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Delivery of liposomes generated from proliposomes using air-jet, ultrasonic, and vibrating-mesh nebulisers

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In this study, particulate-based proliposomes were investigated as a formulation which would form an isotonic liposome dispersion in situ *when nebulised within a medical nebuliser. Transmission electron microscopy and particle size analysis indicated that liposomes were generated from soya phosphatidylcholine/cholesterol/sucrose proliposomes in air-jet (Pari LC Plus), ultrasonic (Liberty) and vibrating-mesh (Omron NE-U22) nebulisers, without the need for a prolonged hydration step. With jet and vibrating-mesh nebulisers, liposomes were efficiently delivered to the second (lower) stage of a two-stage impinger, indicating such liposomes would be predicted to be delivered to the peripheral airways. Phospholipid output from these nebulisers was less than the total mass output, suggesting some accumulation of liposomes in the nebuliser reservoir. The ultrasonic nebuliser delivered less than 6% of available phospholipid to the impinger, demonstrating that this device was inappropriate for delivering a therapeutic proliposome formulation for pulmonary administration. These results show that proliposomes offer a means of producing stable formulations which can readily generate isotonic liposome formulations* in situ *for delivery to the airways using either an air-jet or vibrating-mesh nebuliser.*

Key words: Impinger – Liposome – Nebuliser – Phospholipid – Proliposome.

Nebulisers have been demonstrated to be suitable for the pulmonary administration of liposomes [1-3]. They are simple to use, and capable of delivering a large volume of therapeutic aerosol to sites deep within the lung. Unlike pressurized metered dose inhalers or dry powder inhalers, nebulisers can aerosolize liposomes prepared using established techniques without requiring additional processing.

In air-jet or jet nebulisers, compressed gas is used to convert a liquid (usually an aqueous solution) into a spray [4]. The jet of high velocity gas is passed through a narrow venturi nozzle and an area of negative pressure, where the air jet emerges, draws liquid from a reservoir up a feed tube. The fine filaments of fluid collapse into droplets as a result of surface tension. A small proportion of the resultant (primary) aerosol leaves the nebuliser directly, whilst large droplets impact on baffles or the walls of the nebuliser chamber and are recycled into the reservoir fluid.

Ultrasonic nebulisers use a piezoelectric crystal transducer, vibrating at a high frequency, to produce a fountain of liquid at the liquid surface. Large droplets are emitted from the fountain's apex and small droplets are produced from the lower part. As with jet nebulisers, large droplets are recycled and small droplets released as the inhalable aerosol [4].

Both jet [1-3] and ultrasonic nebulisers [5] have previously been used for liposome delivery, and their performance (e.g. droplet size, nebulisation time, output, etc.) may vary greatly as a result of differences in design and construction as well as changes in the physicochemical properties of the nebuliser fluid. The ideal aerosol characteristics depend on a number of factors, but the consensus is that nebulised droplets with a mean size less than approximately 5 μm are generally considered necessary for drug delivery to the respiratory lung regions [4], whilst aerosol output rate is also an important consideration. Recently, a third type of nebuliser has become commercially available, namely vibrating-mesh nebulisers. These devices employ a vibrating-mesh or plate with multiple apertures, through which liquid is passed to form a fine, low velocity aerosol over short nebulisation times. In some nebulisers, the plate or mesh itself vibrates, moving up and down by a few micrometres, extruding fluid through the apertures to generate the aerosol. In the device employed in this study (Omron NE-U22), high frequency vibrations of a piezoelectric crystal are transmitted to a transducer horn, which is in contact with liquid to be atomised. Movement of the horn induces a "passive" vibration of the mesh plate, through which the liquid passes to produce an aerosol [6].

Whilst liposomes have potential as drug carriers to the airways, solubilizing hydrophobic drugs [3], providing a sustained release of drug [2] or delivering genetic material into airways cells [7], they are relatively unstable colloidal systems. Chemical instability, particularly hydrolysis and oxidation accelerates their breakdown, whilst physical instability results in aggregation and fusion, with associated changes in vesicle size and release of entrapped hydrophilic materials.

Stable, dried liposomal powders can be produced by freeze-drying. Depending on the lipid composition [8] and/or the inclusion of a lyoprotectant such as trehalose [9], such liposomes, following rehydration, can be successfully delivered from nebulisers with a high fine particle fraction. Proliposomes offer an alternative to freeze-drying, for the formulation of liposomes as a stable powder. Proliposomes comprise soluble free-flowing carrier particles coated with phospholipids, which generate liposomes on addition of aqueous phase [10, 11].

This work investigated whether proliposomes could produce potentially inhalable liposomes when hydrated in, and delivered from three commercially available medical nebulisers having different operating principles.

I. MATERIALS AND METHODS

1. Materials

Sucrose, sodium chloride, and chloroform (AnalaR grade) were supplied by BDH Laboratory Supplies, United Kingdom. Cholesterol was supplied by Sigma-Aldrich, United Kingdom. Phosphotungstic acid (analytical reagent grade) was purchased from TAAB Laboratories Equipment, United Kingdom. Soya phosphatidylcholine (Lipoid S-100) was a gift from Lipoid, Germany.

All reagents were used as received.

2. Methods

2.1. Preparation of proliposomes

A pear-shaped glass flask containing sucrose particles (300-500 μm) was attached to a modified rotary evaporator (Büchi Rotavapor, Switzerland) under vacuum at 40°C. A solution (60 mg/ml) of soya phosphatidylcholine and cholesterol (1:1 mole ratio) in chloroform was added portion-wise via a feed-line and then the chloroform was removed under vacuum for 30 min at 40°C to produce proliposomes with lipid:sucrose weight ratio 1:5.

2.2. Determination of liposome formation and size on nebulisation

Proliposomes (0.56 g) were added to 5 ml deionised water at ambient temperature in a Pari LC Plus (air-jet) nebuliser (Pari GmbH, Germany) with Pari Master compressor (Pari GmbH, Germany), Liberty (ultrasonic) nebuliser (Clement Clarke International, United Kingdom), or Omron NE-U22 (vibrating-mesh) nebuliser (Omron HealthCare, United Kingdom). The resultant dispersion was an isotonic formulation containing 15 mg/ml lipid. The Omron was shaken for 1 min before nebulisation to "dryness" (approx. 30 min). Both Pari and Liberty nebulisers were operated to "dryness" (approx. 25 and 12 min, respectively) without shaking. Samples were taken from the reservoir for particle size determination by laser diffraction (Malvern Mastersizer, Malvern Instruments, Malvern, United Kingdom). The instrument's software expresses particle size as the volume median diameter (VMD) and the size distribution is expressed as a Span value [(90% undersize-10% undersize)/50%undersize]. Further samples were taken for visualization by transmission electron microscopy (TEM) throughout nebulisation to dryness.

2.3. Transmission electron microscopy (TEM) analysis

Samples for TEM analysis were deposited on copper grids (400 mesh), negatively stained with phosphotungstic acid (1% w/v aqueous solution) and viewed using a Philips CM 120 BioTWIN microscope (Phillips Electron Optics BV, The Netherlands).

2.4. Determination of the size distribution of generated aerosols

Aerosols produced from the proliposomes were drawn through the laser of a Malvern 2600c (Malvern Instruments, Malvern, United Kingdom) diffraction size analyzer, using a 63-mm lens, and the size distribution of droplets of the aerosol up until dryness was determined.

2.5. Determination of liposome size in the two-stage impinger

The two-stage impinger [12] also known as the Single Stage Glass Impinger (USP) and the twin impinger was set up with a flow rate of 60 l/min, and sodium chloride (0.9% w/v aqueous solution) was used as the collection medium. Following addition of water, proliposomes were nebulised to dryness and samples were taken from both stages of the impinger to measure particle size using laser diffraction (Malvern Mastersizers, Malvern Instruments, Malvern, United Kingdom) and for TEM.

2.6. Mass and phospholipid output

Nebulisers were weighed before and after nebulisation to dryness of the proliposome dispersions to determine % mass output. A phospholipid assay [13] was employed to analyze the phospholipid output from the nebulisers and the distribution of phospholipid within the impinger following nebulisation to dryness.

II. RESULTS AND DISCUSSION

1. Generation of liposomes from proliposomes

Hydration in the nebuliser, and during 1 min shaking within the Omron nebuliser, together with the energy generated within devices for atomization resulted in the production of multilamellar vesicles (MLVs) which were seen in samples taken at all time intervals from each nebuliser (*Figure 1*).

Laser diffraction size analysis indicated a polydispersed size distribution, with evidence of deaggregation and size reduction of liposomes during nebulisation. The measured VMD and Span values of the liposome dispersions in each nebuliser were initially large (*Table I*). The procedure employed allowed a very short time for dissolution of the sucrose carrier particles, hydration of the phospholipids film coating those particle and conversion of the hydrated film to vesicles for aerosol delivery. TEMs however showed no particles of undissolved sucrose to be present, and that liposomes were aggregated. Thus, the large VMDs and Spans measured related to aggregates which may form due to the initial high local concentrations of hydrated and hydrating lipid, particularly in the case of the ultrasonic and vibrating-mesh nebulisers, where there was less agitation to aid dispersion. However, as for liposomes prepared using conventional methodologies aggregates/vesicles present within the nebuliser fluid were reduced in size by shearing as nebulisation progressed [14-17], such that the measured VMD was significantly reduced ($p < 0.05$) for each nebuliser during the first 5 min of nebulisation (*Table I*). Nonetheless, Span values remained high, indicating a wide size distribution and suggesting that the liposomes in the nebuliser were still aggregated, after 5 min atomization.

At the end of nebulisation, the mean measured size was greater in the fluid remaining within the nebulisers than in the impinger (*Table II*), suggesting vesicle aggregation in the nebuliser, and vesicle size reduction during delivery of the liposomes in nebulised aerosol droplets to the impinger. There was no significant difference ($p < 0.05$) in the VMD of liposomes delivered to the first and second stages of

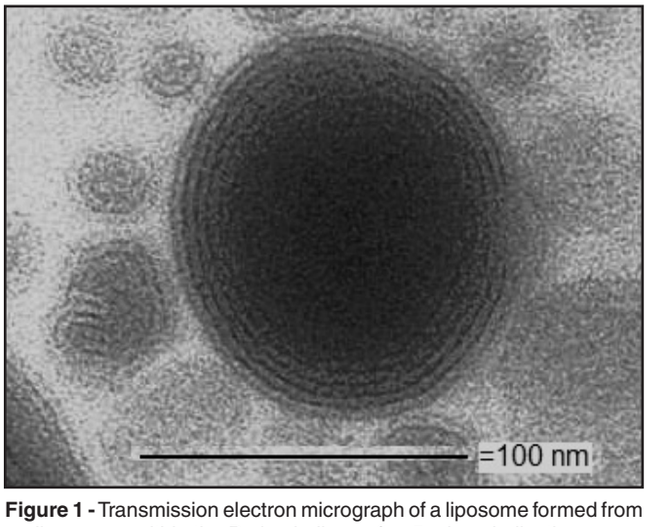

Figure 1 - Transmission electron micrograph of a liposome formed from proliposomes within the Pari nebuliser, after 5 min nebulisation.

Table I - The size of liposome dispersions generated from proliposomes in the 3 nebulisers (mean ± SD, n = 3).

Nebulisation time (min)	Pari		Liberty		Omron	
	VMD (μm)	Span	VMD (μm)	Span	VMD (μm)	Span
1	9.04 ± 4.0.6	9.44 ± 8.54	64.29 ± 37.01	5.47 ± 0.84	49.93 ± 9.63	3.54 ± 0.44
5	5.42 ± 0.04	1.76 ± 0.18	8.75 ± 1.67	6.91 ± 4.94	12.63 ± 2.19	10.63 ± 0.42