate were dependent on liposome size. Liposome aerosols were generated using an air-jet (Pari LC Plus) nebulizer or a customized Aeroneb Pro vibrating-mesh nebulizer with large mesh apertures. Size analysis of liposomes after nebulization showed evidence of liposome instability but was not informative regarding which nebulizer was less disruptive to the vesicles.

HPLC showed that physical stability of liposomes during nebulization was enhanced as the retained entrapment of salbutamol sulfate was improved when the Aeroneb Pro nebulizer was employed compared with the Pari LC Plus nebulizer. Reduction of MLV size by extrusion through 1 μm polycarbonate membranes prior to nebulization markedly enhanced the stability of liposomes when the Aeroneb Pro nebulizer was employed, whilst the enhanced stability was slight for the air-jet nebulizer. Further extrusion of the vesicles through 0.4 μm membranes was not advantageous since the resultant liposomes were less stable, apparently, due to their reduced lamellarity and subsequent reduced robustness. The Aeroneb Pro nebulizer completed nebulization in shorter times and produced higher mass output rates compared with the Pari nebulizer. On the other hand, the Pari nebulizer produced smaller aerosol droplets, and higher mass and drug outputs. Unlike the jet nebulizer, the performance of the Aeroneb Pro was highly dependent on formulation. This study has shown that vibrating-mesh nebulization of liposomes using the customized large aperture Aeroneb Pro is a successful strategy in terms of enhanced liposome stability to nebulization and providing higher output rates and shorter nebulization time compared with air-jet nebulization.

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References

Bridges, P.A., Taylor, K.M.G., 1998. Nebulisers for the generation of liposomal aerosols. Int. J. Pharm. 173, 117–125.

Bridges, P.A., Taylor, K.M.G., 2000. An investigation of some of the factors influencing the jet nebulisation of liposomes. Int. J. Pharm. 204, 69–79.

Bridges, P.A., Taylor, K.M.G., 2001. The effects of freeze-drying on the stability of liposomes to jet nebulisation. J. Pharm. Pharmacol. 53, 393–398.

Caponetti, G., Maggi, L., Zanellotti, L., Veneziani, C., Mora, P.C., 2006. $E_{disp}{}^{TM}$: a new microsuspension formulation approach suitable for the delivery of corticosteroids via soft-mist inhalers and nebulizers. Respir. Drug Deliv., 597–600.

Clark, A.R., 1995. The use of laser diffraction for the evaluation of the aerosol clouds generated by medical nebulizers. Int. J. Pharm. 115, 69–78.

Clay, M.M., Pavia, D., Newman, S.P., Lennard-Jones, T., Clarke, S.W., 1983. Assessment of jet nebulisers for lung aerosol therapy. Lancet ii, 592–594.

Dhand, R., 2002. Nebulizers that use a vibrating mesh or plate with multiple apertures to generate aerosol. Respir. Care 47, 1406–1416.

Dennis, J.H., Stenton, S.C., Beach, J.R., Avery, A.J., Walters, E.H., Hendrick, D.J., 1990. Jet and ultrasonic nebulizer output: use of a new method for direct measurement of aerosol output. Thorax 45, 10–16.

Elhissi, A.M.A., Taylor, K.M.G., 2005. Delivery of liposomes generated from proliposomes using air-jet, ultrasonic, and vibrating-mesh nebulisers. J. Drug Deliv. Sci. Technol. 15, 261–265.

Elhissi, A.M.A., Brar, J., Roberts, S.A., Taylor, K.M.G., 2005. Influence of proliposome hydration medium on the delivery of liposomes using a vibrating-mesh nebuliser. J. Pharm. Pharmacol. 57, S-104.

Elhissi, A.M.A., Karnam, K.K., Danesh-Azari, M.-R., Gill, H.S., Taylor, K.M.G., 2006. Formulations generated from ethanol-based proliposomes for delivery via medical nebulizers. J. Pharm. Pharmacol. 58, 887–894.

Eskandar, F., Steckel, H., Müller, B.W., 2003. A novel formulation for cyclosporin as inhalation suspension. Drug Deliv. Lungs XIV, 67–70.

Ferron, G.A., Kerrebijn, K.F., Weber, J., 1976. Properties of aerosols produced with three nebulizers. Am. Rev. Respir. Dis. 114, 899–908.

Fink, J.B., Simon, M., Heramia, M., Uster, P., 2001a. Does use of a nebulizer result in an increase in drug concentration? Presented at International Respiratory Congress, San Antonio, Texas.

Fink, J.B., Simon, M., Klimowicz, M., Uster, P.S., 2001b. Budesonide administration with a novel aerosol generator: an in vitro evaluation. Presented at American Thoracic Society 97th International Conference, San Francisco, California.

Fink, J.B., Simmons, B.S., 2004. Nebulization of steroid suspension: an in vitro evaluation of the Aeroneb Go and Pari LC Plus nebulizers. Chest 126, 816S.

Hallworth, G.W., Westmoreland, D.G., 1987. The twin impinger: a simple device for assessing the delivery of drugs from metered dose pressurized aerosol inhalers. J. Pharm. Pharmacol. 39, 966–972.

Lesieur, S., Grabielle-Madelmont, C., Paternoster, M.T., Olivon, M., 1991. Size analysis and stability study of lipid vesicles by high-performance gel exclusion chromatography, turbidity and dynamic light scattering. Anal. Biochem. 192, 334–343.

Leung, K.K.M., Bridges, P.A., Taylor, K.M.G., 1996. The stability of liposomes to ultrasonic nebulisation. Int. J. Pharm. 145, 95–102.

McCallion, O.N.M., Taylor, K.M.G., Thomas, M., Taylor, A.J., 1995. Nebulization of fluids of different physicochemical properties with air-jet and ultrasonic nebulizers. Pharm. Res. 12, 1682–1688.

Nerbrink, O., Dahlback, M., Hansson, H.C., 1994. Why do medical nebulizers differ in their output and particle size characteristics? J. Aerosol Med. 7, 259–276.

Niven, R.W., Speer, M., Schreier, H., 1991. Nebulization of liposomes. II. The effects of size and modeling of solute release profiles. Pharm. Res. 8, 217–221.

Niven, R.W., Carvajal, T.M., Schreier, H., 1992. Nebulization of liposomes. III. The effects of operating conditions and local environment. Pharm. Res. 9, 515–520.

O'Callaghan, C., Barry, P.W., 1997. The science of nebulised drug delivery. Thorax 52, S31–S44.

Olson, F., Hunt, L.A., Szoka, F.C., Vail, W.J., Papahadjopoulos, D., 1979. Preparation of liposomes of defined size distribution by extrusion through polycarbonate membranes. Biochim. Biophys. Acta 557, 9–23.

Saari, S.M., Vidgren, M.T., Koskinen, M.O., Turjanmaa, V.M., Waldrep, J.C., Nieminen, M.M., 1998. Regional lung deposition and clearance of 99m Tc-labelled beclomethasone-DLPC liposomes in mild and severe asthma. Chest 113, 1573–1579.

Saari, M., Vidgren, M.T., Koskinen, M.O., Turjanmaa, V.M.H., Nieminen, M.M., 1999. Pulmonary distribution and clearance of two beclomethasone formulations in healthy volunteers. Int. J. Pharm. 181, 1–9.

Saari, S.M., Vidgren, M.T., Herrala, J., Turjanmaa, V.M., Koskinen, M.O., Nieminen, M.M., 2002. Possibilities of formoterol to enhance the peripheral lung deposition of the inhaled liposome corticosteroids. Respir. Med. 96, 999–1005.

Smye, S.W., Jollie, M.I., Cunliffe, H., Littlewood, J.M., 1992. Measurement and prediction of drug solvent losses by evaporation from a jet nebuliser. Clin. Phys. Physiol. Meas. 13, 129–134.

Steckel, H., Eskandar, F., 2003. Factors affecting aerosol performance during nebulization with jet and ultrasonic nebulizers. Eur. J. Pharm. Sci. 19, 443–455.

Szoka, F., Olson, F., Heath, T., Vail, W., Mayhew, E., Papahadjopoulos, D., 1980. Preparation of unilamellar liposomes of intermediate size (0.1–0.2 μm) by a combination of reverse phase evaporation and extrusion through polycarbonate membranes. Biochim. Biophys. Acta 601, 559–571.

Taylor, K.M.G., Taylor, G., Kellaway, I.W., Stevens, J., 1989. The influence of liposomal encapsulation on sodium cromoglicate pharmacokinetics in man. Pharm. Res. 6, 633–636.

Taylor, K.M.G., Taylor, G., Kellaway, I.W., Stevens, J., 1990a. Drug entrapment and release from multilamellar and reverse-phase evaporation liposomes. Int. J. Pharm. 58, 49–55.

Taylor, K.M.G., Taylor, G., Kellaway, I.W., Stevens, J., 1990b. The stability of liposomes to nebulisation. Int. J. Pharm. 58, 57–61.

Waldrep, J.C., Gilbert, B.E., Knight, C.M., Black, M.B., Scherer, P.W., Knight, V., Eschenbacher, W., 1997. Pulmonary delivery of beclomethasone liposome aerosol in volunteers. Tolerance and safety. Chest 111, 316–323.