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Impact of phospholipids, surfactants and cholesterol selection on the performance of transfersomes vesicles using medical nebulizers for pulmonary drug delivery

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ABSTRACT

The aim of this study is to formulate and optimize novel transfersome formulations for pulmonary drug delivery. Transfersome formulations (F1 – F18) were prepared by a thin-film method using three phospholipids (Soya phosphatidylcholine (SPC), Dimyristoly phosphatidylcholine (DMPC) and Hydrogenated soya phosphatidylcholine (HSPC)), in combination with three different surfactants (Tween 80, Span 80 and Span 20) with or without cholesterol, employing Beclomethasone dipropionate (BDP) as the model drug. Nano-transfersome formulations post-extrusion were delivered to a Two-stage Impinger (TSI) *via* three medical nebulizers (i.e. Air-jet, Ultrasonic and Vibrating mesh nebulizer). Based on the physicochemical properties, formulations F1 (SPC and Tween 80), F7 (DMPC and Tween 80) and F13 (HSPC and Tween 80) demonstrated significantly smaller VMD (162.34 ± 6.48, 198.66 ± 6.64, and 183.52 ± 7.34 nm), and significantly higher entrapment efficiency (97.56 ± 2.45, 95.67 ± 4.26 and 95.06 ± 3.38%). Based on nebulization performance, the Ultrasonic nebulizer exhibited the shortest nebulization time for formulations F1, F7 and F13 (i.e. 17.88 ± 2.45, 19.26 ± 2.04 and 19.59 ± 2.12 min), and higher output rate (212.04 ± 11.54, 194.61 ± 10.27 and 192.43 ± 9.84 mg/min), when compared to Air-jet and Vibrating mesh nebulizers. Irrespective of nebulizer type, significantly higher BDP deposition was observed in the lower stage of TSI for the F1 formulation (on average of 61%), whereas a higher amount of BDP was deposited in the upper stage of TSI using the F7 formulations (49%). Moreover, Formulation F1 in combination with Air-jet nebulizer demonstrated higher emitted dose (ED) and fine particle fraction (FPF) (82% and 83%), when compared to the counterpart formulations and nebulizer types investigated. This study has demonstrated that based on nebulizer performance, BDP deposition and formulation type; the F1 formulation in combination with an Air-jet nebulizer is most optimal for lower respiratory tract deposition, whereas the F7 formulation in combination with an Ultrasonic nebulizer is ideal for upper respiratory tract deposition.

1. Introduction

Liposomes/proliposomes, ethosomes, transfersomes/pro-transfersomes, solid lipid nanoparticles, nanostructured lipid carriers and emulsions offer many advantages than their other counterpart delivery systems [1–4]. One of the key advantages of lipid-based formulations is that they encapsulate lipophilic as well as hydrophilic active pharmaceutical agents within the concentric bilayers and central core, simultaneously. Phospholipids self-assemble themselves into vesicles upon direct contact with aqueous medium, when exposed above their phase transition temperature. They are considered both biocompatible and biodegradable due to the nature of phospholipid.

Transfersomes are also referred to as elastic or deformable liposomes due to the presence of surfactants, embedded in the bilayers, making

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Table 1
Transfersome formulations (F1 – F18) were prepared using a lipid phase consisting of three phospholipids (SPC, DMPC and HSPC), three different surfactants (Tween 80, Span 80 and Span 20) and with or without cholesterol inclusion. BDP was used as anti-asthmatic drug in all formulations, n = 3.

Lipid phase							
Formulations	Phospholipid			Surfactant			Cholesterol
F1	SPC	–	–	Tween 80	–	–	–
F2	SPC	–	–	Tween 80	–	–	Cholesterol
F3	SPC	–	–	–	Span 80	–	–
F4	SPC	–	–	–	Span 80	–	Cholesterol
F5	SPC	–	–	–	–	Span 20	–
F6	SPC	–	–	–	–	Span 20	Cholesterol
F7	–	DMPC	–	Tween 80	–	–	–
F8	–	DMPC	–	Tween 80		–	Cholesterol
F9	–	DMPC	–	–	Span 80	–	–
F10	–	DMPC	–	–	Span 80	–	Cholesterol
F11	–	DMPC	–	–	–	Span 20	–
F12	–	DMPC	–	–	–	Span 20	Cholesterol
F13	–	–	HSPC	Tween 80	–	–	–
F14	–	–	HSPC	Tween 80	–	–	Cholesterol
F15	–	–	HSPC	–	Span 80	–	–
F16	–	–	HSPC	–	Span 80	–	Cholesterol
F17	–	–	HSPC	–	–	Span 20	–
F18	–	–	HSPC	–	–	Span 20	Cholesterol

them more flexible and less rigid, in order to pass through the pores as well as offering smaller vesicle size than the traditional liposomes [5]. Essentially, deformability of the concentric bilayers increases when the bilayers of vesicles are destabilized, which are directly related to the surfactant or edge activator containing a single chain with a high radius of curvature. Examples of these surfactants employed in transfersomes include; Tween 80, Tween 20, Span 80, Span 20, dipotassium glycyrrhizinate, sodium deoxycholate and sodium cholate [6,7]. Transfersome vesicles have captured increased interest in both the cosmetics and pharmaceutical industry due to their small vesicle size and improved flexibility. In the cosmetics industry, transfersomes are an ideal vesicular system for encapsulating and delivering active agents, as well as plant extracts as an aqueous suspension or following its incorporation into creams, suspensions, ointments, serums, toners and lotions for skin delivery in order to achieve anti-oxidant, anti-aging, anti-perspirant, skin lightning, moisturizing and photo protective effects. In the pharmaceutical industry, transfersomes have been employed for the delivery of various active ingredients *via* buccal [8], pulmonary [9], transdermal [10], parenteral [11] and topical delivery [12].

The pulmonary system is a non-invasive route of drug administration, offering a large surface area (approximately 100 m^2) [13]. This route has been historically used for the inhalation of *Atropa belladonna* leaves for treating coughs, circa 4000 years ago [14]. Beclomethasone dipropionate (BDP) is a commercially available steroidal drug, used as prophylaxis in asthma *via* inhalation. A number of different brands such as Asmabec are available as a dry powder inhalers, whereas QVAR and Clenil are accessible in pressurised meter dose inhalers, containing BDP. BDP has been studied in a number of lipid-based aqueous formulations, as well as in a dry powder form (prepared *via* spray drying or freeze drying) targeting the pulmonary system including; liposomes [15–18], micelles [19], microspheres [20], niosomes [21], solid lipid nanoparticles [22] and nanostructured lipid carriers [23,24] in order to solubilize the drug and achieve localized effect for longer period of time.

Nebulizers have been employed for a plethora of formulations for pulmonary administration. The three basic types of medical nebulizers are classified based on their mechanism of generating aerosols, these are; Air-jet, Ultrasonic and Vibrating mesh nebulizers. Each class consists of many sub types, working on the same principle, with slight modifications in the design. A high velocity of compressed gas is used in Air-jet nebulizers that convert aqueous suspensions or solutions into aerosols *via* the Bernoulli effect, where after passing the gas through a fine hole (termed a venture nozzle) develops a negative pressure over the formulation, and hence inhalable droplets are generated [25,26]. Additionally, droplet formation is caused by the surface tension of the formulation as well as baffle of the Air-jet nebulizer, where droplets upon generation pass through a mouthpiece for inhalation [13,27]. Ultrasonic nebulizers are comprised of a piezoelectric crystal, which vibrates at a high frequency when connected to a power supply, creating an energy within the stagnant aqueous formulation. This allows the formulation to form a fountain-like structure and hence inhalable droplets are generated at the apex and at the lower part of the fountain [13,25]. As the name indicates, the Vibrating mesh nebulizer consists of a perforated plate, which is also called mesh plate. Vibrations are created by a piezoelectric crystal, which is connected to a horn transducer. Through this transducer these vibrations are transmitted in order to extrude the formulation from the perforated/mesh plate to generate inhalable droplets [13,28]. These three nebulizers and their sub types have been studied extensively using various micro and nano formulations in order to find the best nebulizer as well as formulation to achieve high drug deposition in the pulmonary system without affecting vesicle integrity [2].

In this study, various types of transfersome formulations were prepared and nebulizers were employed for pulmonary drug delivery. The aim of this study was to develop and optimize BDP-loaded transfersome formulations and to investigate the impact of various factors, such as phospholipid type, surfactant type, inclusion or exclusion of cholesterol on the physicochemical properties of transfersome vesicles, including; VMD, polydispersity index (PDI), Zeta potential and entrapment efficiency. Furthermore, different nebulizers were employed for the selected formulations to determine their performance in terms of nebulization time, sputtering time, mass output and output rate. Finally, deposition of BDP in Two-stage Impinger (TSI) was explored to identify the best combination of formulation and nebulizer type.

2. Materials and methods

2.1. Materials

Soya phosphatidylcholine (SPC; Lipoid S-100; 94% purity), Dimyristoly phosphatidylcholine (DMPC; 98% purity) and Hydrogenated soya phosphatidylcholine (HSPC; 98% purity) were purchased from Lipoid, Steinhausen, Switzerland. Beclomethasone dipropionate (BDP, ≥99%), cholesterol (≥99%), Tween 80, Span 80, Span 20 and polycarbonate filters with a pore sizes of 200 and 600 nm, and 25 mm in diameters were bought from Sigma Aldrich, UK. HPLC-grade methanol and absolute ethanol were acquired from Fisher scientific, UK.

2.2. Preparation of transfersomes via thin film method

Transfersomes were prepared *via* a thin-film method, where three different phospholipids (SPC, DMPC or HSPC) and three different surfactants (i.e. Tween 80, Span 80 or Span 20) were used to prepare 18 different transfersome formulations, employing BDP as a model drug in 2 mol% concentration to the lipid phase (Table 1). The lipid phase (250 mg) was comprised of phospholipid, cholesterol (with or without) and surfactant; where phospholipid to surfactant ratio was employed as 75:25 w/w, and this ratio to cholesterol was used in a 1:1 M ratio (Table 1). Phospholipid, cholesterol (with or without), surfactant and BDP were dissolved in 20 ml of absolute ethanol and transferred to a round bottom flask (RBF) (100 ml). The RBF was attached to a rotary evaporator (Buchi Rotavapor R-114, Buchi, Switzerland) and lowered into a water bath (Buchi Water bathe B-480, Buchi, Switzerland) already adjusted to 45 °C. A negative pressure was created to evaporate organic solvent *via* a vacuum pump (Buchi Vac V-501). This process was continued for 1 h with a rotation speed of 270 RPM. Negative pressure was released and the RBF containing a thin film was detached from the rotary evaporator. The thin lipid film in the RBF was hydrated with 10 ml of deionised water and agitated manually for 5 min, followed by 1 h annealing to form stable transfersome vesicles. The phase transition temperature of SPC, DMPC and HSPC is circa −20 °C, 23 °C and 52 °C; therefore hydration and annealing both were conducting above the phase transition temperatures of the phospholipids incorporated. The hydration and annealing temperature for both SPC and DMPC formulations was maintained at 30 °C, whereas for HSPC formulations, this was maintained at 60 °C.

2.3. Size reduction using extrusion method

Transfersome size was reduced using a Lipsofast extruder (Huber Avastin – Lipsofast LF-50, Germany). The extruder was set at temperature of 60 °C and nitrogen (using high pressure approximately 70 psi) was used to force the transfersome suspension (10 ml) through 600 nm filters for 5 cycles followed by passing through 200 nm filters for 5 cycles, to achieve nanoparticles with uniform size distribution.

2.4. Transfersome size and Zeta potential analysis

Size of transfersomes also referred to as volume median diameter (VMD) and polydispersity index (PDI) were measured employing Dynamic Light Scattering (DLS), using a Zetasizer Nanoseries instrument (Malvern Instruments Ltd., UK). The surface charge of transfersomes was also identified using the Zetasizer Nanoseries *via* Laser Doppler Velocimetry (LDV), where transfersome vesicle electrophoretic mobility in the suspension was determined.

2.5. Separation and entrapment efficiency of BDP

The transfersome suspension (0.5 ml) was placed in a Millipore centrifuge tube (10 KD) (Fischer Scientific, UK) and bench centrifugation (Spectrafuge 24D, Labnet International, USA) was employed using a speed of 13,000 RPM (15,500×*g*) for 30 min. This process allowed the separation of unentrapped drug (free drug passing through Millipore filter called filtrate) from the entrapped drug in transfersomes (transfersomes as carrier particles are too large to pass through the Millipore filter). The unentrapped BDP was then analysed *via* HPLC (Agilent 1200 HPLC instrument, UK). For total drug BDP analysis, 1 ml from the transfersome suspension was separately dissolved in 15 ml of methanol and quantified using HPLC in accordance to equation (1).

$$\text{Entrapment Efficiency } (\%) = \left(\frac{\text{Total drug loading} - \text{Unentrapped drug}}{\text{Total drug loading}}\right) \times 100 \quad \text{(Eq. 1)}$$

BDP was assayed *via* HPLC using a mobile phase of methanol and water (75:25 v/v) with a flow rate of 1.7 ml/min and an injection volume of 20 μl. The detection wavelength was set at 239 nm with a temperature adjusted to 40 °C. The HPLC column used was a C-18, 150 mm × 4.6 mm, and 5-μm column (Agilent technology, USA).

2.6. Optimal formulation and nebulization performance

Transfersome formulations were selected on the basis of size, Zeta potential and entrapment efficiency. Each formulation was then tested in three nebulizers: Air-jet (PARI Turboboy 5 air jet, UK), Ultrasonic (Uniclife rechargeable ultrasonic inhaler MY-520B, UK) and Vibrating mesh (Omron Micro-air U22 pocket nebulizer, UK) with a Two-stage Impinger (TSI) i.e. an artificial lung model as suggested by the British Pharmacopoeia. The TSI is comprised of an upper stage and lower stage (representing the upper and the lower airways), containing 7 and 30 ml of deionised water, respectively. The cut-off diameter between the upper and lower stage at 60 L/min is 6.4 μm [2,29]. Transfersome suspensions (5 ml) were transferred to the nebulizer reservoir of each nebulizer and nebulization performance was determined in terms of nebulization time, sputtering time, aerosol mass output and aerosol output rate. Aerosol mass output and aerosol output rate were calculated with the help of the following equations (2) and (3):

$$\text{Mass output } (\%) = \left(\frac{\text{Weight of nebulized formulation}}{\text{Weight of formulation present in the nebulizer prior to nebulization}}\right)\text{X}100 \quad \text{(Eq. 2)}$$

$$\text{Aerosol output rate } (\text{mg}/\text{min}) = \left(\frac{\text{Weight of nebulized formulation}}{\text{Complete nebulization time}}\right) \quad \text{(Eq. 3)}$$

Furthermore, deposition of BDP in the two stages of TSI, as well as the formulation remained as dead or residual volume in the nebulizer reservoir was determined *via* HPLC, post nebulization. Additionally, the amount of BDP deposited in the upper and lower stages was determined as emitted dose (ED). The amount of BDP deposited in the lower stage is also considered as fine particle dose (FPD). The final particle fraction

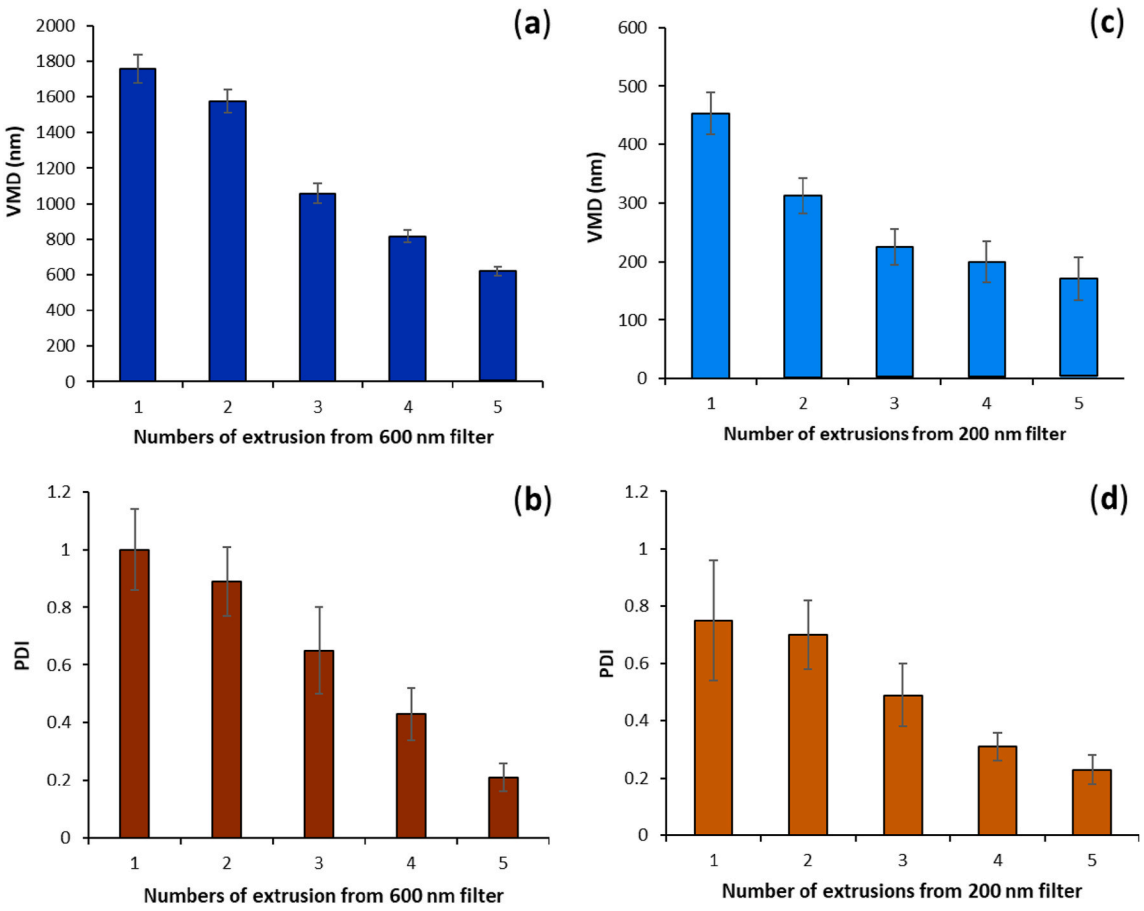

Fig. 1. An average of the following six transfersome formulations (i.e. F1, F2, F7, F8, F13 and F14) were determined (each formulation in triplicate) after passing the vesicles through polycarbonate membrane employing five cycles using a pore size of 600 nm for, (a) volume median diameter (VMD) and, (b) polydispersity index (PDI); followed by passing through polycarbonate membrane employing five cycles using a pore size of 200 nm for, (c) volume median diameter (VMD) and, (d) polydispersity index (PDI). Data are presented as mean ± SD, n = 3.

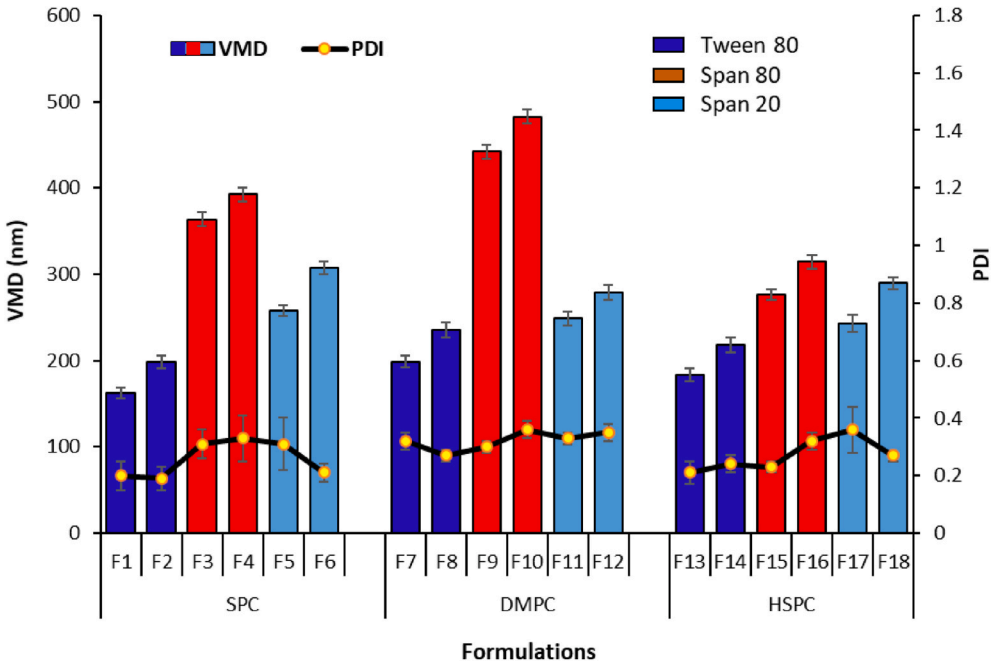

Fig. 2. VMD (vertical bars) and PDI (horizontal lines) of transfersome formulations (F1 – F18), prepared from three different phospholipids (SPC, DMPC and HSPC), three surfactants (Tween 80, Span 80 and Span 20) with and without cholesterol addition. Transfersome formulations prepared from surfactants are highlighted with different colours i.e. Tween 80 (F1, F2, F7, F8, F13, F14), Span 80 (F3, F4, F9, F10, F15, F16) and Span 20 (F5, F6, F11, F12, F17, F18). Data are mean ± SD, n = 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(FPF) was calculated as the percentage of ED deposited in stage 2 (i.e. FPD).

2.7. Morphology studies via transmission electron microscopy (TEM)

For TEM analysis, a drop of transfersome suspension and a drop of negative stain (i.e. 1% w/v phosphotungstic acid) were placed on a carbon coated carbon grid (400 mesh; TAAB Laboratories Equipment Ltd., UK). After drying (1 h), transfersome structures were observed, and a number of images were captured using a Philips CM 120 Bio-Twin TEM (Philips Electron Optics BV, Netherlands).

2.8. Statistical analysis

One way analysis of variance (ANOVA) and Student's *t*-test was performed to compare more than two set and two groups of results. All experiments were performed in triplicate. Values of $p < 0.05$ were considered as a statistically significant difference between compared groups.

3. Results and discussion

3.1. Size reduction and size analysis

There are many benefits associated with smaller vesicle size e.g. offering better uptake by cells [30] and drug deposition. With the same quantity of transfersome-forming materials, reduction of particle size may lead to higher drug entrapment. This may be directly related to the overall surface area, as there are a higher number of transfersomes. As the particle size decreases, the surface area increases and hence drug encapsulation. Therefore, engineering in achieving the required vesicle size of transfersomes is highly significant. Post size reduction, properties of the transfersomes were investigated in terms of size (also known as volume median diameter (VMD)) and size distribution (also denoted as polydispersity index (PDI)). Extrusion was employed for the formulated transfersome formulations (i.e. F1, F2, F7, F8, F13 and F14) in order to reduce transfersome particle size and achieve lower PDI. An average of these formulations with regards to VMD and PDI are shown in Fig. 1. Unlike probe sonication, extrusion of vesicles through polycarbonate membrane filter does not contaminate the formulation with any residue, such as titanium and hence no further purification step is required [2, 24]. Similarly, the time required to reduce vesicle size to approximately 200 nm by bath sonication would be significantly longer than that of probe sonication or the extrusion method. For these reasons extrusion was selected for the reduction of vesicle size in this study. In order to achieve particle size of circa 200 nm, transfersome suspensions were passed through polycarbonate membranes of 600 nm for 5 cycles, where vesicle size as well as PDI were reduced simultaneously after each cycle (Fig. 1a and b). After achieving vesicles sizes up to ~600 nm, these formulations were then passed through polycarbonate membranes of 200 nm pore size. Similarly, reduction in vesicle size and PDI was also observed for 5 cycles (Fig. 1c and d). Whereas, more cycles through 200 nm filters did not reduce the vesicle size, but showed increase in particle size as well as PDI, which may be associated with aggregation, fusion or acquiring electrostatic charges (Data not shown). It is important to know that using the Zetasizer instrument, a PDI value of 1 is considered the maximum particle size distribution within the sample; therefore a sample with PDI value close to 1 indicates that particles are highly polydispersed (i.e. have a broad size distribution) whilst a PDI of 0.1 exhibit a narrow particle size distribution.

Upon comparison of VMD values, all formulations did not achieve particle size of circa 200 nm, when passing through 600 nm and followed by 200 nm polycarbonate membrane for 5 cycles. Moreover, it was found that formulations prepared with cholesterol were larger in size, when compared to formulations without cholesterol [31] (Fig. 2). Upon the addition of cholesterol, the hydrophobicity of particle surfaces may increase. This increase of surface hydrophobicity may cause particle aggregation and subsequently increase particle size. The thickness of phospholipid layer of membrane was also found to increase in the presence of cholesterol by 3 Å, when using low-angle X-ray diffraction spacing [32]. This increase in thickness due to cholesterol may decrease in the area per molecule of phospholipid in the plane of membrane, which ultimately increases the size of the vesicles. Moreover, it was also suggested that both cholesterol and surfactant compete to keep their place in the lipid bilayers and hence increase vesicle size in addition to an increase in bilayer packing density [33,34]. However, it was also identified that lipid vesicles are comparatively more prone to deformation in the absence of cholesterol. The incorporation of cholesterol is known to stabilize vesicles by giving them rigidity and thus maintaining their structure [35,36]. Both SPC and DMPC possess relatively low phase transition temperatures (- 20 °C and 23 °C) and the incorporation of cholesterol stabilizes transfersome vesicles; whereas HSPC has a higher phase transition temperature (52 °C). At room temperature, HSPC becomes more rigid (due to its solidification), and hence stabilizes transfersome vesicles. Additional incorporation of cholesterol molecules fills the gaps within the phospholipid and surfactant molecules (by interacting with the core of membrane during annealing), and makes the transfersome membrane highly rigid; thus, reducing drug leakage from the vesicles [37–39]. This may be related to the change of the fluid phase phospholipid bilayer to a solid phase bilayer, due to the incorporation of cholesterol and hence the ability of transfersome bilayers to retain hydrophobic drugs is enhanced [40,41].

Upon comparison of the VMD values, formulations prepared using SPC as a phospholipid formed significantly smaller vesicles ($p < 0.05$) when compared to vesicles prepared from DMPC and HSPC. This may be related to the lower phase transition temperature of SPC i.e. - 20 °C, when compared to 23 °C and 52 °C of DMPC and HSPC, respectively (Fig. 2). The lower phase transition of the SPC formulation may keep the bilayer membrane more fluid, facilitating size reduction, whilst the higher phase transition temperatures of vesicles made from DMPC or HSPC can increase bilayer rigidity and promote resistance against sonication-driven fragmentation. Furthermore, surfactants are added into the formulation in order to enhance vesicles flexibility. Significantly smaller ($p < 0.05$) VMD of transfersome vesicles was achieved using Tween 80 as a surfactant than the incorporation of Span 80 or Span 20 (Fig. 2). Tween 80 is water-soluble surfactant with a HLB value of 15, and therefore the presence of a water-soluble surfactant in transfersomes enabled lower vesicle size, when compared to the employment of Span 80 or Span 20, which are oil soluble and water dispersible surfactants (i.e. HLB values of 4.3 and 8.6). It is suggested that the hydrophilic surfactant (Tween 80) covers the surface of the vesicles more, due to their hydrophilic moiety (higher HLB value) [42–44], when compared to Span 80 and Span 20. Moreover, Tween 80 may reduce the high interfacial tension of the vesicles (due to the hydrophilic head group) resulting in more consistent VMD and PDI, compared to other non-ionic surfactants used in transfersome formulations [45]. Additionally, Tween 80 contains a longer hydrocarbon chain and, based on other literature reports, this may contribute in generating vesicles with a smaller size. Duangjit et al. have evaluated the effect of hydrocarbon chain length on the size of lipid vesicles and reported that; as the carbon chain length increases (from C4 to C16), particle size decreases [46]. However, contrasting results were also found in literature; where different surfactants demonstrated no significant change in the size of transfersome vesicles [47]. Further investigations by our research group and by other labs are needed to provide a better understanding about the relationship between surfactant structure/geometry and the resultant particle size. Additionally, water soluble surfactants cover the surface moiety of vesicles further, preventing transfersome aggregation. Moreover, the hydrophilicity of the surfactant head group also aids in lowering vesicle size, this may be associated with the smaller/short hydrophobic backbone. These results are in agreement with studies conducted by previous researchers [46,48]. Span 80, cholesterol and BDP are lipophilic

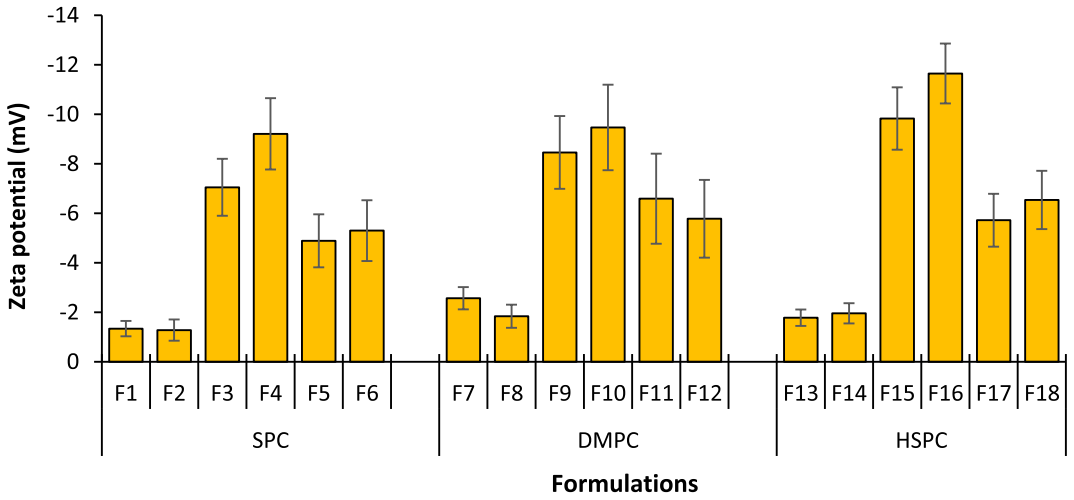

Fig. 3. Zeta potential of transfersome formulations (F1 – F18), prepared from three different phospholipids (SPC, DMPC and HSPC), three surfactants (Tween 80, Span 80 and Span 20) with and without the presence cholesterol. Data are mean ± SD, n = 3.

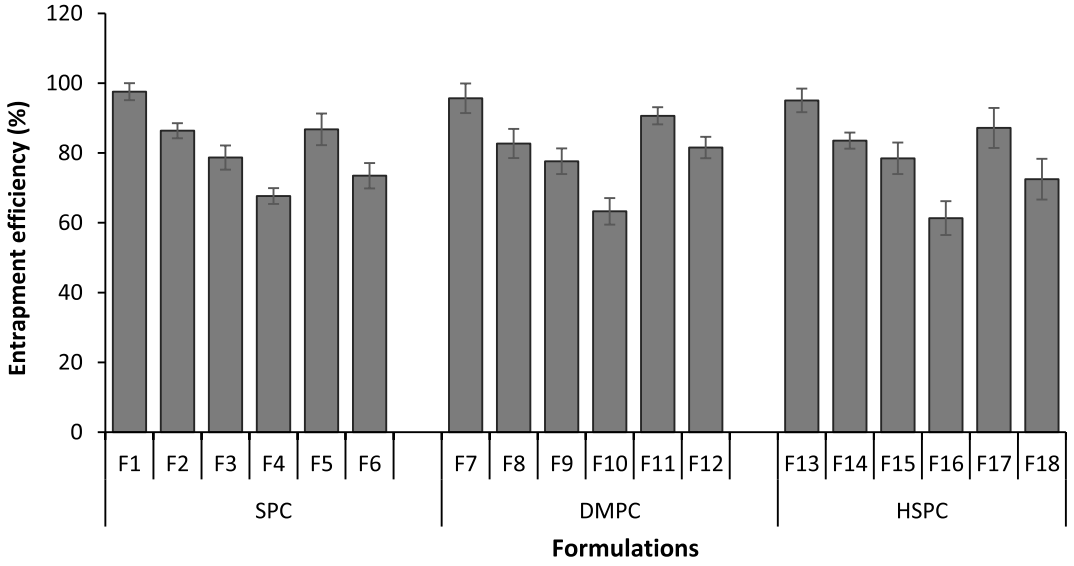

Fig. 4. Entrapment efficiency of BDP in transfersome formulations (F1 – F18), prepared from three different phospholipids (SPC, DMPC and HSPC), three surfactants (Tween 80, Span 80 and Span 20) with and without cholesterol inclusion. Data are mean ± SD, n = 3.

molecules and tend to lodge themselves within the lipid bilayers. Span 80 as an oil-soluble surfactant may interact with the lipophilic chains of the phospholipids in further addition to the presence of lipophilic substances (i.e. cholesterol and BDP), which create a further competition with Span 80 on the phospholipid alkyl chains. The packing of these molecules in the bilayer membrane might be causative of the relatively large vesicle size [33,34]. This effect of increasing particle size was also observed in multilamellar vesicles [49] as well as in unilamellar vesicles [50]. Whereas, water dispersible surfactants (e.g. Span 20) may disperse along with the transfersome vesicles within the dispersion medium, enabling slightly larger VMD, when compared to transfersomes formulated with Tween 80 surfactants (Fig. 2). The effect of surfactant on smaller to larger size of transfersomes vesicles were found in the following rank; Tween 80 < Span 20 < Span 80 (i.e. HLB values from higher to lower). However, by comparing PDI, no significant difference was observed between transfersome formulations (Fig. 2). Post sonication, stability studies were conducted for all transfersome formulations for 2 weeks at room and fridge temperatures (i.e. 25 °C and 6 °C, respectively). No significant differences ($p > 0.05$) were noted in terms of VMD, PDI and entrapment efficiency, and therefore were not included in the manuscript.

In this study, three different phospholipids and three different surfactants were employed to prepare transfersome vesicles with or without cholesterol, in order to examine their impact on particle size and PDI. Criteria determined for the selection of optimum formulation for further studies (i.e. nebulization studies) was smaller particle size and lower PDI (to fit better in the small droplets with minimal fragmentation; this was found in previous investigation to help with increasing deposition of vesicles in the lower stage of the Impinger). An additional key parameter for a good formulation is its ability to entrap the highest possible proportion of the drug. Thus, the best formulation should possess smallest vesicle size, in combination with lowest PDI and maximum drug entrapment. Thus, transfersomes formed using SPC as the phospholipid and Tween 80 as the surfactant without cholesterol demonstrated the best properties in terms of size.

3.2. Zeta potential of transfersome vesicles

Upon investigation of Zeta potential values, it was found that all the formulations had a negative charge (Fig. 3). The three phospholipids

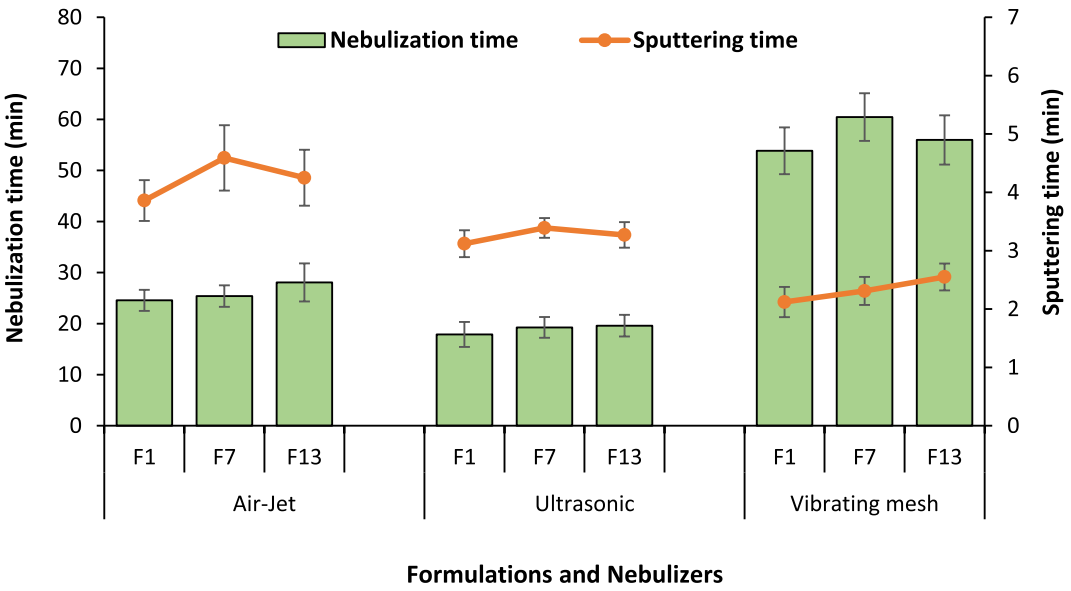

Fig. 5. Nebulization time (vertical bars) and sputtering time (horizontal lines) of BDP-loaded transfersome selected formulations (F1, F7 and F13) were determined using three nebulizers (i.e. Air-jet, Ultrasonic and Vibrating mesh). All these three formulations containing Tween 80 as a surfactant; whereas, in terms of phospholipid, F1, F7 and F13 consist of SPC, DMPC and HSPC respectively. Data are mean ± SD, n = 3.

used (i.e. SPC, DMPC and HSPC) are zwitterion compounds with an isoelectric point of 6–7, meaning that they are neutral compounds, and the surfactant used in the formulations are also non-ionic. It was also identified that some surfactants type and their concentrations greatly affect zeta potential values [7]. Moreover, formulations prepared with Span 80 demonstrated a higher negative charge (circa −7.05 ± 1.15 to −11.65 ± 1.21) than formulations prepared from Span 20 and Tween 80 (Fig. 3). The presence of a charge could be beneficial in order to keep the formulation stable, as electrostatic repulsive forces tend to reduce vesicle aggregation and fusion. However, the negative charges are not substantially high and thus may possibly be attributed to trace amounts of impurities (though excipients used are of pharmaceutical grades, the purity of phospholipids SPC, DMPC and HSPC is 94%, 98% and 98% respectively; thus the percentage impurity of phospholipids may cause a mild surface charge).

3.3. Entrapment efficiency of BDP in transfersome vesicles

Upon comparison of phospholipid effect on entrapment efficiency, no significant difference ($p > 0.05$) was found. This suggests that entrapment efficiency of BDP was not affected by phospholipid type. However, it was recognized that entrapment efficiency was highly affected by the inclusion and exclusion of cholesterol, as well selection of surfactant type (Fig. 4). Cholesterol was incorporated in vesicles in order to make them rigid by filling the gaps between the assembled phospholipids within the bilayers of the liposomes (i.e. to form more ordered membrane) and hence reduce drug leakage [39]. However, it is important to know that both cholesterol and BDP are lipophilic molecules and have a similar steroidal structure, hence compete for their accommodation in the concentric bilayers. Therefore, it is suggested that the incorporation of cholesterol decreases drug entrapment by housing in the bilayers of liposomes [16]. Similar findings were found where formulations without cholesterol exhibited significantly higher ($p < 0.05$) entrapment of BDP, when compared to transfersome vesicles with cholesterol included, irrespective of phospholipid type (i.e. SPC, DMPC and HSPC) (Fig. 4) [9]. Additionally, transfersomes are elastic/flexible vesicles (in the absence of cholesterol) and therefore during extrusion through polycarbonate membrane, they were successfully reduced in size, and hence high entrapment was achieved. Whereas, transfersomes containing cholesterol were more rigid, and hence their extrusion through membrane under pressure may have resulted in vesicle breakage and hence lower entrapment was attained due to drug leakage (Fig. 4). These results are in agreement with previous research conducted by Duangjit et al. [31].

Surfactant selection is also important in order to achieve high drug entrapment. Span 80 in transfersome vesicles demonstrated lower drug entrapment when compared to Span 20 and Tween 80. As discussed in section 3.1, lipophilic surfactants (Span 80) also compete with lipophilic molecules (i.e. BDP and cholesterol) to assemble themselves in the bilayers, which may result in a larger vesicle size, but also leave less space for BDP entrapment, providing an explanation for the low entrapment observed (an average of 78% without cholesterol and 64% with cholesterol) (Fig. 4). Furthermore, it is also suggested that Span 80 contains an unsaturated double bond in their alkyl carbon chain. The presence of double bond may bend the alkyl chain in the bilayers, and therefore lower bilayer domain available to house BDP (lipophilic drug molecule). Analogous results were also demonstrated by previous studies [51,52]. Span 20, a water dispersible surfactant demonstrated lower competition for accommodation into the vesicle structure, whereas, Tween 80 (water soluble surfactant) showed higher drug entrapment with in transfersome vesicles (an average of 96% without cholesterol and 84% with cholesterol) (Fig. 4). Similar results were also found where the employment of hydrophilic surfactants with high HLB values demonstrated high drug entrapment in vesicles [53].

In summary, transfersome vesicles prepared from SPC as a phospholipid and Tween 80 as a surfactant without cholesterol demonstrated the highest BDP entrapment. A trend of higher BDP entrapment based on surfactant was identified as; Tween 80 > Span 20 > Span 80.

3.4. Nebulization performance

Based on aforementioned characterizations, formulations with Tween 80 as a surfactant without cholesterol were carried through for testing with nebulizers. Specially, formulations F1, F7 and F13 were selected for further analysis. Air-jet, Ultrasonic and Vibrating mesh nebulizers were employed for the nebulization performance using 5 ml of transfersome formulation.

3.4.1. Nebulization and sputtering time of transfersome formulations

Nebulization to dryness was determined for transfersome

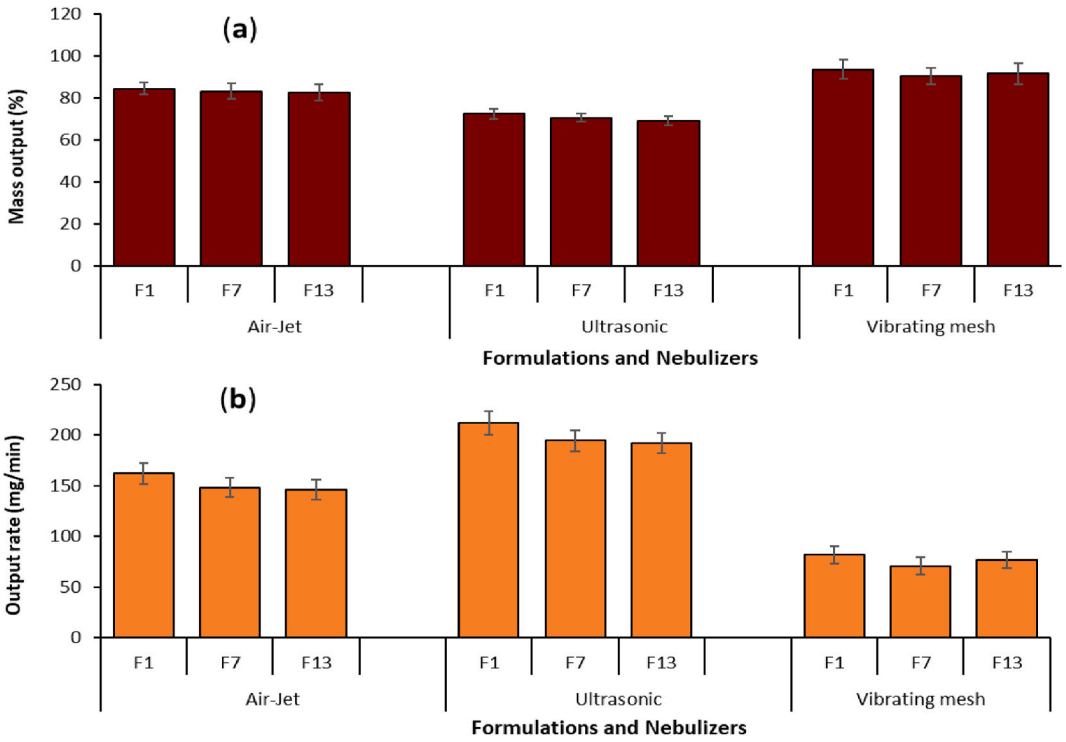

Fig. 6. (a) Mass output and, (b) output rate of BDP-loaded transfersome formulations (F1, F7 and F13) were found using three nebulizers (i.e. Air-jet, Ultrasonic and Vibrating mesh). All these three formulations containing Tween 80 as a surfactant; whereas, in terms of phospholipid, F1, F7 and F13 consist of SPC, DMPC and HSPC respectively. Data are mean ± SD, n = 3.

suspensions for all three formulations, using three nebulizers. Nebulization time is the time required for aerosolization, where the formation of the aerosol becomes erratic. Using Air-jet, Ultrasonic and Vibrating mesh nebulizers for the nebulization time of F1, F7 and F13 formulations, a significant difference was observed in terms of nebulization time ($p < 0.05$), with an order in terms of time as follows; Vibrating Mesh > Air-jet > Ultrasonic nebulizer (Fig. 5). Nebulization performance (e.g. droplet size, nebulization time, etc.) is highly dependent on formulation properties, such as; viscosity, surface tension [54,55] and type of ions in the solution [56]. These effects are interactive with the nebulizer design and its operating mechanism [55]. For vesicle-based formulations, the influence on nebulization performance is complicated and multifactorial, considering that these formulations are neither Newtonian solutions nor traditional non-Newtonian dispersions, but rather are a special type of dispersion made from liquid crystalline constituents, interplaying with a dispersion medium that is made from mainly water and partially other ingredients (e.g. free surfactant molecules that are not associated with the vesicles). For nebulization of the vesicle-based formulations, the main factors influencing nebulization performance are vesicle size, vesicle membrane rigidity (i.e. judged through lipid phase transition temperature), and nebulizer operation mechanism and design [55]. Vesicles of a large size may demonstrate prolonged nebulization time and higher residual volumes, as the vesicles have to be first fragmented by shearing within the nebulizer, in order to fit into the size of the forming aerosol droplets. Additionally, higher membrane rigidity makes it more difficult for nebulizers to break up the vesicles into smaller ones, prolonging nebulization time and reducing output. Large vesicles tend to stay in the reservoir of jet and mesh nebulizers, and block the mesh apertures of mesh nebulizer, resulting in prolongation of nebulization time [2]. Nebulization time is also dependent on nebulizer type and design, with performance of Ultrasonic and Vibrating mesh devices being more formulation-sensitive compared to Air-jet nebulizers. Transfersomes are vesicles with limited reports in literature for nebulization delivery; thus, it is worth investigating whether their particle size would influence the performance of the resultant aerosol, and whether their proposed "ultra-deformability" can ameliorate the negative influence of vesicle size on nebulization performance (time and output). High viscosity of the formulation may slow the aerosolization rate from lipid-based formulations in the nebulizer reservoir and hence can prolong nebulization time [25,55,57,58]. Moreover, the effect of surface tension on nebulization is not well understood. On one hand, it has been shown that low surface tension might be advantageous in shortening the nebulization time [55,59]. On the other hand, the presence of surfactant may cause formation of foam through aerosolization-induced shearing/vigorous mixing, which may increase the nebulization sputtering time [24]. In this study, we have focused on investigating the influence of vesicle size and bilayer composition on aerosol performance.

Employing individual nebulizers i.e. Air-jet, Ultrasonic and Vibrating mesh, no significant difference ($p > 0.05$) was found between F1, F7 and F13 formulations. However, for Vibrating mesh nebulizer, nebulization time was significantly higher ($p < 0.05$) when compared to Air-jet and Ultrasonic nebulizers (Fig. 5). This may be associated with the principle of Vibrating mesh nebulizer, where the low energy of atomization may lengthen the nebulization time [55]. Additionally, it may further cause the aggregation/fusion of nanoparticles, which may cause the blockage of mesh and hence be responsible for longer nebulization time [29].

Upon analysis, the Ultrasonic nebulizer, comparatively exhibited significantly shorter ($p < 0.05$) nebulization time (under 20 min) (Fig. 5). This is suggested to be due to the function of Ultrasonic nebulizer, where the movement generated by the piezoelectric crystal enables aerosol formation without impacting upon the transfersome vesicle size, and hence shorter nebulization time was achieved. Similar results were also reported for a shorter nebulization using an Ultrasonic nebulizer [29,60].

The nebulization time of the Air-jet nebulizer was found to be circa 26 min, significantly higher ($p < 0.05$) than the Ultrasonic nebulizer and significantly lower ($p < 0.05$) than the Vibrating mesh nebulizer (Fig. 5). The Air-jet nebulizer may increase formulation viscosity, which is associated with a decrease in temperature [13,24]. Higher viscosity of

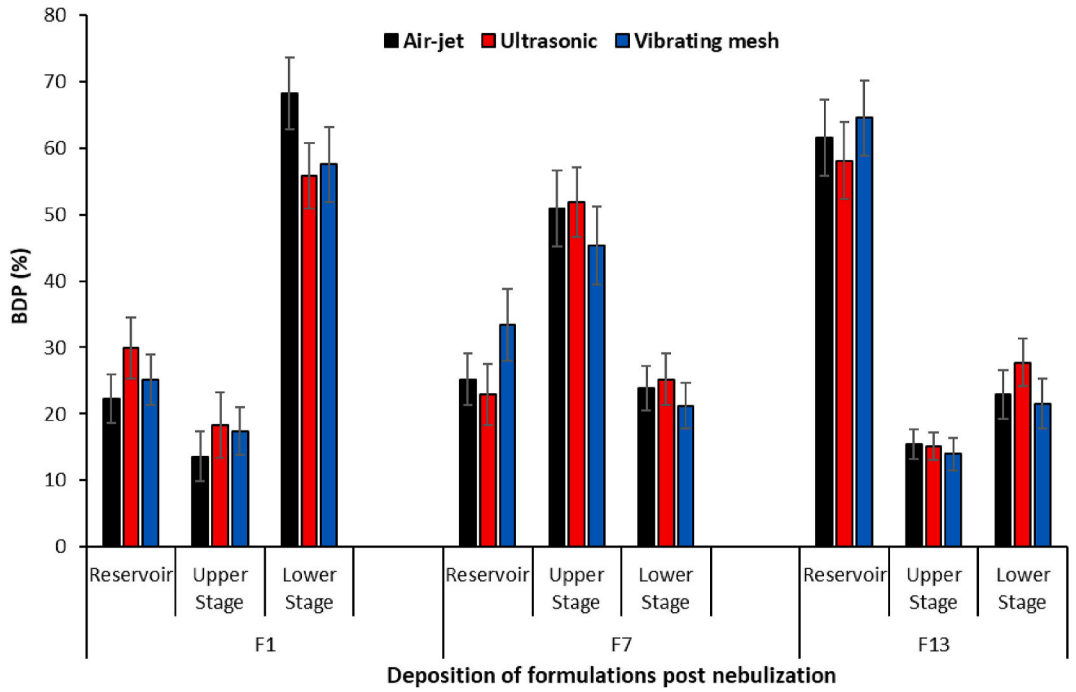

Fig. 7. Deposition of BDP in the nebulizer reservoir, upper stage and lower stage of TSI using transfersome formulations (F1, F7 and F13), employing three nebulizers (Air-jet, Ultrasonic and Vibrating mesh). All these three formulations containing Tween 80 as a surfactant; whereas, in terms of phospholipid, F1, F7 and F13 consist of SPC, DMPC and HSPC respectively. Data are mean ± SD, n = 3.

formulation may lengthen nebulization time, due to the greater adherence of the formulation (aerosol droplets) to the internal walls of Air-jet nebulizer, followed by delayed/slower deflection of formulation to the nebulizer reservoir. Comparable finding in terms of nebulization time were also demonstrated by previous studies [59,61].

Sputtering time also differed significantly ($p < 0.05$) across the different nebulizers used, whereas, no effect was observed by altering the formulation type (Fig. 5). Higher to lower sputtering time by nebulizer's type were ranked in the following order; Air-jet > Ultrasonic > Vibrating mesh. The longer sputtering time by Air-jet nebulizer is related to the higher viscosity caused by the nebulizer during aerosolization, where formulations deposited on the internal walls (after initial aersolization) took longer time for formulation deflection back into the nebulizer reservoir for the re-aerosolization process. The Vibrating mesh nebulizer retained a smaller portion of formulation following nebulization (the majority of the formulation was aerosolized and therefore took longer nebulization time) and hence demonstrated a shorter sputtering time compared to Air-jet and Ultrasonic nebulizers (Fig. 5). The Ultrasonic nebulizer generates aerosol by a mechanism called capillary wave formation followed by fragmentation. However, Ultrasonic nebulizers are not able to convert lower formulations (remaining in the nebulizer reservoir) into aerosol by capillary wave formation and therefore transfersome suspensions remained in the nebulizer (also terms as "dead or residual volume"). As a result a longer sputtering time was observed.

3.4.2. Mass output and aerosol output rate of transfersome formulations

Irrespective of nebulizer type, nebulization was carried out to dryness or complete nebulization time (combined time duration of nebulization time and sputtering time), it is not possible to achieve 100% mass output. Significantly lower ($p < 0.05$) mass output was demonstrated by the Ultrasonic nebulizer (an average of 70%) when compared to Air-jet and Vibrating mesh nebulizers (Fig. 6a). This may be related to the higher amount of residual volume remaining in the nebulizer reservoir, post nebulization. Whereas, higher mass output by the Vibrating mesh nebulizer (circa 92%) is linked with a lower residual volume (Fig. 6a).

Table 2
Nebulization performance of Air-jet, Ultrasonic and Vibrating mesh nebulizers using formulations F1, F7 and F13 employing emitted dose (ED), fine particle dose (FPD) and fine particle fraction (FPF) using TSI. Data are mean ± SD, n = 3.

Formulations	ED (%)	FPD (%)	FPF (%)
Air-jet nebulizer			
F1	81.85 ± 6.10	68.24 ± 5.38	83.37 ± 7.41
F7	74.78 ± 5.88	23.87 ± 3.33	31.92 ± 5.06
F13	38.33 ± 7.24	22.94 ± 3.64	59.83 ± 5.62
Ultrasonic nebulizer			
F1	74.08 ± 6.77	55.84 ± 4.95	75.38 ± 6.35
F7	77.01 ± 5.14	25.19 ± 3.91	32.71 ± 5.54
F13	42.86 ± 6.42	27.75 ± 3.58	64.75 ± 4.67
Vibrating mesh nebulizer			
F1	74.91 ± 7.33	57.53 ± 5.66	76.80 ± 7.17
F7	66.60 ± 6.54	21.24 ± 3.48	31.89 ± 4.38
F13	35.48 ± 5.47	21.54 ± 3.71	60.71 ± 5.54

The lower residual volume of the formulation by the Vibrating mesh nebulizer is directly linked to the design of mesh nebulizer, where a slanting position of reservoir (holding formulation) maximises its dissemination through the mesh in order to generate aerosol. Analogous results were also found by previous studies, where a lower residual volume was found for Vibrating mesh nebulizers when compared to Ultrasonic and Air-jet nebulizers [29,62,63]. Furthermore, similar results were found when liposomes as lipid-based formulations were employed for nebulization (a trend of higher to lower mass output; Vibrating mesh > Jet > Ultrasonic nebulizer) [27]. It was also identified that formulation type did not affect aerosolization and hence mass output was not compromised/affected. These results indicated that method of action and design of nebulizers influenced mass output rather than formulation type.

Upon investigation, the three nebulizers used showed significantly different ($p < 0.05$) output rates. Based on the results obtained in this study, it was identified that nebulizer performance in terms of output rate is directly related to the nebulization time and mass output. Shorter nebulization times or higher mass output, results in higher output rate

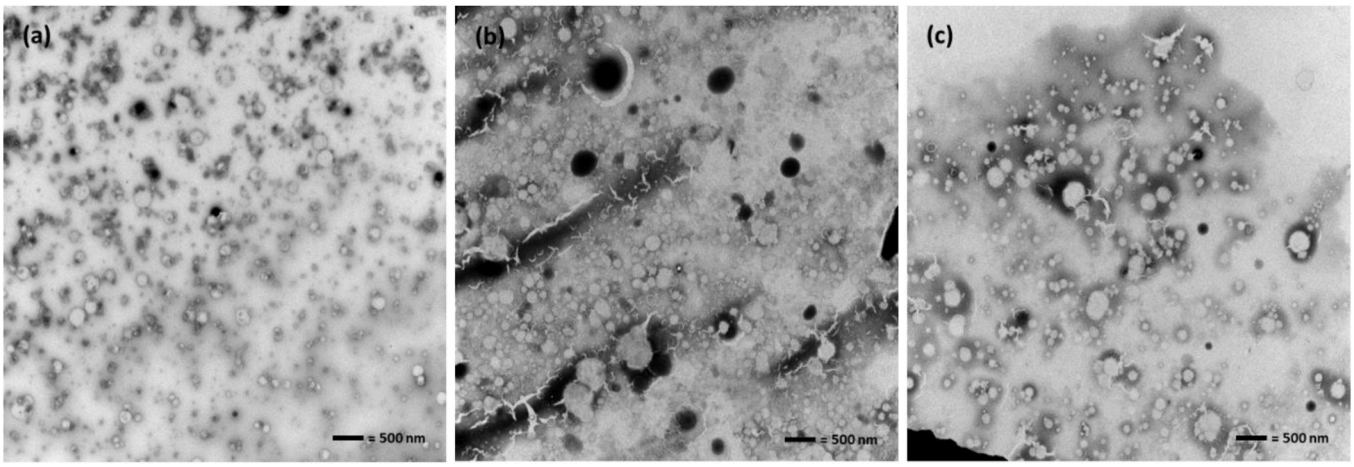

Fig. 8. TEM images of transfersome formulations, (a) F1, (b) F7, and (c) F13. These images are typical of three different experiments.

per min (Figs. 5a and 6a). The Ultrasonic nebulizer demonstrated an average output rate of circa 200 mg/min, which is a significantly higher ($p < 0.05$) output rate than both counterpart nebulizers (Fig. 6b). The Ultrasonic nebulizer demonstrated a shorter nebulization time as well as higher mass output. A significantly lower ($p < 0.05$) output rate was exhibited by the Vibrating mesh nebulizer regardless of formulation type (approximately 76 mg/min), which is 62% lower than the output rate of the Ultrasonic nebulizer (Fig. 6b). As explained, low energy atomization as well as blockage of aperture mesh plate (due to aggregation/fusion) may extend the nebulization time and hence reduce output rate per min [29]. The Air-jet nebulizer showed an output rate higher than the Vibrating mesh and lower than Ultrasonic nebulizer i.e. an average of 152 mg/min (Fig. 6b). This output rate is 24% smaller than the Ultrasonic nebulizer, and 50% higher than the Vibrating mesh nebulizer.

Overall, it was found that Ultrasonic nebulizer is superior with regards to higher mass output and output rate, regardless of formulation type (i.e. F1, F7 or F13), when compared to the Air-jet and Vibrating mesh nebulizers.

3.5. BDP deposition in two-stage Impinger (TSI), post nebulization

Post nebulization, deposition of BDP in both the upper and lower stages (also referred as emitted dose (ED)) of TSI and formulations remaining in the nebulizer reservoir containing BDP was determined. Based on formulation type i.e. F1, F7 and F13, the delivered BDP in the two stages (combined in upper stage and lower stage) regardless of nebulizer type was 77%, 73% and 39%, respectively (Fig. 7 and Table 2). Indicating that both F1 and F7 formulations are more suitable than the F13 formulation. This difference in drug delivery to the two stages of TSI may be associated with the lower phase transition temperatures of SPC and DMPC (- 20 and 23 °C) for formulations F1 and F7, when compared to the higher phase transition temperature of HSPC (i.e. 52 °C) [2]. Moreover, when using the Air-jet nebulizer, formulation F1 demonstrated a higher ED, when compared to the counterpart formulations as well as nebulizers (Table 2). Formulations with a lower phase transition temperature are easier to prepare (in an ambient/room temperature) and are more stable than formulations with a higher phase transition temperature (keeping that temperature throughout their preparation) and hence are associated with less stability (temperature change from elevated to room), which may affect the integrity of the vesicles.

Upon comparison of FPD, formulation F1 (prepared from SPC) showed a significantly higher ($p < 0.05$) BDP deposition in the lower stage of TSI (61%), representing deep lung deposition and considered as a desirable formulation (irrespective of nebulizer type). F7 and F13 formulations deposited less than half of BDP nebulized in the lower stage (Fig. 7 and Table 2). Formulation F7, demonstrated significantly higher ($p < 0.05$) BDP deposition in the upper stage of TSI (49%), and it was thus considered an appropriate formulation for achieving pharmacological action required in the upper respiratory tract (Fig. 7). This may be related to the lower phase transition temperature of the phospholipid SPC, which possibly increases bilayer fluidity and vesicle flexibility, regardless of the type of nebulizer. Whereas, the other phospholipids may make the bilayers more rigid and less flexible (related to their phase transition temperatures), and increase the retention of vesicles in the nebulizer reservoir (Fig. 7, for F13) or cause increased deposition in the upper stage of the Impinger (Fig. 7, formulation F7) for vesicles prepared from HSPC and DMPC. Contrastingly on average, 61% of BDP remained in the nebulizer reservoir for the F13 formulation, post nebulization. Formulation F1 showed significantly higher ($p < 0.05$) FPF for all three nebulizers, when compared to the F7 and F13 formulations. Additionally, a trend of higher FPF value based on nebulizer performance using F1 formulation was identified as; Air-jet > Vibrating mesh > Ultrasonic nebulizer (83%, 77% and 75%) (Table 2).

Overall, it was found that the F1 formulation is ideal for targeting deep lung deposition, and the F7 formulation for deposition in the upper area of the pulmonary system, regardless of nebulizer type.

3.6. Morphology of transfersome vesicles via TEM

TEM was used to determine the morphology of selected transfersome formulations i.e. F1, F7 and F13. Images captured suggested that transfersome vesicles were spherical to slightly oval in shape (Fig. 8). These images confirmed the successful formation of transfersome vesicles.

4. Conclusions

Novel BDP-loaded transfersomes were successfully prepared using various phospholipids (SPC, DMPC and HSPC), surfactants (Tween 80, Span 80 and Span 20) with and without cholesterol, using an extrusion method. The incorporation of Tween 80 demonstrated significantly smaller VMD, PDI, Zeta potential and higher entrapment efficiency. This indicated that physicochemical properties of vesicular system were not affected by the phospholipid type, but surfactant selection without cholesterol inclusion. Based on the aforementioned characterization, only three (i.e. F1, F7 and F13) out of the eighteen formulations developed were selected for nebulization performance. Aerosols were generated from transfersome suspensions, employing; Air-jet, Ultrasonic and Vibrating mesh nebulizers. The Ultrasonic nebulizer showed shorter nebulization time, followed by Air-jet, whereas a significantly longer time was noted for the Vibrating mesh nebulizer. The mass output from the nebulizers were less than the total mass used, suggesting

accumulation/retention of transfersome formulation in the nebulizer's reservoir, related to the design and mechanism of nebulizers aerosol generation. Additionally, output rate of formulations using Air-jet, Ultrasonic and Vibrating mesh nebulizers differed significantly, and were found in the order of Ultrasonic > Air-jet > Vibrating mesh. Based on BDP deposition in stages of TSI, the Ultrasonic nebulizer demonstrated circa 49% and 23% DDP deposition in the upper and lower stages respectively when using formulation F7; Whereas, the Air-jet nebulizer demonstrated approximately 16% and 61% of BDP deposition using F1 formulation in the upper and lower stages, respectively. Moreover, the Air-jet nebulizer exhibited higher ED and FPF, when using the F1 formulation, as compared to counterpart nebulizers and formulations. These results indicated (based on physicochemical properties, nebulizer performance and BDP deposition) that the F1 formulation paired with the Air-jet nebulizer is a suitable combination for peripheral drug deposition, whereas a combination of the F7 formulation with the Ultrasonic nebulizer is ideal for targeting the upper respiratory tract.

CRediT authorship contribution statement

Iftikhar Khan: Conceptualization, Investigation, Validation, Supervision, Writing - Original draft preparation. **Rachel Needham:** Data curation, Methodology. **Sakib Yousaf:** Investigation, Writing - Review & Editing. **Chahinez Houacine:** Investigation. **Yamir Islam:** Resources. **Ruba Bnyan:** Methodology. **Sajid Khan Sadozai:** Visualization. **Mohamed A. Elrayess:** Reviewing & Editing. **Abdelbary Elhissi:** Conceptualization, Writing - Review & Editing.

Declaration of competing interest

The authors declare no conflict of interest.

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