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The stability of liposomes to ultrasonic nebulisation

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Abstract

The stability of multilamellar liposomes to aerosolisation with an ultrasonic nebuliser has been determined by analysing the size of liposomes deposited in a two-stage impinger and those remaining in the nebuliser reservoir. The size of aerosols produced from egg phosphatidylcholine liposomes was independent of concentration, whilst increasing the concentration of egg phosphatidylcholine/cholesterol liposomes resulted in a decrease in aerosol size. The size of liposome remaining in the nebuliser reservoir during nebulisation decreased, most markedly for egg phosphatidylcholine liposomes. There was a good correlation between the size of egg phosphatidylcholine liposomes deposited in each stage of the impinger and the size of aerosols collected in that stage. The behaviour of cholesterol containing liposomes was less predictable in the early stages of nebulisation, possibly due to vesicle aggregation. In the latter stages of nebulisation, the size of liposomes deposited on either stage of the impinger was independent of the size of aerosol droplets. Copyright © 1996 Elsevier Science B.V.

Keywords: Aerosol; Impinger; Liposome; Nebuliser; Stability; Ultrasonic

1. Introduction

Studies of liposomes for pulmonary drug delivery, have shown that liposomes deposited in the peripheral airways are retained for prolonged periods of time (Farr et al., 1985; Barker et al., 1994). This may result in prolonged drug presence in the airways giving sustained plasma levels of drug (Taylor et al., 1989), localised drug action in the respiratory tract (Juliano and McCullough, 1980), and decreased incidence of systemic adverse effects (McCalden et al., 1989).

All reported studies of liposome delivery to the human lung have employed jet nebulisers (Farr et al., 1985; Taylor et al., 1989; Barker et al., 1994; Vidgren et al., 1995). Nebulisers have thus far been preferred to the alternative systems for drug delivery, namely metered dose in-

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halers and dry powder inhalers, because they are capable of delivering large dose volumes and the liposomes can be produced by conventional techniques and usually require no further processing except removal of unentrapped drug where appropriate (Taylor and Farr, 1993). However, jet nebulisers, which use compressed gas to generate an aerosol from aqueous solutions or suspensions, may structurally damage some liposome formulations due to the shearing forces and recycling of liquid that occurs within the nebuliser (Niven and Schreier, 1990; Taylor et al., 1990). The major determinants of liposome stability to jet nebulisation are the size of the liposomes, relative to the size of the aerosolised droplets (Taylor et al., 1990; Niven et al., 1991) and the air pressure employed to generate the aerosol (Niven et al., 1992).

Ultrasonic nebulisers are less widely used than jet nebulisers for drug delivery. With these devices, the energy necessary for the atomisation of liquids is generated by high frequency vibrations in a piezoelectric crystal, which produce a fountain of droplets in the nebuliser chamber, of which the smallest are expelled and inhaled by patients. Operation of ultrasonic nebulisers causes an increase in the liquid reservoir temperature by up to 15°C (Taylor and Hoare, 1993). This can cause chemical breakdown of heat labile materials such as ^{99m}Tc-DTPA (Waldman et al., 1987). Ultrasonic nebulisers have thus generally been avoided for the delivery of liposomes. Barber and Shek (1989) have reported that egg phosphatidylcholine (EggPC) liposomes, with a mean size of 281 nm or smaller were stable to nebulisation in a DeVilbiss Ultra-Neb 99 ultrasonic nebuliser. However, although small dipalmitoylphosphatidylcholine (DPPC) liposomes were stable to nebulisation, vesicles of 499 nm increased in size within the nebuliser reservoir, suggesting fusion of vesicles, which would result in loss of entrapped hydrophilic materials.

In this study the effect of nebulisation on the size of liposomes delivered from and those remaining in an ultrasonic nebuliser were investigated.

2. Materials and methods

2.1. Materials

Unless otherwise stated materials used in this study were AnalaR grade and obtained from BDH (UK). Egg phosphatidylcholine (EggPC; about 90%, Merck, Germany) was purified chromatographically as described by Bangham et al. (1974). Cholesterol (Chol, 99 + %) was obtained from Sigma Chemical Co. Ltd (UK). Water was deionised (Whatman WR50 RO/Deioniser, Whatman UK).

2.2. Preparation of liposomes

The required amount of phospholipid, with equimolar quantities of cholesterol when required, was weighed into a quickfit round bottomed flask and dissolved in a small volume of chloroform. Organic solvent was removed at reduced pressure, on a rotary evaporator at 40°C, to form a thin film on the inner wall of the flask. Deionised water was added at 40°C and the flask maintained at that temperature for 30 min to hydrate the phospholipid film. The flask was then shaken on a mechanical agitator for 10 min, then allowed to stand in a water bath at 40°C for a further 30 min. This method resulted in the production of multilamellar liposomes (MLVs). EggPC and EggPC/Chol liposomes were produced with lipid concentrations of 2.5, 10, 20 and 40 mg lipid component per ml of aqueous phase.

The mean liposome size was reduced to approximately 5 μm by repeated extrusion through polycarbonate membrane filters (Nucleopore Inc., USA, pore size 5 μm) held in 25 mm holders. The size distribution of liposomes in this and subsequent stages of the study, was determined by laser diffraction analysis using a 63 mm lens (Malvern 2600c, Malvern Instruments, UK). The instrument's software gives the volume median diameter (VMD) and the size distribution is expressed as a span value [(90% undersize-10% undersize)/50% undersize].

2.3. Characterization of liposomal aerosols produced by Medix Electronic Nebuliser

Aerosols were produced from 5 ml of EggPC or EggPC/Chol liposomes (lipid concentration 10 mg/ml) atomised from the Medix Electronic ultrasonic nebuliser (Medix, UK) operated at its highest power setting. Aerosol size was measured with a laser diffraction particle size analyser (Malvern 2600c, Malvern instruments, UK). The nebuliser was clamped 25 mm from the centre of the laser beam, and adjusted so that the aerosol traversed the beam at a distance of 5 mm from the 63 mm lens. The liposomes were nebulised and sized for a total of 10 min. At 0, 5 and 10 min, 100 μl samples of liposomes were removed from the nebuliser reservoir and their VMD and span values determined by laser diffraction.

2.4. Characterization of liposomes delivered in aerosols produced by Medix Electronic nebuliser

The aerosol delivered from the Medix Electronic nebuliser was collected by directing the aerosol into a two stage (twin) impinger (TI), a device used routinely for the characterisation of aerosols (Hallworth and Westmoreland, 1987). Aerosols were generated from 5 ml of EggPC or EggPC/Chol liposomes (lipid concentration 10 mg/ml) for 5 min ($t = 0$–5 min) and the aerosol drawn through the TI at 60 l/min by means of a vacuum pump. Alternatively, the nebuliser was run for 5 min (without aerosol being directed into the TI) and then the aerosol was directed into the TI for a further 5 min (i.e. effectively $t = 5$–10 min). The size distribution of deposited liposomes in both stages of the TI was determined by laser diffraction analysis.

3. Results and discussion

3.1. Effects of liposome concentration and duration of nebulisation on aerosol size

For all concentrations of EggPC and EggPC/Chol liposomes, the mean size of the aerosol droplets increased with duration of nebulisation

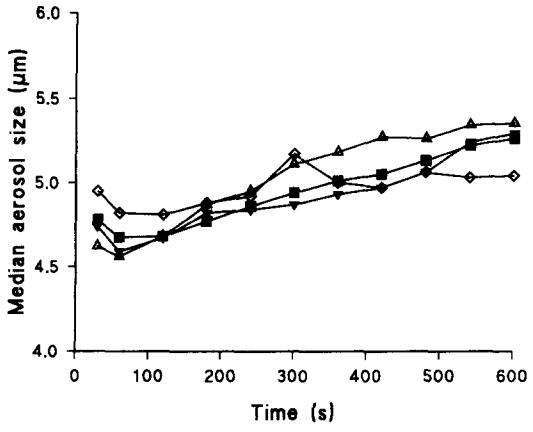

Fig. 1. Median aerosol size-time profiles for EggPC liposomes nebulised in the Medix Electronic nebuliser at 2.5 mg/ml (△), 10 mg/ml (■), 20 mg/ml (▼) and 40 mg/ml (◇). Each point is the mean of four experiments.

(Figs. 1 and 2 respectively). This contrasts with a previous study of dilute suspensions of monodispersed latex spheres (mean sizes ranging from 0.605 to 11.90 μm) of comparable concentrations, atomised with the same nebuliser, which showed no time dependent behaviour (McCallion et al., 1996). The reason for the increase in aerosol size seen in this study with time is unclear, but may be due to the nebulisation of vesicles of approximately 5 μm from a nebuliser producing droplets of approximately 5 μm or due to changes

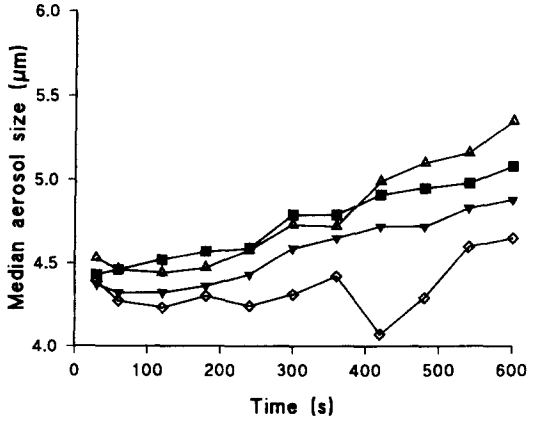

Fig. 2. Median aerosol size-time profiles for EggPC/Chol liposomes nebulised in the Medix Electronic nebuliser at 2.5 mg/ml (△), 10 mg/ml (■), 20 mg/ml (▼) and 40 mg/ml (◇). Each point is the mean of four experiments.

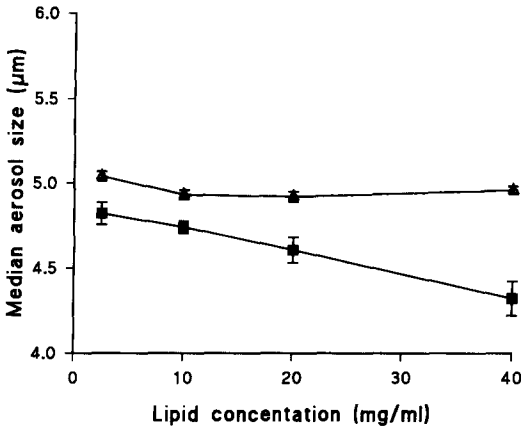

Fig. 3. Median size of aerosols produced over 10 min from varying concentrations of EggPC (△) and EggPC/Chol (■)liposomes. Each point is the mean (± S.E.) of four experiments.

in the liquid viscosity with time due to changes in liposome size or warming of the liquid during nebulisation. With the highest liposome concentration, particularly for the EggPC/Chol formulation (Fig. 2), the aerosol output from the nebuliser, exhibited greater variability. Attempts to nebulise concentrations of 80 mg/ml resulted in a considerably variable and sporadic output and thus were not further investigated. This effect can be related to the viscosity of the formulations being nebulised, since the viscosity of EggPC and EggPC/Chol liposomes increase linearly with concentration (Bridges et al., 1995a) and ultrasonic nebulisers have been reported to be inefficient at delivering high viscosity liquids (McCallion et al., 1995).

The mean volume median diameters of the aerosols produced over 10 min from all liposome preparations were calculated to be between 4.3 and 5.1 μm (Fig. 3). For the EggPC liposome formulations the VMD of the aerosol was independent of lipid concentration. However, for EggPC/Chol liposomes, a significant decrease ($P < 0.05$) in aerosol size occurred when concentrations greater than 10 mg/ml were employed. These results contrast with those found when liposomes of the same size and lipid composition where nebulised from Cirrus, Pari-LC and Sidestream air-jet nebulisers (Bridges et al., 1995b).

Table 1
Size distribution of liposomes remaining in the nebuliser reservoir following nebulisation in a Medix Electronic nebuliser ($n = 3$)

	EggPC	EggPC/Chol
Mean size (± S.E.) of liposomes at $t = 0$ min	5.17 (0.02)	4.71 (0.10)
Mean size (± S.E.) of liposomes at $t = 5$ min	1.20 (0.04)	3.75 (0.06)
Mean size (± S.E.) of liposomes at $t = 10$ min	0.97 (0.05)	4.11 (0.13)

With these devices, there was a direct relationship between concentration and aerosol size for both formulations, with droplet size increasing as concentration was increased, but no significant difference between the two lipid formulations at a particular concentration.

3.2. Effect of nebulisation on the size distribution of liposomes remaining in the nebuliser reservoir

For EggPC liposomes there was a marked reduction in vesicle size throughout the period of nebulisation (Table 1). However, the liposomes containing cholesterol showed less marked reduction in size. The effects on the complete size distribution for EggPC liposomes as measured by laser diffraction are shown in Fig. 4. At $t = 0$ s,

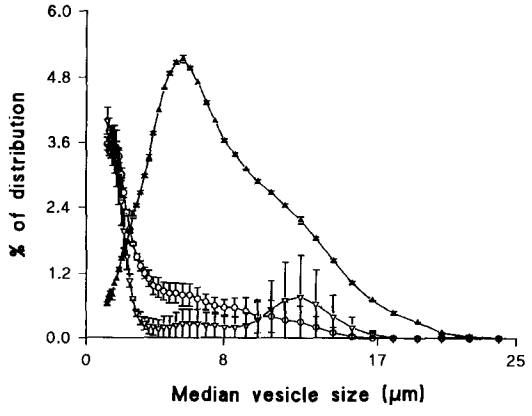

Fig. 4. Changes in the size distribution of EggPC liposomes remaining in the nebuliser reservoir at $t = 0$ s (▲), $t = 300$ s (○) and $t = 600$ s (▽). Each point is the mean (± S.E.) of three experiments.

the VMD was 5.2 μm, with a broad size distribution. The VMD reduced to 1.2 μm as the complete distribution of sizes is shifted to smaller sizes after 5 min and is further reduced to 0.97 μm at 10 min. At these latter times, a significant proportion of vesicles are below the lower limit of detection of the Malvern 2600c laser diffraction analyser (0.5 μm) and thus the quoted figures for median diameter must be treated with caution. However, the overall shift to smaller sizes is clear (Fig. 4). These results can be compared with those of Barber and Shek (1989) who studied the stability of EggPC and DPPC liposomes to ultrasonic nebulisation. They reported that small EggPC and DPPC liposomes sized in the reservoir of the nebuliser were unchanged during nebulisation, but that larger DPPC liposomes increased in size. EggPC has a main phospholipid gel to liquid crystalline temperature of -5 to -15°C. Thus, throughout this study, conducted at ambient temperature, the phospholipid bilayers of EggPC liposomes were in the relatively fluid, liquid crystalline state. Such liposomes have previously been found to be particularly susceptible to damage in jet nebulisers (Bridges et al., 1995b). The results of this study indicate that although ultrasonic and jet nebulisers operate on very different principles and produce aerosol droplets of different sizes (approximately 5 μm for this ultrasonic nebuliser, 2–3.5 μm in the study of Bridges et al., 1995b), EggPC liposomes are similarly affected by both nebulisation processes. Thus, the predominant effect on ultrasonically nebulised EggPC liposomes is to disrupt the liposome structure in the same way that ultrasonic probe sonication, above the phospholipid phase transition temperature, can be used to reduce the liposomal size and number of bilayers. During ultrasonic nebulisation droplets are formed from capillary waves generated at the surface of the liquid (Lang, 1962), from hydraulic shocks produced by imploding cavitation bubbles waves (Gerhenzon and Eknadiosyants, 1964) near the liquid surface or from a combination of these two processes (Boguslavskii and Eknadiosyants, 1969). Any of these processes, combined with the constant recycling of fluid within the nebuliser might be expected to physically disrupt relatively fluid EggPC vesicles. Interestingly, at 10 min there

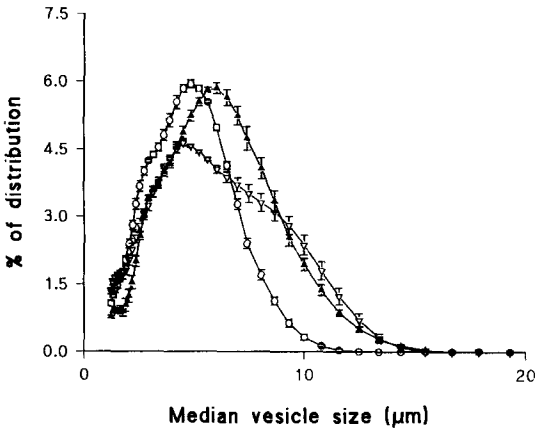

Fig. 5. Changes in the size distribution of EggPC/Chol liposomes remaining in the nebuliser reservoir at $t=0$ s (▲), $t=300$ s (○) and $t=600$ s (▽). Each point is the mean (± S.E.) of 3 experiments.

is a small subpopulation of vesicles with a mean size of approximately 13 μm, which suggests that after a prolonged time, when the reservoir temperature is at its highest, some aggregation and fusion of liposomes may be occurring, as suggested by the study of Barber and Shek (1989).

Compared to EggPC liposomes, EggPC/Chol liposomes showed a less marked change in size during nebulisation, though a shift to a smaller mean size was evident (Table 1 and Fig. 5). Inclusion of 50 mol% cholesterol into liposomal bilayers, as used is this study, has been reported to eliminate the sharp phospholipid gel to liquid crystalline phase transition and modulates the fluidity of bilayers, imparting rigidity to the bilayer that restricts the movement of the phospholipid hydrocarbon chains above their transition temperature (Ladbrooke et al., 1968). The rigidity imparted to the bilayers seems to make them more resistant to the disruptive forces to which liposomes are expose on ultrasonic nebulisation. A previous study has shown that rigid DPPC/Chol liposomes were more resistant to shearing in jet nebulisers than more fluid EggPC liposomes of comparable size (Bridges et al., 1995b). Disruption of liposome bilayers during nebulisation is particularly important for hydrophilic drug entrapped in the liposomal aqueous phase, since bilayer disruption can result in drug release (Tay-

lor et al., 1990). However, it is likely that such disruption will be less significant for hydrophobic materials incorporated into liposomal bilayers (Taylor and Farr, 1993).

3.3. Effect of nebulisation on the size distribution of liposomes deposited in a two-stage impinger

The cut off diameter for stage two of the TI is 6.4 μm, thus liposomes deposited in the upper stage of the TI were aerosolised in droplets having an aerodynamic diameter greater than 6.4 μm and those in the lower stage in droplets less than 6.4 μm. The median size of aerosols produced by the Medix Electronic nebuliser in this study varied between the extremes of 4.1 and 5.5 μm. By definition 50% of the aerosol volume is greater than this size, and the widely distributed nature of nebuliser aerosol clouds (mean span values were 1.57 and 1.53 for 10 mg/ml EggPC and EggPC/Chol aerosols respectively) means that a proportion of the aerosol will be greater than 6.4 μm and will thus be collected in the upper stage of the TI.

In the first 5 min of nebulization the mean size of EggPC liposomes collected in the upper and lower chamber of the impinger was 6.70 and 3.91 μm respectively (Table 2), compared with the initial mean size of 5.14 μm. The fractionation of the aerosol within the TI is thus reflected in the size of vesicles deposited on the respective stages, with the smallest vesicles being delivered in the smaller droplets. The same, fractionating effect is seen with the EggPC liposomes collected between 5 and 10 min. However, the size of deposited vesicles on each stage was increased, with the mean size in the upper and lower chamber of the impinger was 7.60 and 5.26 μm respectively (Table 2). This contrasts with the overall decrease in size occurring in the liposomes remaining in the nebuliser (Table 1), but reflects the time dependent increase in aerosol droplet size with time (Fig. 1). A precise explanation of these findings cannot be given, especially since laser diffraction sizing gives a size distribution based on volume, and hence large liposomes will have a much greater influence on the distribution than smaller liposomes. However, the data suggest that larger liposomes are conveyed in the largest droplets of the aerosol output from a nebuliser, whilst smaller liposomes are restricted to the smaller droplets. Over 10 min the mean size of the aerosol increases permitting delivery of the larger liposomes which are still present in the nebuliser chamber.

In the first 5 min of nebulization the mean size of EggPC/Chol liposomes collected in the upper and lower chamber of the impinger was 4.60 and 8.20 μm respectively (Table 2), compared with the initial mean size of 4.71 μm. This is the opposite trend to that found for EggPC alone. However, in this instance the distributions showed a high degree of bimodality. This suggests considerable aggregation of vesicles and thus the mean measured size is a reflection of this aggregation, rather than the size of individual vesicles. Between 5 and 10 min the mean size of EggPC liposomes collected in the upper and lower chamber of the impinger was 3.35 and 3.36 μm respectively (Table 2) with some degree of bimodality, which compare to the size of residual liposomes of 3.75 and 4.11 μm at 5 and 10 min respectively. Thus, for these less 'fluid' liposomes the changes in the residual lipo-

Table 2
Size distribution of liposomes deposited on the upper and lower stages of the two-stage impinger ($n = 3$)

	EggPC	EggPC/Chol
Mean size ($\pm$S.E.) of liposomes in upper stage 0–5 min	6.70 (0.1)	4.60 (0.02)
Mean span of liposomes in upper stage 0–5 min	1.64	1.62
Mean size ($\pm$S.E.) of liposomes in lower stage 0–5 min	3.91 (0.03)	8.20 (0.04)
Mean span of liposomes in lower stage 0–5 min	1.72	1.48
Mean size ($\pm$S.E.) of liposomes in upper stage 5–10 min	7.56 (0.18)	3.35 (0.01)
Mean span of liposomes in upper stage 5–10 min	1.69	1.56
Mean size ($\pm$S.E.) of liposomes in lower stage 5–10 min	5.26 (0.07)	3.36 (0.01)
Mean span of liposomes in lower stage 5–10 min	1.49	1.57

some size seems to be more important than the changes occurring in the aerosol size with time, and the aerosol droplets deposited in the upper stage of the impinger may have contained one or more liposomes.

4. Conclusions

The size of aerosols produced from EggPC and EggPC/Chol liposomes increased with time and for EggPC/Chol liposomes there was a significant decrease in aerosol size with time. However, all the liposomal aerosols produced in this study had a median size of between 4.1 and 5.5 μm and would be expected to have similar regional distributions if they were administered to the human airways. Thus, within the limitations of the formulations tested aerosol size is relatively insensitive to formulation changes as with jet nebulisers (Barker et al., 1994) and is much more dependent on the design of nebuliser used for delivery. The size of liposome remaining in the nebuliser reservoir during ultrasonic nebulisation decreased for both formulations, but this decrease was much more marked for EggPC liposomes. Reduction of vesicle size is indicative of vesicle break up during the nebulisation process and has previously been correlated with the loss of entrapped hydrophilic materials from liposomes in jet nebulisers (Niven and Schreier, 1990; Taylor et al., 1990). Inclusion of cholesterol in liposomes above the phospholipid phase transition temperature would thus appear desirable to promote vesicle stability during ultrasonic nebulisation. There was a good correlation between the size of EggPC liposomes deposited in the impinger and the size of aerosols collected on each stage. Aerosolised EggPC/Chol liposomes collected in the early stages of nebulisation showed evidence of aggregation, whilst in the latter stages the size of liposomes deposited in the impinger was independent of the size of aerosol droplets and was smaller than the initial size. The results of this study indicate that large liposomes can be delivered from ultrasonic nebulisers and that although some disruption of vesicles occurs, cholesterol containing formulations seem most appropriate for further study.

Acknowledgements

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