

# **A simple approach to predict the stability of phospholipid vesicles to nebulization without performing aerosolization studies**

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## **Abstract**

Membrane extrusion was investigated for predicting the stability of soya phosphatidylcholine liposomes and surfactosomes (Tween 80-enriched liposomes) to nebulization. Formulations were prepared with or without cholesterol, and salbutamol sulphate (SBS) or beclometasone dipropionate (BDP) were incorporated as model hydrophilic or hydrophobic drugs respectively. Formulations were extruded through 5, 2, 1 and 0.4  $\mu\text{m}$  polycarbonate membrane filters to study the influence of membrane pore size on drug retention by the vesicles. Surfactosomes were found to be very leaky to SBS; such that even without extrusion greater than 50% of the originally entrapped drug was lost, and cholesterol minimized drug losses. The smaller the pore size, the greater the leakage of SBS; hence only around 10% were retained in cholesterol-free surfactosomes extruded through 0.4  $\mu\text{m}$  filters. To study the influence of vesicle size on SBS retained entrapment, an excessive extrusion protocol was proposed (51 extrusion cycles through 1  $\mu\text{m}$  filters) to compare the stability of freshly prepared vesicles (i.e. unextruded; approx. 4.5-6.5  $\mu\text{m}$ ) with those previously extruded through 1  $\mu\text{m}$  pores. Cholesterol was essential for minimizing losses from liposomes, whilst for surfactosomes size reduction prior to extrusion was the only way to minimize SBS losses which reached up to 93.40% of the originally entrapped drug when no cholesterol was included. When extrusion was applied to BDP-loaded vesicles, greater proportions of the drug were retained in the vesicles compared to SBS. Even with extrusion through 0.4  $\mu\text{m}$ , BDP retention was around 50-60% with little effect of formulation. Excessive extrusion showed BDP retention using small liposomes (1 $\mu\text{m}$ ) to be as high as 71-87%, compared to 50-66% for freshly prepared vesicles. The findings, based on extrusion, were compared to studies of vesicle stability to nebulization, published by a range of investigators. It was concluded that extrusion is a valid method for predicting the stability of liposomes to nebulization.

**Keywords:** Aerosol, Drug delivery, Drug development, Extruder, Nebulizer

## 1. Introduction

Inhalation of liposomal drug formulations via nebulization can prolong drug residence within the lung, potentially maximizing therapeutic benefit, whilst reducing systemic adverse effects (Taylor et al., 1989; Saari et al., 1999; Fauvel et al., 2012; Gaspar et al., 2012; Cipolla et al., 2013; Clancy et al., 2013). However, damage of the liposome structures during air-jet nebulization can cause loss of the originally entrapped material; thus liposome stability to nebulization-induced damage should be considered (Taylor et al., 1990b; Niven et al., 1991; Elhissi et al., 2006a; Elhissi et al., 2007; Chadha et al., 2012; Nasr et al., 2013). Loss of entrapped hydrophilic agent can be minimized by reducing liposome size before jet-nebulization (Taylor et al., 1990b; Niven et al., 1991) or by inclusion of cholesterol (Taylor et al., 1990b; Tseng et al., 2007; Chadha et al., 2012) or high phase transition phospholipid (Niven and Schreier, 1990).

Vibrating-mesh nebulizers have revolutionized pulmonary delivery of conventional solutions (Dhand, 2002) and novel drug delivery systems, such as liposomes (Elhissi and Taylor, 2005; Kleemann et al., 2007; Nasr et al., 2013; Cipolla et al., 2014; Lehofer et al., 2014). Compared to air-jet nebulizers, vibrating-mesh devices may cause less damage to liposomal structures and hence higher proportions of the originally entrapped hydrophilic drug can be retained during aerosolization (Elhissi et al., 2006a; Elhissi et al., 2007), especially when the vesicles are extruded to the size of 1  $\mu\text{m}$  prior to nebulization, and by using devices with large mesh apertures (Elhissi et al., 2007). Arikace<sup>®</sup> (Insmmed, NJ, USA), a novel nebulizable liposome formulation of the hydrophilic anti-pseudomonal antibiotic amikacin is currently in phase III trials, and has been demonstrated to be well tolerated by

cystic fibrosis patients, with prolonged drug residence in the lung and enhanced penetration through *pseudomonas aeruginosa* biofilms. The success of this formulation has been attributed to the use of appropriate formulation (cholesterol-enriched dipalmitoylphosphatidylcholine with vesicle size around 300 nm) and suitable inhalation device (Pari e-Flow mesh nebulizer) (Clancy et al., 2013; Ehsan et al., 2014; Waters and Ratjen, 2014).

Unlike hydrophilic drugs, the loss of hydrophobic materials (e.g. steroids) from liposomes during nebulization is dependent on lipid bilayer composition and mode of drug interaction with the bilayers (Darwis and Kellaway, 2001; Elhissi et al., 2006b). For example, beclometasone dipropionate (BDP) inhaled in liposomes showed prolonged retention in the respiratory tract of human volunteers, although liposomes underwent marked size reduction during jet-nebulization from 3.49 to 0.83  $\mu\text{m}$  and from 5.07 to 0.91  $\mu\text{m}$  for dilauroylphosphatidylcholine (DLPC; a low  $T_m$  phospholipid) and dipalmitoylphosphatidylcholine liposomes (DPPC; a high  $T_m$  phospholipid) respectively (Saari et al., 1999). Hence, size reduction (i.e. massive disruption) of the liposomes during nebulization did not cause marked loss of the entrapped BDP. These findings are consistent with *in vitro* studies using BDP (Elhissi et al., 2011) and other hydrophobic drugs, such as ciprofloxacin (Desai et al., 2002). Clinical trials have been conducted with Pulmaquin™ (Aradigm Corp., CA, USA), a nebulizable liposomal formulation of ciprofloxacin for inhalation by non-cystic fibrosis bronchiectasis patients (Cipolla et al., 2013; Serisier et al., 2013).

Studies investigating the physical stability of liposomes during nebulization are usually conducted using nebulizers linked with appropriate aerosol collection systems (e.g.

impingers or impactors), followed by analysis to determine drug losses from aerosolized liposomes (Taylor et al., 1990b; Desai et al., 2002; Elhissi et al., 2007; Kamalporn et al., 2014). This approach is laborious and time-consuming, and loss of aerosol to the surrounding environment is a potential hazard, even with standard aerosol-collection models. It has been reported that aerosolized particles to impingers may bounce from the bottom of the collection compartment or be re-aerosolized with liquid bubbles created by the air flowing into the impinger, causing particles to escape with the effluent air, resulting in reduced aerosol collection efficiency (Grinshpun et al., 2007). Moreover, hydrophobic particles might be poorly collected by the impinger's liquid owing to poor particle wettability. The particle "bouncing" phenomenon and reduced collection efficiency is even more significant with dry collection models such as impactors (Xu et al., 1993). The "bouncing" effect has also been reported to be dependent on formulation, impactor design (Mitchell et al., 2003) and angle of particle deposition (Xu et al., 1993).

In this study, we have proposed a convenient, economical, and environment-friendly approach to predict the stability of liposomes during nebulization without conducting aerosolization studies. This was achieved by performing excessive extrusion through polycarbonate membrane filters. The repetitive shearing provided by extrusion (51 cycles through 1  $\mu\text{m}$  pore filters) aimed to simulate that occurring during nebulization. The extrusion approach was evaluated using salbutamol sulphate (SBS) and beclometasone dipropionate (BDP) as vesicle-entrapped hydrophilic and hydrophobic drugs respectively. Drug retention by liposomes upon extrusion was assessed as a stability indicator, using cholesterol as "rigidity" enhancer (Kirby et al., 1980) and Tween 80 as "fluidity" promoter (Young et al., 1983) of the vesicles. Vesicles made using Tween 80 were referred to as "surfactosomes" and were compared to conventional liposomes. The findings of extrusion

studies were appraised in relation to a range of liposome nebulization studies available in the literature.

## **2. Materials and methods**

### **2.1. Materials**

Soya phosphatidylcholine (SPC; Lipoid S-100) was a gift from Lipoid, Switzerland. Cholesterol, beclometasone dipropionate (BDP) and Tween 80 were purchased from Sigma Aldrich, UK. Sodium chloride (ACS, 99.0%), salbutamol sulphate (SBS, 99%), sodium 1-hexane sulfonate monohydrate (99%) and Triton X-100 were all purchased from Alfa Aesar, UK. Glacial acetic acid, chloroform (stabilized with ethanol), water (HPLC-grade) and methanol (HPLC-grade) were all supplied by Fisher Scientific, UK. Ferric chloride and Ammonium thiocyanate were purchased from VWR, UK, and Deuterium oxide (D<sub>2</sub>O; NMR-grade) was purchased from Acros organics, UK.

### **2.2. Methods**

#### **2.2.1. Preparation of liposomes and surfactosomes**

SPC alone or with cholesterol (1:1 mole ratio) were used to prepare liposomes by dissolving the lipids in chloroform (20 mg/ mL) within a round bottomed flask. The organic solvent was removed using a rotary evaporator (Büchi Rotavapor R-215, Büchi, Switzerland) under vacuum for 1 h in a water bath at 37°C using the maximum rotation speed (280 rpm). The resultant thin lipid film was hydrated by adding SBS dissolved in 1 mL NaCl (0.9%) solution followed by manual shaking. The dispersion was left to anneal for 15 min before

further dilution with drug-free NaCl (0.9%) solution, followed by vigorous hand shaking to give a lipid concentration of 15 mg/mL, and further annealing was allowed for 2 h at room temperature. In other batches, the same procedure was repeated by inclusion of BDP in the lipid phase (2.5 mole% of lipid) and the same hydration procedure was followed, using D<sub>2</sub>O for hydration. Surfactosomes were prepared by using SPC with or without cholesterol (1:1 mole ratio) with Tween 80 (15% w/w of total lipid). The lipid and surfactant were dissolved in chloroform in a round-bottomed flask to give a lipid concentration of 20 mg/mL. Following organic solvent removal, the thin film was hydrated as described above using either SBS (15mg/mL; added with the aqueous phase) or BDP (2.5 mole% incorporated into the lipid phase).

### **2.2.2. Extrusion of formulations**

Avestin Liposofast Mini-extruder (GC technologies, UK) was employed to extrude liposomes or surfactosomes using Nucleopore Track-etched polycarbonate membrane filters with pore sizes: 5 µm (11 cycles), 2 µm (11 cycles), 1 µm (7 cycles) and 0.4 µm (7 cycles) (Nucleopore, UK). The number of cycles was chosen based on preliminary optimization experiments to ensure the desired vesicle size was achieved.

### **2.2.3. Vesicle size analysis**

The size distribution of liposomes and surfactosomes was determined by laser diffraction (Malvern Mastersizer 2000, Malvern Instruments Ltd, UK) with the polydisperse mode of analysis. Volume median diameter (VMD; 50% undersize) and Span were recorded. Span is a term, used by Malvern Instrument software to express the polydispersity of particles. Mathematically,  $\text{Span} = (90\% \text{ undersize} - 10\% \text{ undersize}) / \text{VMD}$ .

#### **2.2.4. Determination of SBS entrapment in liposomes and surfactosomes**

Vesicle-entrapped SBS was separated from unentrapped drug by centrifugation (Beckman ultracentrifuge, L8-80M, USA) at 55,000 rpm for 35 min at 6°C. The supernatant was collected to quantify the unentrapped (free) drug. Triton X-100 solution was added to the pellet of liposomes or surfactosomes to disrupt the vesicles and release the entrapped drug for quantification via HPLC. The mobile phase comprised an aqueous solution of sodium 1-hexane sulfonate (5 mM) and methanol (75:25 v/v) with glacial acetic acid added to constitute 1% of the total mobile phase volume. The Agilent 1200 HPLC instrument (Agilent, USA) was set up using a C18 column (Eclipse XDB-C18, 4.6 x 150 mm, Agilent, UK) with mobile phase flow rate 1 mL/min. The temperature was set at 40°C and UV wavelength at 276 nm (Elhissi et al., 2006a).

#### **2.2.5. Determination of BDP entrapment in liposomes and surfactosomes**

Deuterium oxide (D<sub>2</sub>O; density = 1.053 g/mL) was used to separate entrapped and unentrapped BDP, by adapting the method of Batavia et al. (2001). Vesicles containing BDP were separated from free BDP crystals by centrifugation using D<sub>2</sub>O as dispersion medium. The sample was centrifuged within Eppendorf tubes at 13,000 rpm (relative centrifugal force: 15,300 x g) for 90 min at room temperature using a bench-top centrifuge (Jencons-PLS, Spectrafuge 24D, Jencons Scientific Ltd., UK). Following centrifugation, the floating “creamy” layer comprised the lipid vesicles (and entrapped drug), was aspirated and dissolved using methanol to release entrapped BDP for quantification by HPLC. Centrifugation caused BDP crystals to sediment in the bottom of the tubes. The supernatant was separated, and the sedimented BDP was dissolved in methanol and aspirated for quantification of unentrapped drug using HPLC. Methanol and water (3:1 v/v) constituted

the mobile phase, and the flow rate was set at 2 mL/min with a sample injection volume of 50  $\mu$ L and UV detection at 238 nm (Nasr et al., 2014).

### **2.2.6. Validation of separation of entrapped BDP using phospholipid assay and light microscopy**

To ensure that separation of entrapped BDP via centrifugation using D<sub>2</sub>O was effective, phospholipid was quantified in the floating layer, the sedimented spot, and the aqueous phase between the sediment and the floating layer, using the Stewart assay (Stewart, 1980) following the method designed by Elhissi and Taylor (2005). Liposome samples were placed into glass centrifuge tubes and ethanol was added to dissolve the liposomes. The solution was placed in an oven overnight to evaporate the ethanol and yield a dry lipid film. Chloroform (2 mL) was added followed by 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 with water). The tubes were vortexed for 20 s and centrifuged at 4,000 rpm for 10 min at 4°C using a bench centrifuge (Jouan B4i, Thermo Electron Corporation, UK). The chloroformic layer was separated and the concentration of phospholipid was estimated at 485 nm using the Jenway 7315 Spectrophotometer (Jenway, France). The rotation speed used for separation was chosen following extensive optimization aiming to ensure absence of phospholipid in the sediment and absence of BDP crystals in the floating layer. The sedimented material following centrifugation appeared as a spot at the bottom of the tube and was dispersed in deionized water followed by performing light microscopy (Novex B-series microscope, Euromex, The Netherlands) to investigate whether centrifugation using D<sub>2</sub>O was effective for separation of unentrapped BDP crystals from liposome-entrapped drug.

### **2.2.7. Stability of liposomes and surfactosomes on excessive extrusion**

Liposomes and surfactosomes, with or without cholesterol, were centrifuged using a bench centrifuge and the vesicles (containing the entrapped fraction of SBS or BDP) were separated (using the separation methods described above). The freshly prepared (i.e. unextruded) vesicles were collected following centrifugation and re-suspended in fresh drug-free aqueous phase (deionized water for SBS formulations and D<sub>2</sub>O for BDP preparations) to have a theoretically estimated drug entrapment efficiency of 100%. The vesicles were then extruded 51 times through 1 µm polycarbonate membranes. After extrusion, the drug entrapped in the vesicles was analysed using HPLC. The extruded liposomes or surfactosomes were again centrifuged and the medium containing the untrapped drug was replaced with a drug-free medium. These 1µm vesicles, with theoretical 100% drug entrapment efficiency were further extruded 51 times through the same pores size filters, and the drug entrapment was again determined. This experiment was conducted to study the difference in drug retention between un-extruded (freshly prepared) and extruded (1 µm) vesicles using excessive extrusion (51 cycles). We have found that, regardless of formulation, 17 cycles of extrusion were sufficient to cause extensive disruption to liposomes and leakage of the originally entrapped drug (data not shown). In the present study, this number of extrusion cycles was tripled (i.e. using 51 cycles) to ensure that liposomes were massively disrupted, as would happen during nebulization.

### **2.2.8. Saturation solubility of BDP in presence of Tween 80**

To determine the solubility of BDP in water, excess BDP was added to 1 mL water within an Eppendorf tube. The contents were mixed in a water bath for 24 h at 40°C. The Eppendorf tube was centrifuged for 20 min and the supernatant was tested for drug

concentration using HPLC. The same procedure was repeated to analyse the solubility of BDP in the presence of Tween 80 in water (15:85 v/v).

### **2.2.9. Statistical analysis**

All experiments were conducted three times using three different batches, and the resultant data were analysed using the SPSS statistical program (IBM Corporation, New York, USA). Data were presented as mean  $\pm$  standard deviation (SD). The difference between groups was regarded to be statistically significant when  $p$  value was lower than 0.05 using student  $t$ -tests or analysis of variance (ANOVA) to compare two groups or more than two groups respectively.

## **3. Results and discussion**

### **3.1. Entrapment of SBS in liposomes and surfactosomes prior to extrusion**

SBS entrapment in unextruded liposome and surfactosome vesicles was higher ( $p < 0.05$ ) when cholesterol was included (Table 1). The low entrapment of hydrophilic drugs in liposomes can be attributed to the limited aqueous space within the vesicles, with the majority of the drug molecules present in the continuous phase of the dispersion (Taylor et al., 1990a, Shivhare et al., 2012). The entrapment values obtained were higher than those found in other studies for the same drug using the thin film hydration method (Elhissi et al., 2006a), which might be attributable to the two-step hydration protocol adopted in the present study. Cholesterol confers rigidity to liposome bilayers, reducing drug leakage and enhancing liposome stability (Kirby et al., 1980) and its inclusion in bilayers has been reported to increase the entrapment of hydrophilic drugs (Taylor et al., 1990a). Moreover,

the entrapment values for SBS in liposomes might be underestimated due to drug leakage during centrifugation (Bendas and Tadros, 2007).

### **3.2. Size of liposomes and surfactosomes before and after extrusion**

To study the influence of shearing on vesicle stability a range of liposome and surfactosome formulations with defined particle sizes were prepared by extrusion. The appropriate number of extrusion cycles was determined in preliminary experiments (data not shown) to achieve the desired VMD (i.e. when median vesicle size is very similar to the pore size of the membrane pores). For each membrane pore size, the number of extrusion cycles which gave minimum Span and desired VMD was identified and used in subsequent studies. Since cholesterol maximizes vesicle stability and minimizes drug leakage during nebulization (Taylor et al., 1990b), liposomes prepared from SPC and cholesterol (1:1) were used to determine the number of extrusion cycles required. Thus, extrusion via 5 and 2  $\mu\text{m}$  membrane filters was performed 11 times while extrusion through the 1 and 0.4  $\mu\text{m}$  filters was conducted 7 times.

Figure 1 shows the VMD (i.e. median size) and Span (i.e. size distribution) upon extrusion of SPC liposomes (liposomes without cholesterol), SPC:Chol (1:1) (liposomes with cholesterol), surfactant-enriched SPC vesicles (surfactosomes without cholesterol), and surfactant-enriched SPC:Chol (1:1) (surfactosomes with cholesterol). Unextruded vesicles had large VMD and relatively high Span values, indicating that vesicles at this stage had high polydispersity. For unextruded vesicles, liposomes with cholesterol were larger ( $p < 0.05$ ) than surfactosomes with or without cholesterol. Extrusion through 5  $\mu\text{m}$  membrane filters generally resulted in significant reduction in VMD and Span of liposomes and surfactosomes. The VMDs of liposomes and surfactosomes extruded through 5  $\mu\text{m}$

membranes were smaller than the pore size of the membrane used, possibly because of the presence of large populations of vesicles below 5  $\mu\text{m}$  in the samples prior to extrusion. Subsequent extrusion of liposomes and surfactosomes through 2, 1 and 0.4  $\mu\text{m}$  membranes produced vesicles with size similar to that of the filter pores (Fig. 1a), and the Span values were also lower (Figure 1b), indicating that smaller pore size made the vesicles smaller and more homogeneous. It was found that the Span values were reduced significantly when vesicles were extruded through 0.4 $\mu\text{m}$  membrane as compared to un-extruded vesicles ( $p < 0.05$ ). These findings show that extrusion as a means of generating vesicles of uniform size distribution is applicable not only using conventional liposomes, which is established in literature (Olson et al., 1979; Hope et al., 1986), but also with surfactosomes used in the present investigation.

### **3.3. SBS retention in extruded liposomes and surfactosomes**

Untrapped drug (i.e. in continuous phase) was removed by centrifugation and aqueous phase was replaced with drug-free water, to make drug entrapment efficiency having a theoretical estimate of 100%. Thus, extrusion through each membrane was undertaken with vesicles having a theoretical 100% SBS entrapment efficiency. However, before performing the extrusion cycles, experimental determination of drug entrapment efficiency gave values of less than 100%, indicating drug was partially lost from vesicles via the burst effect during the time taken for practical estimation of values (Figure 2). For surfactosomes, almost 50% of the originally entrapped drug was lost from cholesterol-containing surfactosomes, and the loss was even greater from cholesterol-free vesicles (Figure 2), indicating that Tween 80 has made the vesicles very leaky (Young et al., 1983) even when no extrusion was performed.

Compared to extrusion through relatively large pore filters (e.g. 1-5  $\mu\text{m}$ ), extrusion through smaller membrane pores (e.g. 0.4  $\mu\text{m}$ ) caused marked loss of the originally entrapped SBS (i.e. drug retention decreased). In a previous investigation, we studied the influence of vibrating-mesh nebulization on the stability of liposomes and vesicle retention of SBS. Customized nebulizers with large mesh apertures (8  $\mu\text{m}$ ) helped reduce drug loss during nebulization compared to devices having conventional 4 $\mu\text{m}$  apertures (Elhissi et al., 2006a; Elhissi et al., 2007), indicating correlation of membrane extrusion findings with previous vibrating-mesh nebulization studies. The membrane extrusion findings presented here correlate with previous vibrating-mesh and jet-nebulizer studies; greater drug loss occurs from liposomes forced through the smaller pores as the greater shearing forces are applied to the vesicles. In jet-nebulizers, the shearing force can be increased by increasing the flow rate of gas used to convert the nebulizer fluid into aerosols (O'Callaghan and Barry, 1997). Niven and co-workers (1992) have studied the influence of increased jet nebulizer shearing on liposome stability by increasing the flow rate of gas employed to convert liposomes into aerosol. They demonstrated that liposomes exhibited an air pressure-dependent leakage of the originally entrapped hydrophilic marker, with losses being in the range of 1.3% to 88.2% using gas flow rates of 2.4 to 11.1 L/min respectively (Niven et al., 1992).

Liposomes and surfactosomes containing cholesterol exhibited lower drug loss (i.e. highest drug retention) on extrusion, than those without, including vesicles extruded through 0.4 $\mu\text{m}$  pore membranes (Figure 2). Previous studies have shown that cholesterol produced a protective effect, helping phosphatidylcholine vesicles withstand shear stresses provided by magnetic field (Tseng et al., 2007) or jet-nebulization (Taylor et al., 1991; Bridges and Taylor, 2000), indicating a correlation of these extrusion findings with previous aerosolization studies. The liposomal formulation of Amikacin (Arikace<sup>®</sup>) is currently in

advanced stages of development and is actually enriched with cholesterol as bilayer composition (Cipolla et al., 2013; Ehsan et al., 2014), further highlighting the importance of cholesterol in nebulizable liposome formulations.

Drug loss was greater for vesicles enriched with surfactant (i.e. surfactosomes) even prior to extrusion (Figure 2), demonstrating that bilayers have become more permeable and/or susceptible to damage during shearing, as a result of surfactant inclusion in the formulation. Lehofer and co-workers (2014) have reported greater losses from PEGylated liposomes during nebulization when compared to conventional cholesterol-enriched vesicles, indicating the PEG polymer has interfered with the integrity of the liposome bilayers. Differential scanning calorimetry studies on liposomes have shown that amphiphilic materials can interfere with liposome stability by inducing phase separation in the bilayers and changing the packing patterns of the phospholipid molecules (Castile et al., 1999; Castile et al., 2001; Tasi et al., 2003). These findings indicate that surfactants included with the aim of promoting the “ultradeformability” of liposomes to enhance vesicle stability may actually interfere with the integrity of the bilayers and promote drug leakage from the vesicles (Elhissi et al., 2012). For this reason, our surfactant-enriched liposomes were referred to as “surfactosomes” rather than “ultradeformable” liposomes which are commonly used for enhancing transdermal drug delivery (Verma et al., 2003; Dubey et al., 2006; Subongkot et al., 2014).

#### **3.4. Stability of SBS-entrapped liposomes and surfactosomes using excessive extrusion**

Large liposomes are “processed” during air-jet or vibrating-mesh nebulization, as they are exposed to excessive, repeated shearing and undergo size reduction to gain the optimum size for delivery from the nebulizer (i.e. to be incorporated into the generated aerosol droplets)

(Elhissi and Taylor, 2005; Kleemann et al., 2007). In jet-nebulizers, the reservoir contents undergo extensive shearing before being released in fine aerosol droplets for inhalation (O'Callaghan and Barry, 1997; Kleemann et al., 2007).

Niven and co-workers (1991) showed that that extent of soluble material loss from liposomes was dependent on the duration of nebulization, with longer processing within the nebulizer causing multiple times of shearing cycles within the nebulizer reservoir, and subsequently greater vesicle damage and drug losses. In an attempt to simulate these shearing conditions, we have designed an excessive shearing procedure by subjecting freshly prepared non-extruded (i.e. large) liposomes and surfactosomes to 51 cycles of extrusion through 1  $\mu\text{m}$  membrane filters, followed by studying SBS entrapment in the vesicles. In order to investigate whether the resultant extruded vesicles (1  $\mu\text{m}$  diameter) would retain the drug upon applying further shearing, the untrapped drug, as a result of extrusion damage was removed and replaced with drug-free deionized water and further 51 cycles of extrusion through the same size filter was conducted, followed by determination of the drug entrapment (Table 2).

Following extrusion 51 times through 1 $\mu\text{m}$  polycarbonate membrane filters, liposomes or surfactosomes containing cholesterol retained significantly greater SBS proportions than cholesterol-free formulations (Table 2;  $p < 0.05$ ), with liposomes retaining markedly greater drug proportions than surfactosomes ( $p < 0.05$ ). When the extruded vesicles, after separation of the untrapped drug (i.e. to theoretically yield 100% drug entrapment efficiency) were further extruded through 1 $\mu\text{m}$  membranes, further SBS loss occurred, but drug retention was significantly higher than observed with the large vesicles (Table 2). This clearly indicates that smaller liposomes had greater ability to retain entrapped hydrophilic materials

compared to large liposomes (Table 2), agreeing with nebulization studies of liposomes (Taylor et al., 1990; Niven et al., 1991; Elhissi et al., 2007; Kleemann et al., 2007; Lee et al., 2013).

Table 2 indicated that the influence of extrusion on drug retention was mainly affected by two factors, namely vesicle size and bilayer composition. For surfactosomes, the inclusion of cholesterol and using small vesicles (1 $\mu$ m) were needed to minimize drug losses, whilst for liposomes cholesterol inclusion was more important at minimizing the loss of entrapped SBS; this justifies the use of cholesterol in the liposomal formulation of the hydrophilic antibiotic amikacin (Arikace<sup>®</sup>) (Cipolla et al., 2013). Regardless of bilayer composition and vesicle size, surfactosomes exhibited lower retention for SBS (Table 2). Surfactant-enriched vesicles were found to be very unstable during nebulization, regardless of aerosolization mechanism, causing extensive losses of the hydrophilic drug SBS (Elhissi et al., 2012). This suggests that the use of extensive membrane extrusion, introduced in this study, has applicability in predicting vesicle stability during nebulization.

### **3.5. Entrapment of BDP by liposomes and surfactosomes prior to extrusion**

Whilst vesicle shearing within nebulizers may cause loss of liposome-entrapped hydrophobic drugs, this generally occurs to a much lesser extent compared to hydrophilic materials (Desai et al., 2002). Determination of BDP entrapment in liposomes and surfactosomes requires reliable separation of the untrapped drug from the vesicle-entrapped BDP. BDP tends to form crystals during centrifugation in aqueous media, usually resulting in simultaneous sedimentation of the BDP-entrapping vesicles and the free drug crystals (Batavia et al., 2001; Khan et al., 2015). However, concomitant sedimentation of drug crystals and liposomes did not occur when vesicles were dispersed in high density

water (i.e. deuterium oxide; D<sub>2</sub>O) (Batavia et al., 2001; Khan et al., 2005); thus in the present work, the thin lipid films containing BDP were hydrated with D<sub>2</sub>O instead of deionized water.

As demonstrated in Figure 3, by using D<sub>2</sub>O and an optimum centrifugation speed, BDP crystals were sedimented while liposomes containing incorporated drug constituted a floating creamy layer. Some dissolved BDP might be present in the middle region of the centrifugation tube (i.e. between the floating liposomes and BDP sediment), and this fraction was considered with the sedimented fraction of the drug as the total proportion of untrapped steroid (Figure 3). The sedimented crystals appeared as a “spot” at the bottom of the tube; this fraction was ascertained to be BDP analytically using HPLC and visually using light microscopy (Figure 4). Light microscopy also confirmed that the floating layer comprised liposomes (Figure 4). The Stewart phospholipid assay (Stewart, 1980) indicated that the sediment contained negligible phospholipid, confirming successful separation of the vesicle-entrapped drug from the free BDP crystals. BDP tends to crystallize during storage due the incompatible steric fit between the steroid and the liposome bilayers, restricting incorporation of this drug into liposomes (Batavia et al., 2001), though BDP interaction with bilayers is highly dependent on the excipients used, and the liposome preparation method employed (Elhissi et al., 2006b).

Figure 5 shows the proportion of lipid in each layer of the centrifuged cholesterol-containing liposomes and surfactosomes. The amount of lipid in the top layer was considerably greater than that in the middle layer and sedimented material (Figure 5), indicating that separation of BDP-entrapping liposomes from free BDP was successful and

agreeing with our recent findings using a slurry method to prepare liposomes from proliposome powders (Khan et al., 2015).

BDP entrapment was determined using HPLC (Table 3). Drug entrapment and loading were dependent on formulation, and inclusion of cholesterol has enhanced the drug entrapment ( $p < 0.05$ ; Table 3). The limited entrapment of BDP in phospholipid vesicles (Table 3) has been reported in a range of previous investigations (Fildes and Oliver, 1978; Batavia et al., 2001; Darwis and Kellaway, 2001). When liposomes were compared with surfactosomes, Table 3 has shown that BDP entrapment was influenced by cholesterol ( $p < 0.05$ ) whilst Tween 80 had no effect ( $p > 0.05$ ) on the steroid entrapment.

### **3.6. BDP incorporation and retention in extruded liposomes and surfactosomes**

Liposomes and surfactosomes were separated from the dispersion medium by centrifugation, then re-suspended in drug-free D<sub>2</sub>O to make the theoretical entrapment efficiency of the drug 100%. On extrusion, as the pore size of polycarbonate membrane was reduced, drug entrapment decreased, with greatest loss occurred when vesicles were extruded through 0.4  $\mu\text{m}$  membranes ( $p < 0.05$ ) (Figure 6). The reduction in entrapment of BDP as a result of extrusion (Figure 6) was less than that of SBS (Figure 2). Liposomes with cholesterol retained significantly more BDP than those without cholesterol ( $p < 0.05$ ). Conversely, cholesterol did not significantly affect drug retention in surfactosomes during extrusion, regardless of filter pore size ( $p > 0.05$ ). Following extrusion of cholesterol-containing vesicles, it can be generally seen that the entrapment efficiency of BDP in liposomes and surfactosomes was not greatly different (Figure 6).

When cholesterol was included in the formulations prior to extrusion, liposomes tended to provide higher BDP retention compared to cholesterol-free liposomes. By contrast, inclusion of cholesterol in surfactosome formulations did not enhance the retention of BDP during extrusion (Figure 6). We hypothesise here that cholesterol might have displaced a fraction of Tween 80 molecules from the bilayers to the continuous phase. The free surfactant molecules have possibly solubilized some BDP in the continuous phase, causing less entrapment of the steroid in the bilayers. To investigate whether this hypothesis is valid, a simple solubility study of BDP in Tween 80 solution versus solubility in pure deionized water was conducted. In presence of Tween 80, greater quantities of BDP became soluble (Table 4). This possibly means that formulations with surfactosomes had greater proportions of BDP solubilized in the continuous phase by the free surfactant molecules, hence, less incorporation of BDP in the lipid bilayers of surfactosomes has occurred. Whilst some investigators have reported lower drug entrapment in the bilayers as a result of enhancing fluidity of liposome membranes (Young et al., 1983), other reports have demonstrated enhanced entrapment of hydrophobic drugs (e.g. BDP) when liposomes with more fluid membranes were prepared (Darwis and Kellaway, 2001).

### **3.7 Stability of BDP-entrapped liposomes and surfactosomes using excessive extrusion**

Freshly prepared BDP formulations (i.e. unextruded liposomes) were extruded 51 times through 1  $\mu\text{m}$  polycarbonate membrane filters. Vesicles containing cholesterol retained significantly greater BDP proportions than those without cholesterol ( $p < 0.05$ ; Table 5). Earlier in this study cholesterol did not improve the retention of BDP in surfactosomes using the normal extrusion procedure (Figure 6), leading to the hypothesis that cholesterol has displaced Tween 80 from the bilayers, leading to enhanced steroid solubility in the continuous phase and reduced drug incorporation into the bilayers. However, the findings

with excessive extrusion were different since cholesterol significantly enhanced BDP retention in surfactosomes ( $p < 0.05$ ; Table 5). It is possible that excessive extrusion and repetitive shearing have enhanced the intermixing of phospholipid with Tween 80, hence reducing the proportion of the free surfactant in the continuous phase and enhancing BDP retention in the bilayers. Further studies are needed to evaluate the influence of cholesterol on the stability of surfactant-enriched phospholipid vesicles.

After separation of the untrapped drug from the 1  $\mu\text{m}$  vesicles and replacing that with drug-free dispersion medium (i.e. to reach a theoretical entrapment of 100%), the vesicles were again extruded 51 times through 1 $\mu\text{m}$  polycarbonate membranes. In this case, BDP retention was greater than values obtained upon extruding the freshly prepared vesicles. Moreover, when the small size vesicles were extruded the advantage of cholesterol at retaining greater BDP proportions was diminished, especially for liposomes (Table 5). Excessive jet-nebulizer shearing of BDP-liposomes made with different lipid compositions did not compromise the controlled release property of the formulations inhaled by healthy volunteers (Saari et al., 1999). This correlates well with findings employing high sensitivity differential scanning calorimetry to evaluate BDP interaction with the liposome bilayers following jet-nebulization; it was found that drug interaction with the bilayers was preserved despite the aggressive shearing of liposomes (Elhissi et al., 2011).

To our best knowledge, the present work is the first of its kind that has correlated extrusion of liposomes with *in vitro* and *in vivo* literature findings of stability of drug-loaded nebulized liposomes. The use of polycarbonate membrane extrusion to predict the stability of vesicle-based systems to nebulization can save time, reduce cost in the formulation and manufacturing development of nebulizable liposomes and minimize potential hazards

associated with reduced collection efficiency of liposomal aerosols during nebulization. It is important to bear in mind that these experiments employing mini-extruders have been used here to predict the general trend of vesicle stability to nebulization, rather than correlating with vesicle stability findings using specific nebulizer designs or operating mechanisms. Future studies will investigate the possible optimization of extrusion conditions to correlate with particular nebulizers operating mechanisms at particular operating conditions. Future investigations may also involve large extruders attached to gas cylinders in order to investigate the effect of gas flow rate on drug retention in vesicles. The extrusion approach introduced here will not eliminate the ultimate need for performing aerosolization studies but would rather markedly reduce them, by providing a screening tool for formulations that are best candidates for nebulization experiments.

#### **4. Conclusion**

Shearing studies via membrane extrusion have demonstrated greater retention of the hydrophobic drug BDP into liposomes and surfactosomes when compared to the hydrophilic drug SBS. This correlates with previously reported findings of vesicle stability to nebulization-induced shearing published by our research group and several other investigators. Hence, membrane extrusion may offer a means for predicting the stability of liposomes to aerosolization, avoiding the time consuming procedures of nebulization, and minimizing the possibility of laboratory contamination with unsuccessfully collected aerosols during development of nebulizable liposome formulations. Currently, Arikace<sup>®</sup> and Pulmaquin<sup>™</sup> are in late stage development and further nebulizable liposome formulations are in the pipeline. We expect future development of inhalable liposomes to benefit from this study and consider extrusion as a tool for predicting liposome stability to nebulization.

## Acknowledgements

We thank Lipoid, Switzerland for the gift of SPC (Lipoid S-100).

## References

Batavia, R., Taylor, K.M.G., Craig, D.Q.M., Thomas, M., (2001). The measurement of beclometasone dipropionate entrapment in liposomes: a comparison of a microscope and an HPLC method. *Int J Pharm* 212, 109-119.

Bendas, E.R., Tadros, M.I., 2007. Enhanced transdermal delivery of salbutamol sulfate via ethosomes. *AAPS PharmSciTech* 8, E107.

Bridges, P.A., Taylor, K.M.G., 2000. Factors influencing the jet nebulisation of liposomes. *Int J Pharm* 204, 69-79.

Castile, J.D., Taylor, K.M.G, Buckton, G., 1999. *Int J Pharm*. A high sensitivity differential scanning calorimetry study of the interaction between poloxamers and dimyristoylphosphatidylcholine and dipalmitoylphosphatidylcholine liposomes. *Int J Pharm* 182, 101-10.

Castile, J.D., Taylor, K.M.G, Buckton, G., 2001. The influence of incubation temperature and surfactant concentration on the interaction between dimyristoylphosphatidylcholine liposomes and poloxamer surfactants. *Int J Pharm* 221, 197-209.

Chadha, T.S., Chattopadhyay, S., Venkataraman, C., Biswas, P., 2012. Study of the charge distribution on liposome particles aerosolized by air-jet atomization. *J Aerosol Med Pulm Drug Deliv* 25, 355-364.

Cipolla, D., Gonda, I., Chan, H.K., 2013. Liposomal formulations for inhalation. *Ther Deliv* 4, 1047-1072.

Cipolla, D., Wu, H., Gonda, I., Chan, H.K., 2014. Aerosol performance and long-term stability of surfactant-associated liposomal ciprofloxacin formulations with modified encapsulation and release properties. *AAPS PharmSciTech* 15, 1218-1227.

Clancy, J. P., Dupont, L., Konstan, M. W., Billings, J., Fustik, S., Goss, C. H., Lymp, J., Minic, P., Quittner, A. L., Rubenstein, R. C., Young, K. R., Saiman, L., Burns, J. L., Govan, J. R., Ramsey, B., Gupta, R. & Arikace Study, G., (2013). Phase II studies of nebulised Arikace in CF patients with *Pseudomonas aeruginosa* infection, *Thorax* 68, 818-25.

Darwis, Y., Kellaway, I.W., 2001. Nebulisation of rehydrated freeze-dried beclometasone dipropionate liposomes. *Int J Pharm* 215, 113-121.

Desai, T.R., Hancock, R.E., Finlay, W.H., 2002. A facile method of delivery of liposomes by nebulization. *J Control Release* 84, 69-78.

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

Dubey, V., Mishra, D., Asthana, A., Jain, N.K., 2006. Transdermal delivery of a pineal hormone: melatonin via elastic liposomes. *Biomaterials*, 27, 3491-6.

Ehsan, Z., Wetzel, J.D., Clancy, J.P., 2014. Nebulized liposomal amikacin for the treatment of *Pseudomonas aeruginosa* infection in cystic fibrosis patients. *Expert Opin Investig Drugs* 23, 743-749.

Elhissi, A.M.A., Taylor, K.M.G., 2005. Delivery of liposomes generated from proliposomes using air-jet, ultrasonic, and vibrating-mesh nebulisers. *Journal of Drug Delivery Science and Technology* 15, 261-265.

Elhissi, A.M., Karnam, K.K., Danesh-Azari, M.R., Gill, H.S., Taylor, K.M., 2006a. Formulations generated from ethanol-based proliposomes for delivery via medical nebulizers. *J Pharm Pharmacol* 58, 887-94.

Elhissi, A.M., O'Neill, M.A., Roberts, S.A., Taylor, K.M.G, 2006b. A calorimetric study of dimyristoylphosphatidylcholine phase transitions and steroid-liposome interactions for liposomes prepared by thin film and proliposome methods. *Int J Pharm* 320, 124-30.

Elhissi, A.M., Faizi, M., Naji, W.F., Gill, H.S., Taylor, K.M., 2007. Physical stability and aerosol properties of liposomes delivered using an air-jet nebulizer and a novel micropump device with large mesh apertures. *Int J Pharm* 334, 62-70.

Elhissi, A.M.A., O'Neill, M. Ahmed, W., Taylor, K.M.G., 2011. High-sensitivity differential scanning calorimetry for measurement of steroid entrapment in nebulised liposomes generated from proliposomes. *Micro Nano Letters* 6, 694 – 697.

Elhissi, A.M., Giebultowicz, J., Stec, A.A., Wroczynski, P., Ahmed, W., Alhnan, M.A., Phoenix, D., Taylor, K.M., 2012. Nebulization of ultradeformable liposomes: The influence of aerosolization mechanism and formulation excipients. *Int J Pharm* 436, 519-526.

Fauvel, M., Farrugia, C., Tsapis, N., Gueutin, C., Cabaret, O., Bories, C., Bretagne, S., Barratt, G., 2012. Aerosolized liposomal amphotericin B: prediction of lung deposition, in vitro uptake and cytotoxicity. *Int J Pharm* 436, 106-110.

Fildes, F.J.T., Oliver, J.E., 1978. Interaction of cortisol-21-palmitate with liposomes examined by differential scanning calorimetry. *J Pharm Pharmacol* 30, 337-342.

Gaspar, M.M., Radomska, A., Gobbo, O.L., Bakowsky, U., Radomski, M.W., Ehrhardt, C., (2012). Targeted delivery of transferrin-conjugated liposomes to an orthotopic model of lung cancer in nude rats, *J Aerosol Med Pulm Drug Del* 25, 310-318.

Grinshpun, S.A., Willeke, K., Ulevicius, V., Jouzaitis, A., Terzieva, S., Donnelly, J., Stelma, G.N., Brenner, K.P., 1997. Effect of impaction, bounce and reaerosolization on the collection efficiency of impingers. *Aerosol Sci Technol* 26, 326-342.

Hope, M.J., Bally, M.B., Mayer, L.D., Janoff, A.S., Cullis, P.R., 1986. Generation of multilamellar phospholipid vesicles. *Chemistry and Physics of Lipids*, 40, 89-107.

Kamalaporn, H., Leung, K., Nagel, M., Kittanakom, S., Calvieri, B., Reithmeier, R.A., Coates, A.L., 2014. Aerosolized liposomal Amphotericin B: a potential prophylaxis of invasive pulmonary aspergillosis in immunocompromised patients. *Pediatr Pulmonol* 49, 574-580.

Khan, I., Yousaf, S., Subramanian, S., Korale, O., Alhnan, M.A., Ahmed, W., Taylor, K.M.G., Elhissi, A., 2015. Proliposome powders prepared using a slurry method for the generation of beclometasone dipropionate liposomes. *Int J Pharm* (accepted subject to successful revision).

Kirby, C., Clarke, J., Gregoriadis, G., 1980. Effect of the cholesterol content of small unilamellar liposomes on their stability in vivo and in vitro. *J Biochem* 186, 591-598.

Kleemann, E., Schmehl, T., Gessler, T., Bakowsky, U., Kissel, T., Seeger, W., 2007. Iloprost-containing liposomes for aerosol application in pulmonary arterial hypertension: formulation aspects and stability. *Pharm Res* 24, 277-287.

Lee, J-H., Cheng, K.T., Malinin, V., Li, Z., Yao, Z., Lee, S-J., Gould, C.M., Olivier, K.N., Chen, C., Perkins, W.R., Paik, C.H., 2014. <sup>99m</sup>Tc-labeled therapeutic inhaled amikacin loaded liposomes. *J Liposome Res* 23, 336-342.

Lehofer, B., Bloder, F., Jain, P.P., Marsh, L.M., Leitinger, G., Olshewski, H., Leber, R., Olshewski, A., Prassl, R., 2014. Impact of atomization technique on the stability and transport efficiency of nebulized liposomes harbouring different surface characteristics. *Eur J Pharm Biopharm* 88, 1076-1085.

Mitchell, J.P., Nagel, M.W., Wiersema, K.J., Doyle, C.C., 2003. Aerodynamic particle size analysis from pressurized metered-dose inhalers: comparison of Anderson 8-stage cascade impactor, next generation pharmaceutical impactor, and model 3321 aerodynamic particle sizer aerosol spectrometer. *AAPS PharmSciTech* 4, E54.

Nasr, M., Taha, I., Hathout, R.M., 2013. Suitability of liposomal carriers for systemic delivery of risedronate using pulmonary route. *Drug Deliv* 20, 311-318.

Nasr, M., Nahlah, M. D'Emanuele, A., Elhissi, A., 2014. PAMAM dendrimers as aerosol drug nanocarriers for pulmonary delivery via nebulization. *Int J Pharm* 461, 242-250.

Niven, R.W., Schreier, H., 1990. Nebulization of liposomes. I. Effects of lipid composition. *Pharm Res* 7, 1127-1133.

Niven, R., 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, Suppl 2, S31-S44.

Olson, F., Hunt, C.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, M., Vidgren, M.T., Koskinen, M.O., Turjanmaa, V.M., Nieminen, M.M., 1999. Pulmonary distribution and clearance of two beclomethasone liposome formulations in healthy volunteers. *Int J Pharm* 181, 1-9.

Serisier, D.J., Bilton, D., De Soyza, A., Thompson, P.J., Kolbe, J., Greville, H.W., Cipolla, D., Bruinenberg, P., Gonda, I., ORBIT-2 investigators, 2013. Inhaled, dual release liposomal ciprofloxacin in non-cystic fibrosis bronchiectasis (ORBIT-2): a randomised, double-blind, placebo-controlled trial. *Thorax* 68, 812-817.

Shivhare, U.D., Surse, P.B., Thombare, V.S., 2012. Formulations and In-Vitro Evaluation of Salbutamol Sulphate Liposomes. *Am J Pharm Res* 2, 970-981.

Stewart, J.C.M., 1980. Colorimetric determination of phospholipids with ammonium ferrothiocyanate. *Anal Biochem* 104, 10-14.

Subongkot, T., Pamornpathomkul, B., Rojanarata, T., Opanasopit, P., Ngawhirunpat, T., 2014. Investigation of the mechanism of enhanced skin penetration by ultradeformable liposomes. *Int J Nanomed* 9, 3539-3550.

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

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.

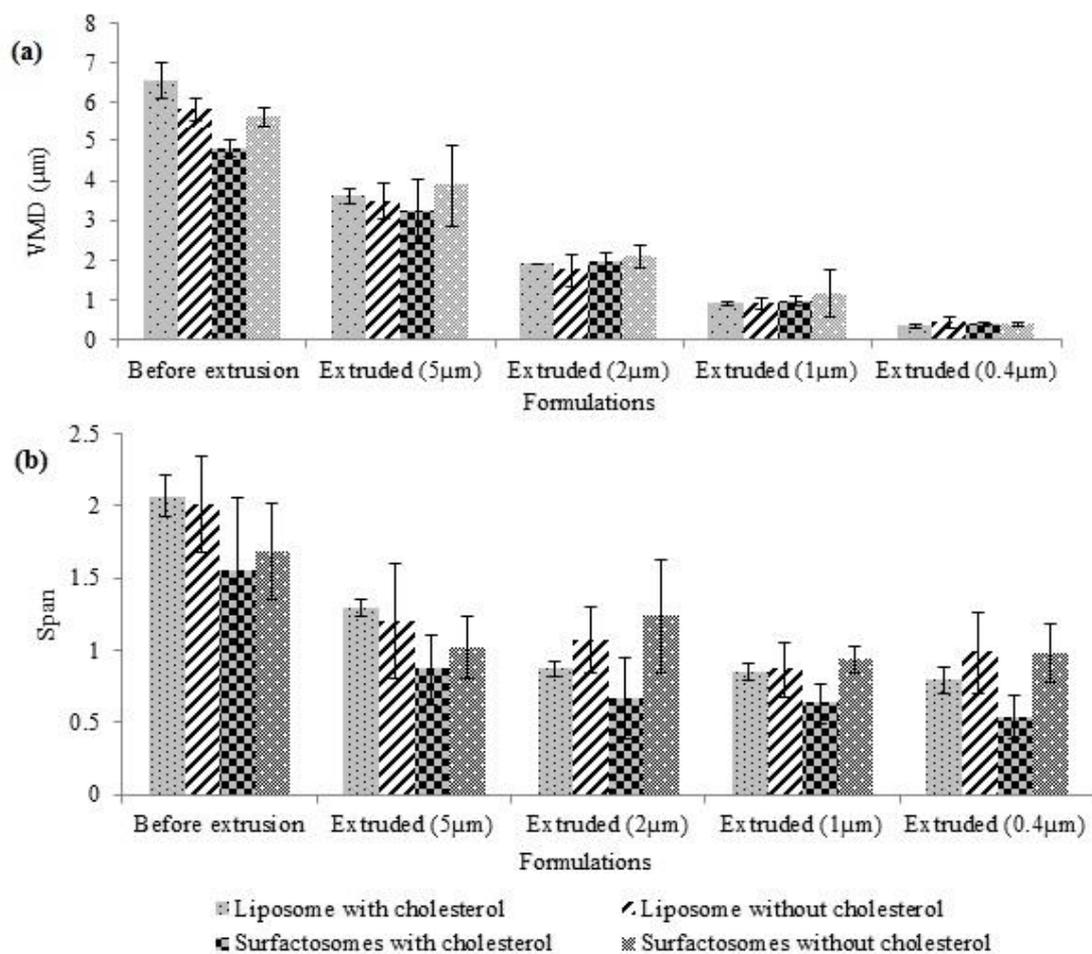
Tseng, L.P., Liang, H.J., Chung, T.W., Huang, Y.Y., Liu, D.Z., 2007. Liposomes incorporated with cholesterol for drug release triggered by Magnetic field. *J Med Biol Eng*, 27, 29-34.

Verma, D.D., Verma, S., Blume, G., Fahr, A., 2003. Particle size of liposomes influences dermal delivery of substances into skin. *Int J Pharm* 258, 141-51.

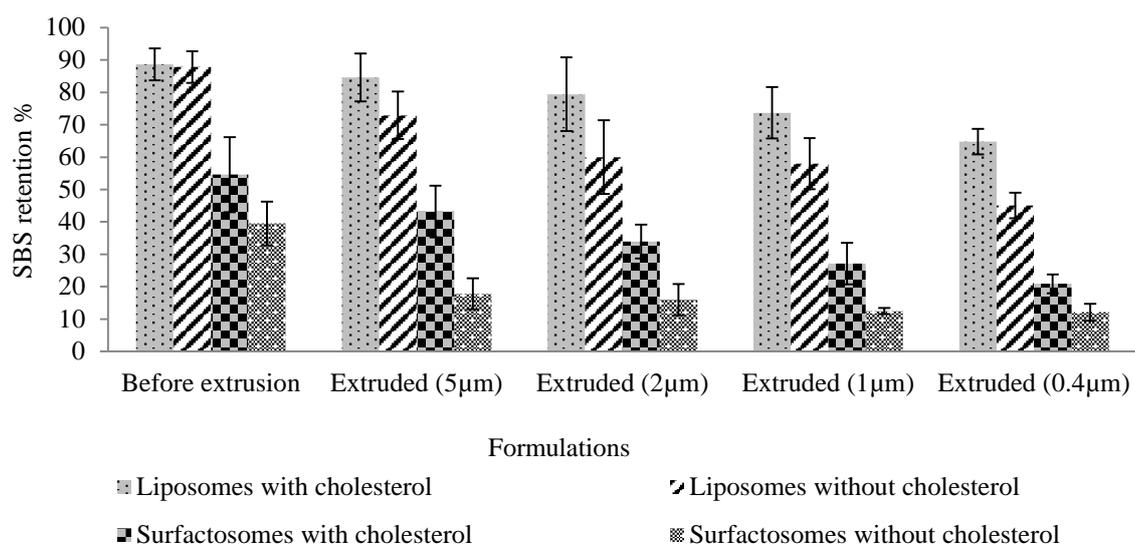
Young, M., Dinda, M., Singer, M., 1983. Effect of Tween 80 on lipid vesicle permeability. *Biochim Biophys Acta* 735, 429-432.

Waters, V., Ratjen, F., 2014. Inhaled liposomal amikacin. *Expert Rev Respir Med* 8, 401-409.

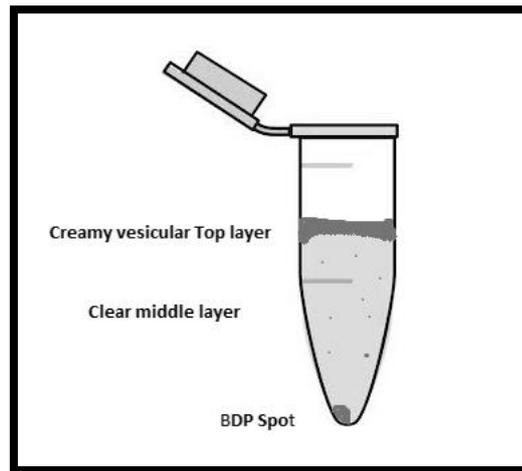
Xu, M., Willeke, K., Biswas, P., Partsinis, S.E., 1993. Impaction and rebound of particles at acute incident angles. *Aerosol Sci Technol* 18, 143-155.



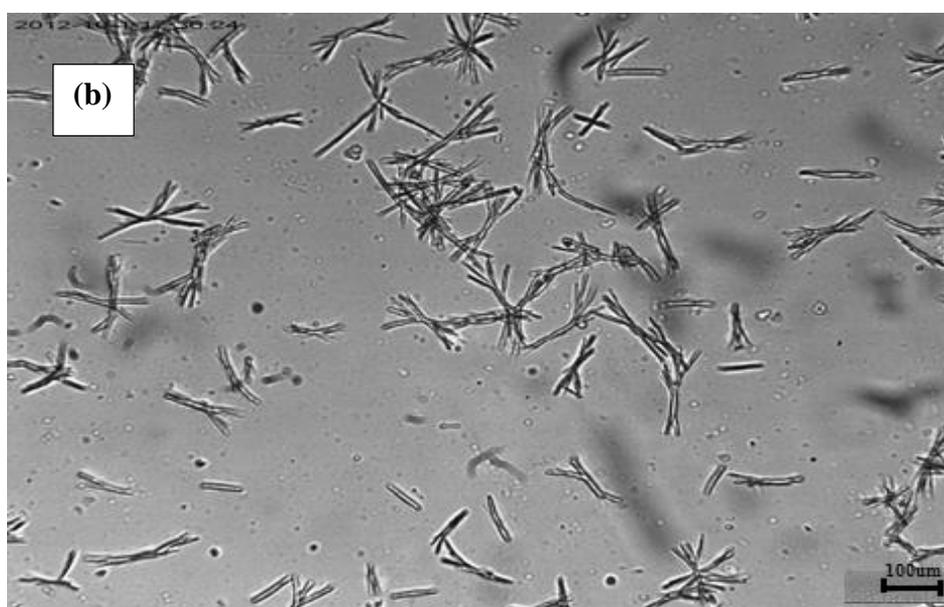
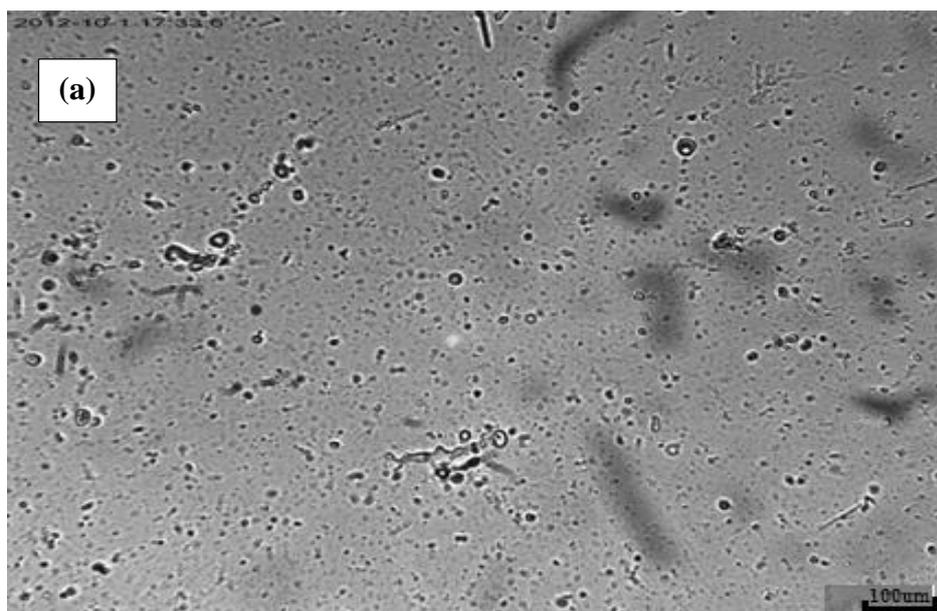
**Figure 1. VMD (a) and span (b) of liposomes and surfactosomes prepared with or without cholesterol, and with or without extrusion through 5, 2, 1 or 0.4  $\mu\text{m}$  polycarbonate membrane filters ( $n = 3 \pm \text{SD}$ )**



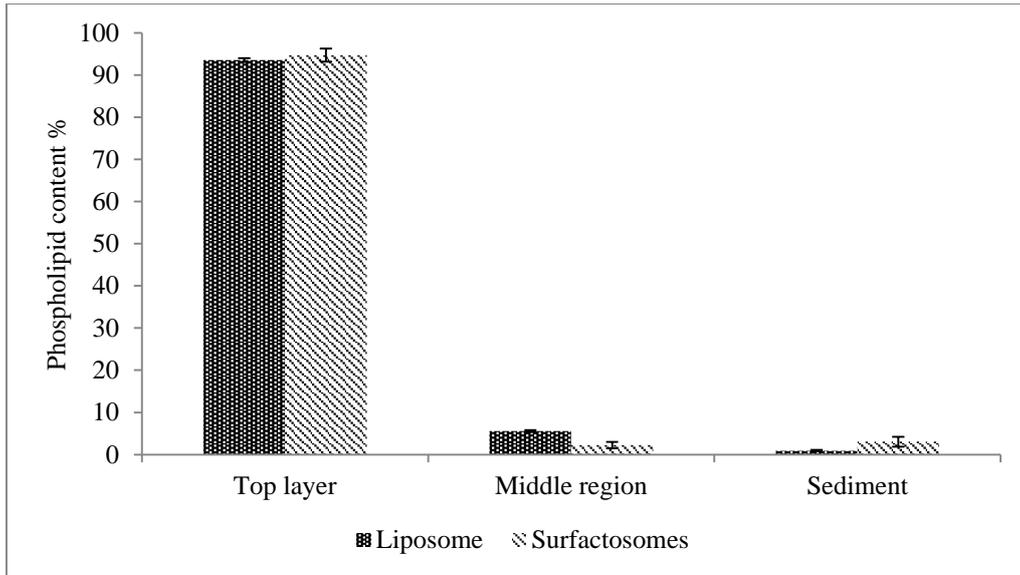
**Figure. 2. SBS retention in liposomes and surfactosomes with or without cholesterol, before or after passing the vesicles through polycarbonate membrane filters of pore size 5, 2, 1 or 0.4 μm (n=3 ± SD)**



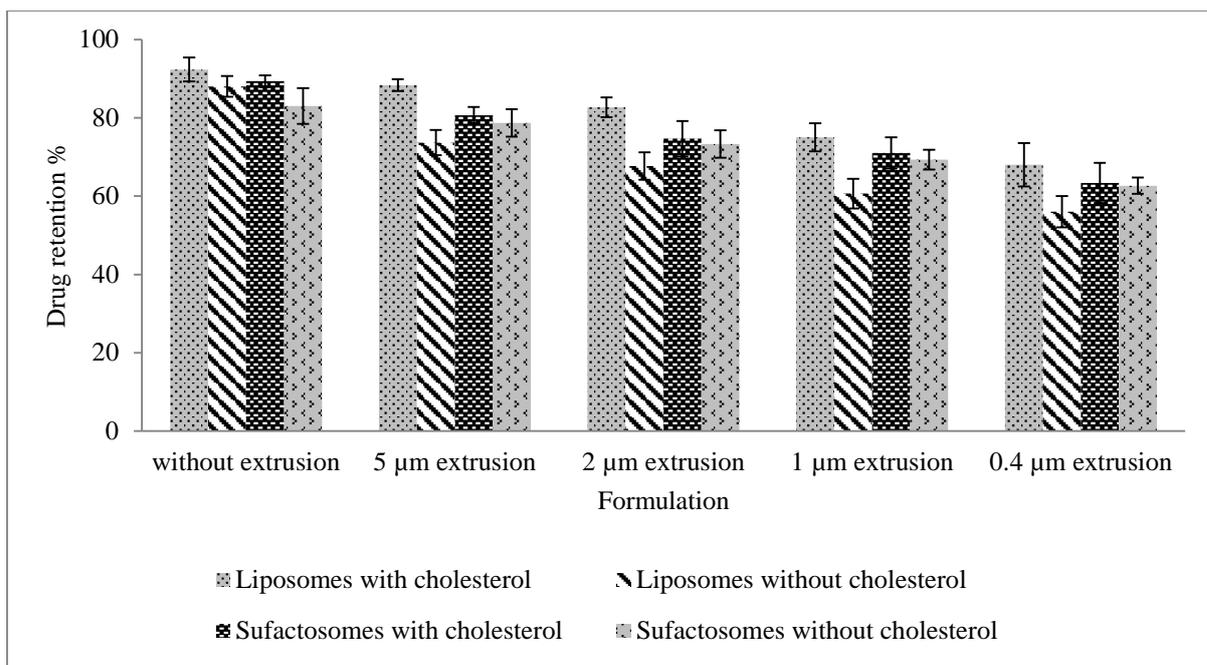
**Figure 3. Eppendorf tube following centrifugation of liposomes in D<sub>2</sub>O medium. Three regions were apparent: the upper floating layer ( liposomes with entrapped drug), the sediment (free BDP crystals) and middle region (D<sub>2</sub>O-soluble fraction of BDP)**



**Figure 4. Light microscopy images for samples collected from the floating creamy layer (a) and the sedimented material (b). Samples revealed that the creamy layer constituted of liposomes whilst the sediment was mainly BDP crystals. The samples were typical of 3 different experiments**



**Figure 5. Phospholipid content in the three regions, following centrifugation of cholesterol-containing liposome and surfactosome BDP preparations (n=3 ±SD)**



**Figure. 6. BDP entrapment in liposomes and surfactosomes, with or without cholesterol, before extrusion or after passing through polycarbonate membrane filters of pore size 5, 2, 1 or 0.4 μm (n=3 ± SD)**

**Table 1. Entrapment efficiency and loading of SBS in unextruded liposomes and surfactosomes, as determined by HPLC (n = 3 ± sd)**

| <b>Formulation</b>                | <b>Entrapment efficiency (%)</b> | <b>Drug loading (mg drug/100 mg lipid)</b> |
|-----------------------------------|----------------------------------|--|
| Liposomes with cholesterol        | 30.03 ± 1.69                     | 2.00 ± 0.11                                |
| Liposomes without cholesterol     | 23.25 ± 0.75                     | 1.55 ± 0.05                                |
| Surfactosomes with cholesterol    | 29.7 ± 7.26                      | 1.98 ± 0.48                                |
| Surfactosomes without cholesterol | 21.36 ± 5.5                      | 1.42 ± 0.37                                |

**Table 2. Stability of SBS liposomes and surfactosomes using excessive extrusion through 1 $\mu$ m polycarbonate membrane (n = 3  $\pm$ sd)**

| <b>Formulation</b>                | <b>SBS retention after 51 extrusion cycles of large freshly prepared vesicles through 1 <math>\mu</math>m membrane pores (%)</b> | <b>SBS retention after further 51 extrusion cycles of previously extruded vesicles through 1 <math>\mu</math>m membrane pores (%)</b> |
|-----------------------------------|--|---|
| Liposomes with cholesterol        | 60.10 $\pm$ 3.67   | 67.27 $\pm$ 1.86  |
| Liposomes without cholesterol     | 45.06 $\pm$ 2.95   | 63.77 $\pm$ 1.65  |
| Surfactosomes with cholesterol    | 14.60 $\pm$ 1.04   | 52.00 $\pm$ 1.17  |
| Surfactosomes without cholesterol | 6.60 $\pm$ 0.98  | 47.9 $\pm$ 3.30   |

**Table 3. Entrapment efficiency and loading of BDP in unextruded liposomes and surfactosomes, as determined by HPLC (n = 3 ± sd)**

| <b>Formulation</b>                | <b>Entrapment efficiency (%)</b> | <b>Drug loading (mg drug/100 mg lipid)</b> |
|-----------------------------------|----------------------------------|--|
| Liposomes with cholesterol        | 31.66 ± 3.05                     | 2.10 ± 0.20                                |
| Liposomes without cholesterol     | 24.66 ± 2.82                     | 1.64 ± 0.23                                |
| Surfactosomes with cholesterol    | 30.66 ± 3.21                     | 2.04 ± 0.21                                |
| Surfactosomes without cholesterol | 22.66 ± 4.72                     | 1.5 ± 0.31                                 |

**Table 4. Solubility of BDP in water and Tween 80 solution (n = 3 ± sd)**

| <b>Medium</b>             | <b>Saturated solubility of BDP</b> |
|---------------------------|------------------------------------|
| Deionized water           | 0.14 µg/ml                         |
| Tween 80 aqueous solution | 12.67 µg/ml                        |

**Table 5. Stability of BDP liposomes and surfactosomes using excessive extrusion through 1 $\mu$ m polycarbonate membrane (n = 3  $\pm$ sd)**

| <b>Formulation</b>                | <b>BDP retention after 51 extrusion cycles of large freshly prepared vesicles through 1<math>\mu</math>m membrane pores (%)</b> | <b>BDP retention after 51 further extrusion cycles of previously extruded vesicles through 1<math>\mu</math>m membrane pores (%)</b> |
|-----------------------------------|---|--|
| Liposomes with cholesterol        | 65.8 $\pm$ 1.79   | 87.13 $\pm$ 1.8  |
| Liposomes without cholesterol     | 55.27 $\pm$ 2.97  | 84.10 $\pm$ 1.10   |
| Surfactosomes with cholesterol    | 55.30 $\pm$ 3.08  | 78.47 $\pm$ 1.45   |
| Surfactosomes without cholesterol | 49.50 $\pm$ 2.36  | 71.03 $\pm$ 2.15   |