JPP Journal of Pharmacy and Pharmacology

JPP 2006, 58: 887–894

Received August 16, 2005
Accepted March 14, 2006
DOI 10.1211/jpp.58.7.0002
ISSN 0022-3573

Formulations generated from ethanol-based proliposomes for delivery via medical nebulizers

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Abstract

Multilamellar and oligolamellar liposomes were produced from ethanol-based soya phosphatidylcholine proliposome formulations by addition of isotonic sodium chloride or sucrose solutions. The resultant liposomes entrapped up to 62% of available salbutamol sulfate compared with only 1.23% entrapped by conventionally prepared liposomes. Formulations were aerosolized using an air-jet nebulizer (Pari LC Plus) or a vibrating-mesh nebulizer (Aeroneb Pro small mesh, Aeroneb Pro large mesh, or Omron NE U22). All vibrating-mesh nebulizers produced aerosol droplets having larger volume median diameter (VMD) and narrower size distribution than the air-jet nebulizer. The choice of liposome dispersion medium had little effect on the performance of the Pari nebulizer. However, for the Aeroneb Pro small mesh and Omron NE U22, the use of sucrose solution tended to increase droplet VMD, and reduce aerosol mass and phospholipid outputs from the nebulizers. For the Aeroneb Pro large mesh, sucrose solution increased the VMD of nebulized droplets, increased phospholipid output and produced no effect on aerosol mass output. The Omron NE U22 nebulizer produced the highest mass output (approx. 100%) regardless of formulation, and the delivery rates were much higher for the NaCl-dispersed liposomes compared with sucrose-dispersed formulation. Nebulization produced considerable loss of entrapped drug from liposomes and this was accompanied by vesicle size reduction. Drug loss tended to be less for the vibrating-mesh nebulizers than the jet nebulizer. The large aperture size mesh (8 μm) Aeroneb Pro nebulizer increased the proportion of entrapped drug delivered to the lower stage of a twin impinger. This study has demonstrated that liposomes generated from proliposome formulations can be aerosolized in small droplets using air-jet or vibrating-mesh nebulizers. In contrast to the jet nebulizer, the performance of the vibrating-mesh nebulizers was greatly dependent on formulation. The high phospholipid output produced by the nebulizers employed suggests that both air-jet and vibrating-mesh nebulization may provide the potential of delivering liposome-entrapped or solubilized hydrophobic drugs to the airways.

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Acknowledgements: We thank Lipoid (Germany) for their gift of soya phosphatidylcholine. We thank Aerogen (now Nektar, CA, USA), Omron Healthcare (UK) and Pari GmbH (Germany) for supplying us with the nebulizers used in this study. We thank Mr Dave McCarthy, Microscopy Unit, School of Pharmacy, University of London for electron microscopy.

Introduction

Nebulizers have been used for the pulmonary administration of liposomes to man (Farr et al 1985; Taylor et al 1989; Saari et al 1999). Medical nebulizers are of three general types: air-jet, ultrasonic and vibrating-mesh.

Air-jet nebulizers use compressed gas to convert aqueous solutions or suspensions into a spray. A jet of high-velocity gas passes through a narrow venturi nozzle. Liquid is drawn from a reservoir up a feed tube and emerges as fine filaments that collapse into aerosol droplets (O'Callaghan & Barry 1997). In ultrasonic nebulizers, a piezoelectric crystal vibrating at high frequency produces a fountain of liquid at the liquid surface from which large droplets are emitted at the apex, and a 'fog' of small droplets is generated from the lower part. With both air-jet and ultrasonic nebulizers, large droplets are recycled and small aerosol droplets are released for inhalation (O'Callaghan & Barry 1997).

Recently, vibrating-mesh nebulizers have become commercially available. These generate aerosols by passing liquids through a vibrating-mesh or plate with multiple apertures. The energy of vibration is derived from a vibrating piezoelectric crystal

attached to a horn transducer to transmit vibrations and extrude liquid through a perforated plate with up to 6000 tapered holes, approximately 3 μm in diameter (e.g. Omron NE U22 nebulizer) or from an aerosol generator, which comprises a domed aperture plate with up to 1000 holes and a vibrational element that contracts and expands to generate the aerosol (e.g. Aeroneb Pro nebulizer) (Dhand 2002). Vibrating-mesh nebulizers have been shown to be as efficient as air-jet nebulizers in delivering ciclosporin (Eskandar et al 2003) and steroid (Fink & Simmons 2004) suspensions, and demonstrated the capability to leave a negligible volume of solutions intended for delivery (Dhand 2002; Ismail & Chrystyn 2004).

Liposomes are unstable to jet nebulization as shearing may result in vesicle disruption and subsequent loss of the entrapped hydrophilic material (Taylor et al 1990; Niven et al 1991). However, liposome-entrapped hydrophobic materials may retain their sustained release property after shearing caused by jet nebulization (Saari et al 1999). Nebulization performance may be enhanced using liposomes made from low phase transition lipids compared to high phase transition lipids (Waldrep et al 1993; Saari et al 1999). This suggests that for delivery of liposome-entrapped hydrophobic drugs, finding the appropriate delivery technology to convert liposome formulations into inhalable aerosols may be more important than the issue of liposome instability caused by jet nebulization.

Conventional liposomes entrap hydrophilic agents poorly and are difficult to manufacture on a large scale. Rehydrated freeze-dried liposomes have been successfully nebulized when lipid composition is appropriate (Darwis & Kellaway 2001) and/or lyoprotectant, for example trehalose, is included in the formulation (Bridges & Taylor 2001). Proliposome methods may provide convenient, cheap and simple alternatives to freeze-drying. Moreover, proliposome methods may offer a potential for manufacturing liposomes on a large scale (Chen & Alli 1987; Turánek et al 1997). Proliposomes may be classified into two main types. First, particulate-based proliposomes comprise soluble free-flowing carrier particles coated with phospholipids that generate liposomes on addition of aqueous phase (Payne et al 1986). These are capable of generating liposomes in-situ when nebulized from air-jet, ultrasonic and vibrating-mesh nebulizers (Elhissi & Taylor 2005). Second, an alcohol-based proliposome method offers a relatively simple means of generating liposomes with a high entrapment of hydrophilic agents, by the addition of aqueous phase to a concentrated alcoholic solution of phospholipids (Perrett et al 1991). The final liposome dispersion may be similar in composition to that produced by the ethanol-injection method (Batzri & Korn 1973), which has been widely investigated in a range of therapeutic applications (Justo & Moraes 2005; Lendemans et al 2005).

In this study, liposomes generated from ethanol-based proliposome formulations containing cholesterol were used to entrap the hydrophilic bronchodilator drug salbutamol sulfate. Nebulization performance of proliposome formulations and entrapment of the drug in the recovered liposomes after nebulization were investigated using air-jet and vibrating-mesh nebulizers. The fraction of phospholipid delivered from nebulizers was estimated as a means of predicting the potential of these formulations in delivering entrapped or solubilized hydrophobic drugs.

Materials and Methods

Materials

Sucrose, sodium chloride (NaCl), absolute ethanol, ammonium thiocyanate, ferric chloride, chloroform, and glacial acetic acid were AnalaR grade and purchased from BDH, UK. Cholesterol (>99%), Triton X-100 and sodium hexane sulfonate were purchased from Sigma-Aldrich, UK. Phosphotungstic acid (analytical reagent grade) was purchased from TAAB Laboratories Equipment Ltd, UK. Salbutamol sulfate (99%) was supplied by Avocado Research Chemicals Ltd, UK. Soya phosphatidylcholine (SPC; Lipoid S-100) was a gift from Lipoid, Germany. Water and methanol used for chromatography were of high performance liquid chromatography (HPLC) grade and purchased from Fisher Scientific Ltd, UK. Aeroneb Pro (4 and 8 μm mesh) nebulizers were supplied by Aerogen, Inc., USA. The Omron NE U22 nebulizer was supplied by Omron Healthcare UK Ltd, UK. Pari LC Plus nebulizers and Pari Master Compressor were supplied by Pari, GmbH, Germany. All materials were used as received.

Generation of liposomes from proliposomes

The method was modified from that of Perrett et al (1991) by inclusion of a high cholesterol concentration within the lipid phase, and increasing the ethanol to phospholipid ratio in order to aid the dissolution of lipid. Lipid phase (50 mg) comprising SPC and cholesterol (1:1 mole ratio) was dissolved in 60 mg absolute ethanol at 70°C for 1 min in a glass vial to yield a clear lipid solution (i.e. the proliposomes). Heating the components to 70°C was necessary to dissolve the high concentration of cholesterol included in the lipid phase. Salbutamol sulfate (5 mg) dissolved in isotonic NaCl (0.9%) or sucrose (9.25%) solution (100 μL) was added to the ethanolic solution of phospholipid and mixed gently for 1 min to generate concentrated liposome dispersion. The addition of the aqueous phase was performed while the ethanolic solution was hot (70°C) in order to avoid lipid phase solidification before aqueous phase addition. The dispersion was made up to 5 mL with the appropriate drug-free isotonic solution and vigorous hand shaking took place for 1 min to yield diluted liposomes (10 mg mL^{-1}). The procedure was repeated using SPC without cholesterol in a 100:80 w/w SPC:ethanol ratio (Perrett et al 1991).

Preparation of liposomes by the thin-film method

Lipid phase (50 mg) comprising SPC and cholesterol (1:1 mole ratio) was dissolved in chloroform (60 mg mL^{-1}) in a

100-mL pear-shaped flask and attached to a rotary evaporator (Büchi, Switzerland) under vacuum for 1 h while the flask was immersed in a water bath (40°C). The thin film formed was hydrated by adding isotonic NaCl solution (5 mL) containing salbutamol sulfate (1 mg mL^{-1}) followed by hand shaking for 10 min to yield a liposome dispersion having a lipid concentration of 10 mg mL^{-1}.

Liposome size analysis

The size distribution of liposomes was measured using laser diffraction (Malvern Mastersizer S; Malvern Instruments Ltd, UK). The median size and size distribution were calculated by the instrument as volume median diameter (VMD) and Span respectively, where Span = (90% undersize − 10% undersize)/VMD.

Transmission electron microscopy analysis

Samples of liposomes were deposited on carbon-coated copper grids (400 mesh), negatively stained with phosphotungstic acid (1% w/v) and viewed with a Philips CM 120 BioTwin electron microscope (Phillips Electron Optics BV, The Netherlands).

Determination of size and morphology of liposomes delivered to a twin impinger

The twin impinger (Hallworth & Westmoreland 1987), also known as the two-stage impinger or the Single Stage Glass Impinger, was set up with a flow rate of 60 L min^{-1} and isotonic NaCl (0.9%) as the collection medium. Cholesterol-containing liposomes prepared from proliposomes by dispersion in isotonic sucrose solution (5 mL) were placed in the nebulizer with the mouthpiece directed into the 'throat' of the impinger. Nebulization was commenced and continued to 'dryness', which was the point at which measurable aerosol ceased for at least 30 s. Samples were taken from the nebulizer reservoir and the upper and lower stages of the impinger for liposome size analysis using laser diffraction, and for morphological study of vesicles using transmission electron microscopy.

Determination of aerosol droplet size distribution

Following dispersion of proliposomes, aerosols produced from cholesterol-containing liposome formulations were drawn through the laser beam of a Malvern 2600c laser diffraction sizer (Malvern Instruments Ltd, UK) and the size distribution of aerosol droplets was determined using a 63-mm lens at the mid-point of nebulization to 'dryness'.

Determination of aerosol mass and phospholipid output and output rates

Cholesterol-containing liposomes were produced from proliposomes using sucrose or NaCl solutions for dispersion, and nebulized to 'dryness'. Aerosol mass output (%) was calculated by weight difference of preweighed nebulizers before and after nebulization. Phospholipid output (%) was estimated by washing nebulizers with deionized water followed by dilution to 1 L. Samples of diluted liposomes (4 mL) were placed in 15-mL glass tubes and 1 mL absolute ethanol was added to each in order to convert liposomes into ethanolic solution of phospholipid. Solvent was evaporated from samples overnight in an oven at 90°C to yield dried lipid deposited in the tubes. The amount of dried lipid was estimated analytically (Stewart 1980) by dissolving the dried samples in 2 mL chloroform followed by the addition of an equal volume of ammonium ferrothiocyanate solution (prepared by dissolving 6.76 g ferric chloride and 7.6 g ammonium thiocyanate in deionized water made up to 250 mL) and vigorous vortex mixing for 1 min. Tubes were spun for 5 min using a 3K30 bench centrifuge (Sigma Laboratory Centrifuges, Germany) at 300 *g* and 4°C. The lower (chloroformic) layer was aspirated using a Pasteur pipette and phospholipid complexed with ammonium ferrothiocyanate was estimated colorimetrically at 488 nm (Stewart 1980). The amount of phospholipid delivered was calculated by subtracting the amount of lipid that remained in the nebulizer from the lipid amount originally intended for nebulization. The output rates of the aerosol mass and phospholipid were also calculated.

Separation of entrapped and unentrapped drug

Salbutamol sulfate-containing liposomes dispersed in NaCl solution were centrifuged for 50 min at 40 000 *g* and 4°C using the 3K30 bench centrifuge. The supernatant was collected and liposome pellets in the centrifuge tubes were redispersed in NaCl (0.9%) solution (10 mL) to remove drug adsorbed onto the surface of vesicles. Centrifugation was repeated for 50 min and supernatant again collected. The combined supernatants were assayed by HPLC to allow quantification of unentrapped drug. Liposomes in the pellet were solubilized in Triton X-100 (0.5% w/v) and the drug released was analysed as entrapped fraction. Drug entrapment was expressed as entrapment efficiency (% of available drug entrapped in liposomes) and as mg of drug entrapped per 100 mg of phospholipid.

HPLC analysis of salbutamol sulfate

A buffer comprising sodium hexane sulfonate (5 mM) in water was mixed with methanol (75:25 v/v) to produce the mobile phase to which glacial acetic acid was added to constitute 1% of the total mobile phase volume. The HPLC (HP 1050 with UV detector; Hewlett-Packard Co., USA) was set up with a Symmetry C18 column (150 mm × 4.6 mm, 5 μm; Waters Ltd, UK) and samples were analysed at 276 nm. The mobile phase flow rate was adjusted to 1 mL min^{-1} at 40°C, and the volume of automatically injected sample set to 10 μL. A calibration curve of ascending drug concentrations was made and drug in

samples collected from liposomes before and after nebulization to the twin impinger was accordingly quantified. Retained entrapment of the drug was calculated in aerosol collected in the impinger stages and in the fluid remaining in the nebulizers. Retained entrapment = (fraction of entrapped drug after nebulization/fraction of entrapped drug before nebulization) × 100%.

Statistical analysis

Statistical analysis of the effects of nebulizer type and formulation on nebulization performance was performed using the Kruskal–Wallis multicomparisons test. Dunnett's test was employed when using one of the groups as a control. Where only two data sets were compared, the Mann-Whitney *U*-test was used. A value of $P < 0.05$ denotes a statistically significant difference for all statistical tests used. All experiments were undertaken in triplicate.

Results and Discussion

Liposome size analysis and morphology

Hydration of proliposomes by manual shaking resulted in the formation of oligolamellar and multilamellar vesicles from all formulations (Figure 1). Changes in the hydration medium, cholesterol inclusion, or ethanol to phospholipid ratio resulted in no significant differences ($P > 0.05$) in the measured VMD or Span of the liposomes formed (Table 1). Thus, changes in phospholipid composition or hydration medium resulted in no apparent effects on the size distribution of liposomes generated from ethanol-based proliposomes.

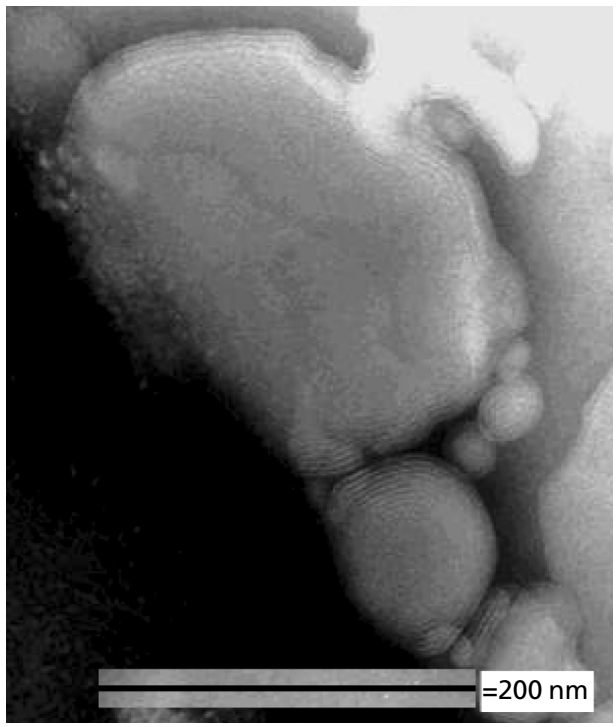

Figure 1 Transmission electron micrograph of multilamellar and oligolamellar liposomes formed from proliposomes dispersed in isotonic sucrose after 1 min hand shaking.

Table 1 Size distribution of liposomes generated from proliposomes dispersed in isotonic sodium chloride and sucrose (mean ± s.d., n = 3)

Formulation	Volume median diameter (μm)	Span
SPC/NaCl	4.20 ± 0.32	1.66 ± 0.07
SPC:cholesterol (1:1)/NaCl	4.92 ± 0.34	1.98 ± 0.21
SPC:cholesterol (1:1)/sucrose	4.59 ± 0.37	2.31 ± 0.66

SPC, soya phosphatidylcholine.

Size and morphology of liposomes delivered to the twin impinger

For all nebulizers, the VMD of liposomes remaining in the nebulizer reservoirs was significantly larger ($P < 0.05$) than that of vesicles delivered to the impinger stages. Moreover, except for the Omron nebulizer, vesicles delivered to the lower impinger stage had a smaller VMD ($P < 0.05$) than those delivered to the upper stage of the impinger, which collects aerosols of larger aerodynamic diameter (Table 2). Such 'size fractionation' has previously been reported for liposomes delivered from an ultrasonic nebulizer (Leung et al 1996) and air-jet nebulizers (Bridges & Taylor 2001). The reduction in the measured VMD is likely to result from fragmentation of large liposomes in the dispersions and deaggregation of vesicle aggregates (Taylor et al 1990; Niven et al 1991).

The size of liposomes generated from proliposomes (Table 1) suggests that such vesicles are too large for efficient nebulization to the respiratory tract. However, liposomes are not conventional particles, since they may be processed to smaller sizes during nebulization to a size to be appropriate for delivery to the lower stage of the twin impinger (Table 2). The absence of the 'size fractionation' of liposomes within the impinger using the Omron nebulizer suggests that the mesh of this device offered a better control of the size distribution of nebulized vesicles compared with

Table 2 Measured volume median diameter (μm) of liposomes generated from proliposomes in the nebulizer reservoir and twin impinger stages (mean ± s.d., n = 3)

Nebulizer	Reservoir	Upper stage	Lower stage
Pari LC Plus	5.20 ± 1.11	3.60 ± 0.17	2.83 ± 0.18
Aeroneb Pro (4 μm)	6.42 ± 2.45	3.19 ± 0.08	2.50 ± 0.05
Aeroneb Pro (8 μm)	6.38 ± 1.00	3.32 ± 0.29	2.43 ± 0.06
Omron NE U22	4.75 ± 0.48	3.19 ± 0.06	2.84 ± 0.36

the baffles of the jet nebulizer or the mesh of the Aeroneb Pro nebulizers.

Transmission electron microscopy indicated that liposomes delivered to the lower impinger stage by all nebulizers were predominantly oligolamellar and multilamellar liposomes, suggesting that delivered vesicles preserved their morphology, although the VMD was reduced during nebulization.

Determination of aerosol droplet size distribution

Statistical analysis has shown that both VMD and Span were significantly different ($P < 0.05$) between the nebulizers, regardless of hydration medium. The VMD of aerosol droplets generated by the Pari air-jet nebulizer was significantly smaller ($P < 0.05$) than that of droplets generated by the other nebulizers, regardless of the hydration medium (Table 3). However, size distribution (Span) of the aerosol droplets generated by the Pari nebulizer was significantly larger ($P < 0.05$) than that of droplets generated by the vibrating-mesh nebulizers. This indicates that the mesh of the vibrating-mesh nebulizers offered a more robust control on the size distribution of the droplets generated compared with the air-jet and baffles of the Pari nebulizer. The Omron nebulizer produced the best control of droplet size distribution, as it showed the lowest Span values (Table 3). This correlates with the absence of 'size fractionation' of liposomes delivered from this device, suggesting that vesicles extruded from the mesh of this device were the least polydispersed.

While aerosol VMD for the Pari was not affected by the dispersion medium, the VMD for the vibrating-mesh nebulizers was significantly smaller ($P < 0.05$) for droplets generated from liposomes dispersed in NaCl compared with sucrose (Table 3). However, compared with VMD, Span was generally less dependent on hydration medium for all the nebulizers studied, since mean differences are statistically significant ($P < 0.05$) only for the Pari and Omron nebulizers. The Aeroneb Pro, customized for this investigation by the manufacturer to generate aerosols by passing fluid through an 8-μm mesh, produced droplets of significantly larger VMD ($P < 0.05$) compared with the conventional 4-μm vibrating-mesh device. However, no significant difference ($P > 0.05$) in the Span was found between the small and large mesh Aeroneb nebulizers, indicating that the control of droplet size distribution offered by this technology of aerosol generation was independent of the mesh aperture size. The size of the emitted droplets tended to approximate the size of the large mesh (8 μm) only when sucrose solution was used as a dispersion medium. This is probably due to the slower nebulization of liposomes dispersed in sucrose solution compared with NaCl-dispersed formulations, resulting in slower moving aerosols and hence reduced impaction of the large droplets to the mouthpiece positioned perpendicularly to the aerosol generator. However, although the aerosol generated from the nebulizers used in this study was primarily dependent on nebulizer design and liposome dispersion medium, all devices have generated aerosols that are likely to deposit in the peripheral airways, that is, less than 5 or 6 μm (O'Callaghan & Barry 1997).

Aerosol mass and phospholipid output and output rate

Nebulization of the preparations to 'dryness' did not result in complete atomization, and hence some fluid was left within the nebulizers. Some liquid remains as the 'dead' or 'residual' volume, associated with the baffles, internal structures and walls of the nebulizer (Clay et al 1983). Except for the Aeroneb Pro (8 μm) device, nebulizers produced a significantly higher mass output ($P < 0.05$) for NaCl-dispersed formulation compared with formulation dispersed in sucrose solution (Table 3). However, the Omron not only produced a higher mass output ($P < 0.05$) than the other nebulizers but also showed a slight difference in mass output between the two formulations (Table 3). Mass output produced by the Omron approximated 100%, regardless of formulation, agreeing with previous reports of very small residual volumes for this device (Dhand 2002; Ismail & Chrystyn 2004). Overall, the formulation effect on the mass output was greatest for the Aeroneb Pro nebulizers compared with the Pari or Omron nebulizers (Table 3).

Table 3 Effect of nebulizer and dispersion medium on aerosol size, mass and phospholipid output, and output rates (mean ± s.d., n = 3)

Nebulizer/dispersion medium	Aerosol size analysis		Mass and phospholipid output and output rate			
	Volume median diameter (μm)	Span	Mass output (%)	Phospholipid output (%)	Mass output rate (mg min^{-1})	Phospholipid output rate (mg min^{-1})
Pari LC Plus/sucrose	2.48 ± 0.20	2.74 ± 0.23	85.58 ± 2.24	74.02 ± 2.70	191.02 ± 6.66	1.50 ± 0.16
Pari LC Plus/NaCl	2.69 ± 0.12	2.47 ± 0.06	93.18 ± 0.38	80.26 ± 7.11	177.06 ± 14.58	1.50 ± 0.06
Aeroneb Pro (4 μm)/sucrose	4.59 ± 0.64	1.83 ± 0.13	60.13 ± 7.09	48.98 ± 13.03	221.95 ± 54.92	1.92 ± 0.68
Aeroneb Pro (4 μm)/NaCl	3.40 ± 0.03	1.98 ± 0.09	89.94 ± 2.24	75.68 ± 6.68	318.54 ± 10.99	2.56 ± 0.49
Aeroneb Pro (8 μm)/NaCl	4.63 ± 0.14	1.97 ± 0.07	82.95 ± 9.32	34.72 ± 8.37	478.68 ± 84.06	2.13 ± 0.37
Aeroneb Pro (8 μm)/sucrose	6.74 ± 0.71	2.09 ± 0.15	64.29 ± 9.80	67.24 ± 7.51	251.20 ± 113.56	1.66 ± 0.78
Omron NE U22/sucrose	5.23 ± 0.09	1.34 ± 0.04	97.81 ± 1.15	62.91 ± 5.85	88.12 ± 2.87	0.55 ± 0.06
Omron NE U22/NaCl	4.91 ± 0.14	1.57 ± 0.05	99.93 ± 0.05	90.15 ± 2.85	174.48 ± 6.51	1.53 ± 0.03

Phospholipid output of NaCl-dispersed liposomes was significantly ($P < 0.05$) greater than that of sucrose-dispersed liposomes for the Aeroneb Pro (4 μm) and the Omron nebulizers (Table 3). However, for the air-jet (Pari) nebulizer, phospholipid output was not significantly different ($P > 0.05$) between NaCl and sucrose-dispersed formulations (Table 3). Unlike the other nebulizers, the Aeroneb Pro (8 μm) produced higher phospholipid output ($P < 0.05$) from the sucrose-dispersed formulation (Table 3). It is possible that the longer nebulization time (14.93 ± 5.18 min) of this formulation using this nebulizer permitted sufficient opportunity for large vesicles and aggregates to pass through the large mesh. By contrast, the higher nebulization rate (shorter time; 8.87 ± 0.79 min) for the NaCl-dispersed formulation did not sufficiently break up large vesicles and aggregates, while the NaCl continuous phase was rapidly removed and hence vesicles were retained within the large mesh device.

When NaCl solution was used to disperse liposomes, nebulizers, except for the Aeroneb Pro (8 μm), produced a higher mass output than phospholipid output, indicating accumulation of phospholipid in the nebulizers (Table 3). However, when sucrose solution was used to disperse liposomes, only the Pari and Omron nebulizers produced higher mass output than lipid output ($P < 0.05$) (Table 3). This might be attributed to the variation between output experiments when sucrose-dispersed formulation was nebulized from the Aeroneb small and large mesh nebulizers. Accumulation of phospholipid within nebulizers may be due to solvent evaporation from the air-jet nebulizer (Ferron et al 1976) and/or retention of large liposomes or liposome aggregates in the nebulizers, as has been previously reported for conventional multilamellar vesicles delivered from air-jet nebulizers (Bridges & Taylor 2000).

The rate of mass and phospholipid output from the vibrating-mesh nebulizers tended to be greater for formulations dispersed in NaCl solutions compared with those dispersed in sucrose solutions, while no detected difference ($P > 0.05$) was shown for the jet nebulizer (Table 3). This indicates that the performance of the vibrating-mesh nebulizers was more affected by formulation properties than the air-jet nebulizer. Although mass output of the Omron approached 100% regardless of formulation, the delivery rate of aerosols was much lower ($P < 0.05$) when sucrose was used to disperse liposomes (Table 3). Aerosol size and output for conventional liquids atomized in jet and ultrasonic nebulizers is dependent on their physicochemical properties, particularly viscosity and to a lesser extent surface tension (McCallion et al 1995). While further investigation is merited, it seems likely that small differences in viscosity or surface tension of the suspending media is responsible for observed changes in the output and aerosol size for the vibrating-mesh nebulizers, using sucrose and NaCl-based dispersions.

The use of a large mesh nebulizer increased VMD of the nebulized droplets, shortened the nebulization time and affected output and output rate in a manner dependent on formulation (Table 3). This suggests that the employment of such a large mesh device in liposome delivery should be considered, taking into account the physicochemical properties of the formulation, the region required for targeting within the respiratory tract and patient compliance.

Salbutamol sulfate entrapment in liposomes

SPC/cholesterol vesicles generated from ethanol-based proliposomes by dispersion in NaCl solution had much higher entrapment efficiency and entrapment capacity for salbutamol sulfate than conventional liposomes produced by the thin-film hydration method (Table 4). Entrapment efficiency of carboxyfluorescein (a water-soluble marker) in liposomes prepared from alcohol-based proliposomes has been shown to be over the range of approximately 65 to 80% depending on phospholipid composition (Perrett et al 1991) and approximately 30 to 85% depending on hydration procedure (Turánek et al 1997). Moreover, liposomes generated from ethanol-based proliposomes may be superior to vesicles generated from particulate-based proliposomes in entrapping hydrophilic materials. For instance, Ahn et al (1995) have reported that the entrapment efficiency of the hydrophilic drug propranolol hydrochloride in liposomes generated from particulate-based proliposomes was between 4 and 10%. The superior entrapment of water-soluble materials using ethanol-based proliposomes compared with the thin-film and the particulate-based proliposome methods may be attributed to the fewer bilayers of liposomes generated from the ethanol-based proliposomes, as well as the two-step dispersion protocol, which optimizes liposome encapsulation of available drug (as a concentrated aqueous solution) as they form.

Salbutamol sulfate entrapment after nebulization

All nebulizers had a detrimental effect on liposome stability, resulting in significant loss of previously entrapped salbutamol sulfate (Figure 2; Table 5). Thus, although dispersed liposomes produced from proliposomes had 62.1% entrapped salbutamol sulfate, only 6.9%, 10.7%, 10.4%, and 16.1% of salbutamol sulfate delivered to the lower impinger stage was entrapped for the Pari, Omron NE U22, Aeroneb Pro (4 μm), and Aeroneb Pro (8 μm)

Table 4 Entrapment of salbutamol sulfate in soya phosphatidylcholine:cholesterol liposomes dispersed in sodium chloride (0.9%), prepared by proliposome and thin-film methods (mean ± s.d., n = 3)

Method	Entrapment efficiency (%)	Entrapment (mg/100 mg lipid)
Proliposome	62.07 ± 3.07	5.91 ± 0.43
Thin-film	1.24 ± 0.17	0.12 ± 0.01

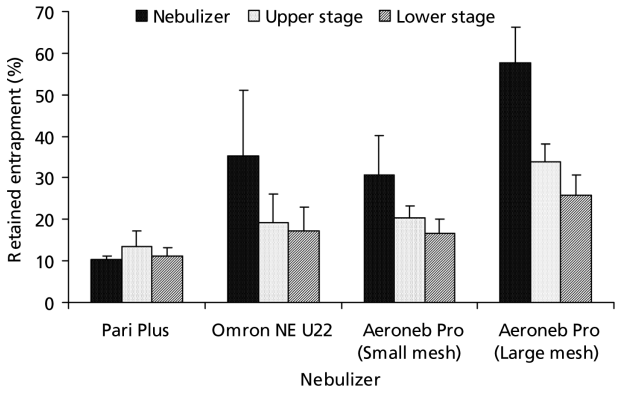

Figure 2 Effect of nebulization on the retained entrapment of salbutamol sulfate in liposomes retained in nebulizers and delivered to the twin impinger (mean ± s.d., n = 3).

Table 5 Liposome-entrapped and unentrapped salbutamol sulfate delivered from nebulizers to the lower stage of the twin-impinger (mean ± s.d., n = 3)

Nebulizer	Entrapped drug (mg)	Unentrapped drug (mg)
Pari LC Plus	0.239 ± 0.036	3.24 ± 0.15
Aeroneb Pro (4 μm)	0.344 ± 0.072	2.97 ± 0.10
Aeroneb Pro (8 μm)	0.263 ± 0.057	1.37 ± 0.02
Omron NE U22	0.380 ± 0.139	3.17 ± 0.21

nebulizers, respectively. The two-stage centrifugation procedure used to separate entrapped and unentrapped salbutamol sulfate may have caused some loss of entrapped drug. However, the large VMDs of liposomes produced by these proliposome formulations (Table 2) and subsequent size reduction during nebulization (Table 3) means that loss of entrapped hydrophilic materials during nebulization is likely. Previously it has been demonstrated that atomization in air-jet nebulizers causes size reduction of large liposomes, and leakage of entrapped hydrophilic material (Taylor et al 1990; Niven et al 1991), as fluid recycling and high shear forces result in air-jet mixing with fluid and possible subsequent disruption to the bilayers of undelivered vesicles. However, for liposome-associated hydrophobic drug, vesicle disruption by jet nebulization has less effect on entrapment (Saari et al 1999) and thus the proliposome formulations developed may have application for delivering such drugs.

There was a trend for all the vibrating-mesh nebulizers to lose less entrapped drug in the nebulizer and impinger stages. The lower stage of the impinger is most important since it collects the aerosol in the fine particle fraction, which is predicted to be 'therapeutically useful' for this particular drug. Compared with the air-jet nebulizer (Pari), the Omron and Aeroneb Pro (4 μm) devices showed a trend of less loss of the previously entrapped drug, with no statistical differences being detected ($P > 0.05$) (Figure 2). However, the large mesh Aeroneb Pro (8 μm) delivered liposomes to the lower impinger stage with a higher retained entrapment ($P < 0.05$) than the air-jet nebulizer (Figure 2). Thus, the method of aerosol generation may influence the extent of liposome disruption, with extrusion of liposome dispersion through the mesh pores of the vibrating-mesh devices showing a trend of less disrupting effect on vesicles compared with the air-jet and baffling system within the Pari nebulizer. The strategy of employing the customized vibrating-mesh nebulizer with large mesh apertures (i.e. Aeroneb Pro, 8 μm mesh) provided an enhanced retained entrapment of salbutamol sulfate compared with the conventional method of delivering liposomes (i.e. jet nebulization).

Although nebulization resulted in marked loss of the entrapped salbutamol sulfate, drug doses delivered by all nebulizers to the lower stage of the impinger were therapeutically relevant when this proliposome approach was used (Table 5). This reveals the superiority of the ethanol-based proliposome method over the conventional film hydration method, which provided very low entrapment of the drug even without applying any nebulization procedure (Table 4). However, in spite of the advantages provided by this approach to liposome formulation, further work is required to enhance the stability to nebulization of liposomes entrapping hydrophilic materials, generated from these proliposomes.

Conclusions

An ethanol-based proliposome approach to formulation provided a simple means of generating isotonic liposome preparations that were deliverable to a twin impinger using air-jet or vibrating-mesh nebulizers. Unlike the air-jet nebulizer, the formulation had a large effect on the performance of all vibrating-mesh nebulizers. The proliposome method provided a greater entrapment of the hydrophilic drug salbutamol sulfate than the traditional film-hydration method. All the nebulizers studied produced aerosol droplets predicted to deliver deep into the airways (O'Callaghan & Barry 1997), however they all caused liposomes disruption and a concomitant leakage of the entrapped hydrophilic drug. This was less for all the vibrating-mesh nebulizers than the jet nebulizer. The use of a large aperture size mesh nebulizer increased the proportion of entrapped drug delivered to the lower impinger stage, but total output and phospholipid output tended to decrease. Thus, this study has demonstrated the potential of using an ethanol-based proliposome approach to generating liposomes for pulmonary administration, and that the relatively new vibrating-mesh nebulizers offer advantages for liposomal delivery compared with a conventional jet nebulizer, in terms of liposome physical stability and output rate. The current proliposome formulation may be appropriate for delivery of hydrophobic drugs such as beclometasone, where stability of liposomes to nebulization may not be essential (Saari et al 1999). Further work is required to reduce the amount of entrapped hydrophilic

drug lost on nebulization, if the system is to be optimized for clinical applications.

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