Proliposome Powders Prepared Using A Slurry Method For The Generation of Beclometasone Dipropionate Liposomes

[1,2]Iftikhar Khan, [1,2]Sakib Yousaf, [1,2]Sneha Subramanian, [1,2]Oshadie Korale, [1,2]Mohamed Albed Alhnan, [1,3]Waqar Ahmed, [1,4]Kevin M.G. Taylor, *[5]Abdelbary Elhissi

[1]Institute of Nanotechnology and Bioengineering, [2]School of Pharmacy and Biomedical Sciences and [3]School of Medicine and Dentistry, University of Central Lancashire, Preston PR1 2HE, United Kingdom

[4]Department of Pharmaceutics, UCL School of Pharmacy, University College London, 29-39 Brunswick Square, London WC1N 1AX, London, United Kingdom

[5]Section of Pharmaceutical Sciences, College of Pharmacy, Qatar University, P.O. Box 2713, Doha, Qatar

***Corresponding author:**

Dr Abdelbary Elhissi

Pharmaceutical Sciences Section

College of Pharmacy, Qatar University

P.O. Box 2713, Doha, Qatar

T: +974 4403 5632

E: aelhissi@qu.edu.qa or aelhissi@gmail.com

Abstract

A novel “slurry method” was described for the preparation of proliposome powders using soya phosphatidylcholine (SPC) with cholesterol (1:1) and for incorporation of beclometasone dipropionate (BDP) at 2 mole% of the total lipid phase. Proliposomes made with a range of lipid to sucrose carrier ratios were studied in terms of surface morphology using scanning electron microscopy (SEM) and thermal properties using differential scanning calorimetry (DSC). Following hydration of proliposomes, the resultant vesicles were compared to liposomes made using the traditional proliposome method, in terms of vesicle size and drug entrapment efficiency. SEM showed that sucrose was uniformly coated with lipid regardless of lipid to carrier ratio. Liposomes generated using the slurry proliposome method tended to have smaller median size than those generated with the conventional proliposome method, being in the range of 4.72 - 5.20 µm and 5.89 - 7.72 µm respectively. Following centrifugation of liposomes using deuterium oxide (D_2O) as dispersion medium, vesicles entrapping BDP were separated as a floating creamy layer, whilst the free drug was sedimented as crystals. Drug entrapment was dependent on formulation composition and preparation method. When 1:15 w/w lipid to carrier was used, liposomes generated using the slurry method had an entrapment efficiency of 47.05% compared to 18.67% for those generated using the conventional proliposome method. By contrast, liposomes made by the thin-film hydration method had an entrapment efficiency of 25.66%. DSC studies using 50 mole% BDP demonstrated that the drug was amorphous in the proliposome formulation and tended to crystallize on hydration, resulting in low drug entrapment. In conclusion, a novel approach to the preparation of proliposomes using a slurry method has been introduced, offering higher entrapment for BDP than liposomes made using the conventional proliposome method and those prepared by thin-film hydration technique.

Keywords: Characterization, Drug development, Proliposome, Liposome, Manufacture

1. Introduction

Novel inhalation therapies have been introduced to treat pulmonary disorders, particularly asthma and chronic obstructive pulmonary disease (COPD) (Momin et al. 2011). Inhalation of glucocorticoids aims for maximum deposition in the pulmonary system, leading to localised therapeutic effect in the lung and minimised systemic adverse effects (Hochhaus 2004). Liposomes have been extensively used in drug delivery for sustained-release applications (Gregoriadis 1980; Safinya and Ewert 2012). Hydrophobic drugs are typically incorporated into the lipid bilayers, whilst hydrophilic drugs are encapsulated into the aqueous spaces of liposomes (Tripathi et al. 2013).

Entrapment of hydrophobic drugs, such as steroids, in liposomes is highly dependent on chemical structure of the drug (Radhakrishnan 1990; Radhakrishnan 1991). Beclometasone dipropionate (BDP) is an inhaled steroid with well-established clinical indications. The entrapment of BDP in liposomes is highly dependent on type of phospholipid used (Batavia et al. 2001; Darwis and Kellaway 2001; Elhissi et al. 2006), type of excipients included in the formulation, preparation procedure of liposomes (Elhissi et al. 2006; Elhissi et al. 2011a) and additional processing of the formulation, such as size reduction (Darwis and Kellaway 2001; Gala et al. 2015).

Liposomes exhibit chemical instability due to oxidation and hydrolysis of the liposomal phospholipids (Hunt and Tsang 1981), resulting in leakage of the entrapped drug, and aggregation or fusion of the vesicles (Wong and Thompson 1982). These instabilities may markedly shorten the shelf-life of liposome formulations. In order to overcome such stability problems and capitalise on the potential of liposomes, proliposome technologies have been developed (Payne et al. 1986a; Payne et al. 1986b; Perrett et al. 1991). Proliposomes are

either particulate-based (Payne et al. 1986a) or ethanol solution-based (Perrett et al. 1991) formulations of phospholipid that generate liposomes upon addition of aqueous phase under appropriate conditions (e.g. above the phase transition temperature of the phospholipid chosen). Particulate-based proliposomes are powder formulations comprising carbohydrate particles coated or loaded with phospholipid to generate liposomes prior to administration (Payne et al. 1986a). The production of such proliposomes has been achieved through a number of methods, including spray drying (Alves and Santana 2004) and fluidized bed coating (Chen and Alli 1987; Kumar et al. 2001; Gala et al. 2015). However, the manufacture of proliposomes on a small scale is traditionally achieved via the feed-line method, utilising a modified rotary evaporator with a feed-line tube for the step-wise addition of an organic phospholipid solution, to coat carbohydrate carrier particles placed in a round-bottomed flask, followed by organic solvent evaporation (Payne et al. 1986a). This technique is time-consuming and may cause marked lipid losses in the feeding tube; thus, finding a simple proliposome manufacturing method that minimises wastage of drug and excipients would be highly advantageous.

In this study, a facile approach to manufacturing BDP proliposomes has been developed using a novel "slurry-based proliposome method" whereby carbohydrate carrier was dispersed in an alcoholic solution of phospholipid followed by solvent evaporation under negative pressure, to yield lipid-coated carbohydrate granules, referred to as “proliposomes”. Moreover, the influence of formulation on the entrapment of BDP in liposomes following hydration of the proliposomes has been determined and critically evaluated using deionised water (DW) or deuterium oxide (D_2O) as dispersion media.

2. Materials and methods

2.1. Materials

Sucrose, deuterium oxide (D_2O; density = 1.105 g/ml), beclometasone dipropionate (BDP) and cholesterol were purchased from Sigma-Aldrich, UK. Soya Phosphatidylcholine (SPC) (Lipoid S-100) was a gift from Lipoid, Switzerland. Ferric chloride and ammonium thiocyanate were purchased from VWR, UK. Absolute ethanol, chloroform, HPLC-grade water and HPLC-grade methanol were all supplied by Fischer Scientific Ltd., UK.

2.2. Proliposome formulation using the slurry-based proliposome method

Sucrose (particle size 300 - 500 µm) was transferred to a 100 ml round-bottomed flask. The lipid phase constituting a total amount of 250 mg (SPC and cholesterol in a 1:1 mole ratio; i.e. 166.66 mg and 83.33 mg respectively) were dissolved in absolute ethanol (175 mg/525 µl) to make a total volume of 4.51 ml. Three proliposome formulations were prepared with a range of lipid to carrier ratios (1:5, 1:10 and 1:15 w/w; i.e. 250 mg of lipid to 1250, 2500 and 3750 mg respectively). BDP was incorporated into the lipid solution at a concentration of 2 mole% (4.48 mg) of the total lipid phase, and the mixture was added to the flask. Subsequently, the flask was attached to a rotary evaporator (Buchi Rotavapor R-114, Buchi, Switzerland) and partially immersed in a water bath (45°C) (Buchi WaterbatheB-480, Buchi, Switzerland). A vacuum pump (Buchi Vac V-501) was used to facilitate the evaporation of the ethanol at a maximum rotation speed of 280 rpm for 2 h to achieve uniform coating of lipid onto the sucrose particles (i.e. formation of proliposomes). After 2 h, the negative pressure was released, the flask was detached from the rotary evaporator and the resultant proliposome granules were harvested and stored at -18°C for subsequent studies. The

relatively long time of solvent evaporation was conducted in order to ensure removal of ethanol from the formulations. Ethanol, if present above certain concentrations in liposome preparations, may cause interdigitation of the liposome bilayers (Simon and McIntosh 1984; Almeida et al. 1986), and possibly minimise entrapment of steroids such as BDP (Elhissi et al. 2006).

2.3.Proliposome formulation using feed-line (conventional) proliposome method

Sucrose (particle size 300 - 500 µm) was transferred to a 100 ml round-bottomed flask which was attached to a rotary evaporator. SPC and cholesterol were utilised as the lipid phase dissolved in ethanol (175 mg/525 µl), followed by addition of BDP (2 mole% of the lipid phase). The resultant ethanolic solution was then added in portions of 0.5 - 1 ml via the feed-line, and ethanol was removed under vacuum for 2 h at 45°C. Initially via this method, a dry proliposome formulation, at a 1:5 w/w lipid to carrier ratio was produced; subsequently, 1:10 and 1:15 w/w ratio formulations were also produced (by maintaining the lipid and BDP concentration, and increasing the carrier concentration). The proliposomes were collected and stored at -18°C for subsequent studies.

2.4. Thin lipid film formation

In the thin-film method, the same concentration of lipid phase was utilised with 2 mole% of BDP. A concentration of 175 mg/525 µl of lipid was produced using ethanol and transferred to a round-bottomed flask, which was subsequently attached to the rotary evaporator at 45°C and under vacuum conditions. Ethanol was allowed to evaporate completely over 2 h, resulting in formation of a thin lipid film for immediate hydration with aqueous phase.

2.5. Study of proliposome morphology using Scanning Electron Microscopy

Scanning electron microscopy (SEM) was employed to study the surface morphology of proliposomes. Proliposome samples or sucrose particles were sprinkled evenly onto aluminium microscopy stubs, and the surface was gold-coated with a sputter coater (JF1200 Fine Coater JEOL, Japan). Subsequently, surface morphology of the samples was examined using a scanning electron microscope (Quanta-200, FEI, Holland) and samples were photographed using the instrument's software.

2.6. Hydration of proliposomes and thin lipid films

Proliposomes prepared by the slurry-based or feed-line methods, were hydrated in a given amount of DW or D_2O at room temperature. In order to produce a liposome suspension, 50 µl of the aqueous phase was added to 30 mg proliposome powder, followed by vortex-mixing (Fisons WhirliMixer, Fisons Scientific Equipment, UK) for 2 min. The liposomes were diluted with an aqueous phase volume of 950 µl, followed by vortex-mixing for 1 min to ensure complete dissolution of the carrier particles and hydration/dispersion of the lipid. Liposomes were left for 2 h in order to anneal at room temperature. For thin-film hydration liposomes, the hydration was conducted in the same way in order to yield the same lipid concentration as that of the hydrated proliposomes. Annealing of hydrated liposomes is performed by leaving the liposomes without shaking or disruption at a temperature above the phase transition of the lipid mixture used. Annealing may help overcome possible structural defects of the liposome bilayers following lipid phase hydration (Lawaczeck et al. 1976).

2.7. Separation of liposomes via centrifugation, and visualisation via light microscopy

Following hydration in the respective media, the resultant liposomal suspensions were centrifuged using a bench centrifuge (Spectrafuge 24D, Jencons, UK). This process was carried out to aid the separation of the BDP-loaded liposomes from the unentrapped drug. Separation was optimized at 15,500 g, and light microscopy (Novex, Holland) was used to investigate whether BDP crystals were present in the samples collected from the supernatant and pellet.

2.8. Liposome size analysis by laser diffraction

The volume median diameter (VMD; 50% undersize) and span (i.e. size distribution) were measured by laser diffraction using the Malvern Mastersizer 2000 (Malvern Instruments, UK). Span was used to express the size distribution (i.e. polydispersity) of liposomes, and was calculated mathematically by the software of the instrument according to equation 1:

Span = (90% undersize – 10% undersize) / VMD Eq.1

2.9. Separation and determination of BDP incorporation into liposomes using high performance liquid chromatography (HPLC)

Following hydration in the respective medium (D_2O or DW), the resultant liposomal suspensions were centrifuged at 15,500 g (i.e. 13,000 rpm), using a bench centrifuge aiming to separate drug-loaded liposomes from the unentrapped BDP (either dissolved or as free crystals). Following centrifugation in D_2O, the BDP-loaded liposomes were isolated from the

unentrapped drug crystals and subsequently analysed by HPLC (Agilent 1200 HPLC instrument, UK). Methanol was used to disrupt the liposomes and liberate the encapsulated BDP. The drug was then assayed via HPLC by employing a mixture of methanol and DW (3:1 w/w) as the mobile phase, at a flow rate of 1.7 ml/min, and UV detection wavelength of 239 nm. An Agilent column (15 cm X 4.6 mm C-18; Agilent technology, USA) was used, and the temperature was set at 40°C with an injection volume of 20 µl. The HPLC method was adapted from those described in previous reports (Zeng et al. 2000; Batavia et al. 2001; Nasr et al. 2014).

2.10. Crystallinity studies using differential scanning calorimetery (DSC)

Differential scanning calorimetry (DSC) was used to investigate the solid state properties of components in the proliposome formulations. The thermal behaviour of each individual ingredient was analysed with respect to the proliposome formulation by DSC (DSC 823^{e}, Mettler Toledo, Switzerland). Prior to sample analysis, indium was used to calibrate the unit in triplicate. Samples were weighed individually in 40 µl aluminium pans and sealed using the provided lids (Mettler Toledo, Switzerland). Thermal data consisted of endothermic peaks and melting points (onset temperatures); the onset temperatures of each of the peaks were used to identify the specific ingredients in proliposome formulation.

2.11. Lipid quantification using the Stewart assay

The Stewart assay (Stewart 1980) was performed in order to determine the lipid content in the liposome formulations following separation of the liposomes from the continuous phase, by adapting the analytical protocol described previously (Elhissi and Taylor 2005; Elhissi et

al. 2011b). Lipid (10 mg) was dissolved in chloroform in a 100 ml round-bottomed flask, and the solvent removed using a rotary evaporator. The thin film formed in the flask was hydrated with 1 ml DW, followed by addition of 1 ml chloroform. Subsequently, the sample was dried overnight in an oven (90°C), after which it was dissolved using 100 ml chloroform to construct a calibration curve with a lipid concentration range of 80 - 160 µg/2ml (i.e. 40 - 80 µg/ml). This experiment was conducted on the liposomes dispersed in DW or D_2O following centrifugation of the dispersions.

2.12. Statistical analysis

One-way analysis of variance (ANOVA) or Student's *t-tests* were performed to allow statistical assessment of difference between more than two groups, or two sets of data respectively, using SPSS software. A *p*-value less than *0.05* was an indicator of statistically significant differences between the groups compared.

3. Results and discussion

3.1. Proliposomes morphology

SEM images of proliposomes produced using the slurry method, showed sucrose particles to be coarse, non-porous and of irregular shape (Figure 1a). The lipid to carrier ratio appeared to make the carrier surfaces smoother, especially at high lipid to carrier ratios: 1:5 w/w (Figure 1b) and 1:10 w/w (Figure 1b). These results are consistent with the observations of formulations prepared by Elhissi et al. (2011a) using the traditional feed-line proliposome technique. However, with the slurry method it was additionally observed that continuous

coating has happened even at low lipid to carrier ratio (1:15), whilst for the traditional feed-line technique, coating was continuous only at high lipid loading levels (Elhissi et al. 2011a). This suggests that the slurry method provided a more uniform coating compared to the conventional feed-line proliposome method. The incomplete coating with the traditional method might be attributed to partial loss of the lipid in the feed-line during coating or uneven distribution of the lipid dripped/sprayed onto the carrier particles via the feeding tube (Elhissi et al. 2011a).

3.2.Size analysis of liposomes generated from proliposomes

Liposomes produced following hydration and dispersion of sucrose-based proliposomes manufactured via the slurry method ranged in median size from 4.72 to 5.2 µm (Table 1). No statistically significant difference ($p>0.05$) in VMD was found between formulations having different lipid to carrier ratios (Table 1). By contrast, the VMDs of liposomes generated from proliposomes prepared using the traditional feed-line method were significantly different ($p<0.05$), exhibiting a smaller size for the 1:5 w/w ratio formulation (5.89 $\pm$ 0.39 µm), in comparison to the 1:10 and 1:15 (7.72 $\pm$ 0.24 and 7.10 $\pm$ 0.25 µm respectively). The difference in the measured liposome size between the traditional proliposome and the slurry methods might be attributed to the different coating patterns of the carrier as reported in the previous section. Liposomes generated by thin-film hydration had mean VMD of 5.51 $\pm$ 0.56 µm. Median liposome size and variability between formulations were found to be smallest when the slurry method was used, which might be attributed to the uniform carbohydrate coating with the lipid, facilitating efficient hydration upon addition of the aqueous phase (Table 1; Figure 1). Consequently, the slurry method seems very useful for manufacturing proliposomes that, on hydration, they can generate liposomes having relatively small and

consistent size measurements compared to the other two methods. Considering size distribution (i.e. span measurements) a negligible difference ($p>0.05$) was noted between the formulations examined, indicating similar heterodispersed size irrespective of the method used to prepare the liposomes (Table 1). Noteworthy, other investigators have shown that BDP liposomes in the micrometres size range (similar to the size of liposomes used in the present investigation) can be effectively delivered by jet-nebulisation to the lungs of healthy human volunteers (Saari et al. 1999). Recent *in vitro* nebulisation studies using aqueous suspensions have shown that nebuliser output and "respirable" fractions of the delivered aerosol were highly dependent on particle size of the suspension and mechanism of operation of the nebulizer (Najlah et al. 2014). In general, larger particles are more resistant to nebulisation, especially when ultrasonic nebulisers are used (McCallion et al. 1996; Najlah et al. 2014). Further studies should be conducted in the future to investigate whether size differences of liposomes made using the slurry method compared to the traditional proliposome technique could translate into different performance profiles *in vivo*, especially in the field of pulmonary delivery.

3.3.Separation of BDP-loaded liposomes from free BDP crystals

As shown in Figure 2a, proliposomes are typically hydrated and annealed using deionised water (DW). Following centrifugation for separation in DW, liposomes were found to sediment at the bottom of the centrifuge tube, allowing for their collection for analysis (Figure 2b) (Meisner et al. 1989; Taylor et al. 1990; Ma et al. 1991). When BDP is used in liposome formulations, DW as a dispersion medium may cause the unentrapped BDP crystals and liposomes (containing the entrapped drug) to sediment simultaneously upon centrifugation (Batavia et al. 2001). This may result in erroneous inclusion of the free crystals

as part of the drug entrapped, resulting in an over-estimation of BDP entrapment. Previous literature postulated that a higher density dispersion medium may aid at separation of liposomes from BDP crystals upon centrifugation. Deuterium oxide (D_2O), also referred to as heavy water (density at 20°C is 1.053 g/ml) has higher density than DW (density at 20°C is 0.9982 g/ml). Thus, D_2O was proposed as a potentially suitable liposome dispersion medium that can allow more reliable separation for accurate quantification of the entrapped and unentrapped drug proportions (Weast 1988; Batavia et al. 2001). It is important to bear in mind that deuterium oxide (D_2O) is toxic in high quantities (e.g. in amounts exceeding 20% of animal's body weight) (Kushner et al. 1999), and its use in the present study was confined to determination of drug entrapment and within limited volumes of 1.5 ml. If further investigations are to be carried out on cell lines or *in vivo*, established aqueous media such as saline or buffer solutions should be used to hydrate the proliposomes.

Analysis of liposomes, separated by centrifugation in D_2O, showed clear differences in the distribution of the formulation components, with liposomes being separated as a floating "creamy" layer (Figure 2c). By contrast, dispersion in DW followed by centrifugation caused the liposomes to sediment as a pellet at the bottom of the tube (Figure 2b). BDP was preferentially accumulated as a pellet (also referred to as a spot (i.e. BDP crystals) at the bottom of the tube when D_2O was employed (Figure 2c); this is attributed to the crystals having higher density than D_2O dispersion medium while liposomes have a lower density. This mode of separation (i.e. distinct layers) was observed for liposomes generated using all three different methods (Figure 2).

3.4.Confirmation of separation via light microscopy

Light microscopy was employed to substantiate the proposal of D_2O being a superior separation medium than DW. For this reason, analysis of the three defined layers (Figure 2c) was conducted, showing a clear separation of liposomes (loaded with BDP) in the floating layer, and complete sedimentation of BDP crystals as a spot at the bottom of the tube, post-centrifugation. Figure 3a clearly shows free sedimented BDP crystals, whilst Figure 3b shows liposomes from the upper layer (with the entrapped BDP), indicating that separation was successful using D_2O as dispersion medium.

The middle layer (Figure 2c) was subjected to the Stewart assay to determine the content of phospholipid in the D_2O-dispersed samples (Figure 2c), to ensure that the separation conditions using D_2O were optimal. Three different centrifugation forces were employed in this investigation (i.e. 11,100, 13,200 or 15,500 g for 90 min; i.e. 11,000, 12,000 or 13,000 rpm respectively) to determine which speed would provide the most complete separation between liposomes (with the entrapped drug fraction) and BDP crystals and water-dissolved drug (representing the unentrapped proportion of BDP). The minimum concentration of lipid was established via the Stewart assay in the middle layer, indicating that liposomes were not any more present in the bulk of the dispersion. Centrifugation under these parameters demonstrated optimum separation of BDP-loaded liposomes from the unentrapped free BDP crystals. It is proposed that the middle layer may contain dissolved or free BDP crystals which failed to sediment.

As shown in Table 2, the lowest centrifugation speed (equivalent to 11,000 g) may not be able to achieve complete separation since the relatively greatest proportion of lipid was still in the bulk of the dispersion. The Stewart assay conducted to quantify the lipid concentration in the middle aqueous region, following centrifugation in D_2O, indicated that 15,500 g for 90

min was optimal for separating liposomes from unentrapped (i.e. free) BDP crystals (Table 2). These conditions showed an insignificant amount of lipid in the unentrapped component following centrifugation in D_2O.

While taking samples for light microscopy study or HPLC analysis, it is important to aspirate the samples slowly and carefully using a Gilson Pipette. Quick or careless aspirations of the floating "creamy" layer may result in mixing of the middle aqueous phase with the floating liposomes or can cause disruption of the sedimented BDP crystals, which can lead to inaccurate findings.

3.5.Determination of BDP crystallinity via DSC

DSC was employed to investigate whether BDP in the proliposome formulations was crystalline or amorphous. The sharp endothermic peaks of individual components of the proliposome formulation: BDP, cholesterol and sucrose are shown in Figure 4. Peaks indicating melting point, were observed at 186.60°C for sucrose and 104.59°C for BDP in addition to three polymorph endothermic peaks which were observed for cholesterol, at 95.96, 122.61 and 147.01°C. The initial endothermic peaks for cholesterol may have exhibited loss of water; however the liquid crystalline phase is stable over the temperature range of 122.61 to 147.01°C. Whilst the presence of three endothermic peaks were in concordance with findings by Loomis et al. (1979), the actual values differed (86, 123 and 157°C). This might be attributed to differences in the experimental conditions or equipment used, considering that the present investigation has been done using a modern instrument compared to that used by Loomis and co-workers (1979). Cholesterol was incorporated in order to enhance formulation stability, since cholesterol may fill the gaps between the assembled phospholipid within the bilayers; this may reduce drug leakage (Kirby et al. 1980).

The DSC scan for proliposomes exhibited an endothermic peak for sucrose at 198.69°C, and three peaks associated with cholesterol were observed at 134.04, 141.23 and 172.46°C (Figure 4). Low BDP concentration, i.e. 2 mole%, in the proliposome formulation did not produce a visible endotherm on the DSC trace. Consequently, the concentration of BDP was increased to 50 mole% to clarify whether the absence of a BDP endothermic peak was concentration dependent. Even at this high drug concentration, the melting point for BDP was not detectable, possibly indicating its amorphous structure in the proliposome formulation. Additionally, the shift change in melting points for cholesterol and sucrose might be attributed to the presence of other constituents in the formulations. Adherence of BDP to the surface of lipid coating the sucrose particles could inhibit the crystallanity of BDP. This might be attributed to the solubility of the steroid in the high lipid concentration used (Guan et al., 2011). This further confirms that on hydration of proliposomes, BDP has crystallized, resulting in sedimentation upon centrifugation. The employment of light microscopy and DSC has provided a valuable insight into the behaviour of BDP in hydrated and dry formulations respectively.

3.6.Determination of drug entrapment efficiency using D_2O or DW

In the traditional approach to quantifying entrapment efficiency in liposomes, the sedimented pellet is usually assumed to comprise liposomes and the entrapped fraction of the drug, while the unentrapped drug remains in the supernatant. When DW was used as the dispersion medium, the unentrapped BDP present in the supernatant constituted less than 5% of the total BDP originally included in the sample (Figure 5); this was attributed to concomitant sedimentation of BDP crystals with the liposomes upon centrifugation, hence the high

apparent entrapment efficiency was a result of inadequate separation of free drug from liposome-incorporated BDP.

By contrast, when D_2O was used to disperse liposomes, much better separation was achieved, as demonstrated using light microscopy and the Stewart assay. The complete separation of drug loaded in liposomes from unentrapped BDP crystals (Figure 2b and c), provided a means for reliable and accurate determination of drug entrapment following centrifugation. The middle layer (representative of a proportion of the unentrapped drug fraction in D_2O) contained less than 10% of the originally included BDP in all formulations (Table 2). Figure 6 compares the effect of preparation method on BDP entrapment efficiency. Although the thin film hydration method did not use carbohydrate carriers, values generated in terms of entrapment efficiency were still directly compared to entrapment efficiencies of formulations prepared from the feed-line and slurry proliposome methods. Both feed-line and slurry methods utilised carbohydrates and lipid in the manufacture of proliposomes. In this research, whilst lipid phase (1:1 ratio of SPC and cholesterol) and BDP concentration (2 mole%) were kept constant, carbohydrate carrier concentration was varied, producing formulations with lipid to carrier ratios of 1:5, 1:10 and 1:15 w/w.

HPLC analysis for 1:5 lipid to carrier ratio, identified the pellet composition for the feed-line method as having the lowest BDP proportion (51.25 $\pm$ 2.81%), followed successively by the thin-film (72.61 $\pm$ 1.84%) and slurry-based methods (74.16 $\pm$ 8.66%) (Figure 6). The difference in pellet BDP incorporation for the feed-line method was significantly lower ($p<0.05$) than values for the slurry-based proliposomes and thin-film methods. In general, the high proportion of unentrapped BDP may be attributed to the poor stearic fit of BDP within

the liposome bilayers (Radhakrishnan 1990; Radhakrishnan 1991); and may be affected by excipients and method used to manufacture liposomes (Elhissi et al. 2006).

With 1:10 lipid to carrier ratio, higher concentrations of free BDP crystals were found in the pellet, with BDP incorporation for the thin film method being significantly higher ($p<0.05$) than that of the feed-line and slurry methods. For the 1:15 w/w ratio, a higher ($p<0.05$) entrapment and lower free BDP crystal concentration was found in liposomes prepared using the slurry-based method, when compared to liposomes generated from the thin film and feed-line method (Figure 6). The layer of unentrapped BDP (i.e. soluble drug in the middle region) showed that BDP proportion was less than 5% for all three lipid concentrations used, irrespective of liposome manufacturing method. The entrapment efficiency values were dependent on lipid to carrier ratio, hence, compared to the other two methods, the slurry proliposome method provided superior drug entrapment at the lowest lipid to carrier ratio (1:15 w/w) (Figure 6). Thus, the slurry method could provide higher drug entrapment when formulation was optimized. By contrast, BDP entrapment by liposomes made by the thin film method was least dependent on lipid to carrier ratio (Figure 6).

All formulations exhibited entrapment efficiency values in excess of 95% when DW was employed as a separation media (Figure 5). The entrapment efficiency values expressed when DW was used has been demonstrated not to be reflective of the actual entrapment efficiency. This is due to the presence of unentrapped BDP crystals in the sedimented liposomes at the bottom of the tube, following centrifugation. The presence of these crystals may not only be attributed to the type of media used, but also to the size similarities exhibited by both BDP crystals and liposomes, which are subjected to the same relative centrifugation force (Batavia et al. 2001).

Overall, the slurry-based proliposomes constitutes a superior approach to generating liposomes, exhibiting enhanced BDP entrapment in the resulting vesicles, giving rise to higher entrapment values for the slurry-based method, followed by feed-line proliposome and thin-film hydration methods. However, the superior entrapment provided by liposomes prepared using the slurry proliposome approach was dependent on formulation, particularly lipid to carbohydrate carrier ratio. Further investigations in the future are needed to explore the potential of slurry-based proliposomes using hydrophilic drugs, amphipathic molecules (e.g. peptides) and other hydrophobic therapeutic agents in various drug delivery applications.

4. Conclusions

A slurry proliposome method has been described, involving coating soya phosphatidylcholine and cholesterol (1:1) onto sucrose carrier particles. The resultant liposomes, following hydration and dispersion, were smaller than those generated with the conventional feed-line proliposome method, and the proportion of drug entrapped, upon formulation optimization, was higher in these vesicles compared to those generated from conventional proliposomes and vesicles produced by the thin-film method. Following this study the potential of slurry-based proliposomes should be explored in various fields of drug delivery such as pulmonary, nasal and transdermal.

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References

Almeida LM, Vaz WL, Stumpel J, Madeira VM, 1986. Effect of short-chain primary alcohols on fluidity and activity of sarcoplasmic reticulum membranes. Biochemistry 25, 4832-4839.

Alves GP, Santana MHA. 2004. Phospholipid dry powders produced by spray drying processing: Structural, thermodynamic and physical properties. Powder Technology 145, 139-148.

Batavia R, Taylor KMG, Craig DQM, Thomas M. 2001. The measurement of beclomethasone dipropionate entrapment in liposomes: a comparison of a microscope and an HPLC method. Int J Pharm 212, 109-119.

Chen CM, Alli D. 1987. Use of fluidized bed in proliposome manufacturing. J Pharm Sci 76, 419.

Darwis Y, Kellaway IW. 2001. Nebulisation of rehydrated freeze-dried beclomethasone dipropionate liposomes. Int J Pharm 215, 113-121.

Elhissi A, Taylor KMG. 2005. Delivery of liposomes generated from proliposomes using air-jet, ultrasonic and vibrating-mesh nebulisers. J. Drug Del. Sci.Tech 15, 261-265.

Elhissi AM, O'Neill MA, Roberts SA, Taylor KMG. 2006. A calorimetric study of dimyristoylphosphatidylcholine phase transitions and steroid-liposome interactions for liposomes prepared by thin film and proliposome methods. Int J Pharm 320, 124-130.

Elhissi AMA, Ahmed W, McCarthy D, Taylor KMG. 2011a. A study of size, microscopic morphology, and dispersion mechanism of structures generated on hydration of proliposomes. J Dispers Sci Tech 33, 1121-1126.

Elhissi A, Gill H, Ahmed W, Taylor KMG. 2011b. Vibrating-mesh nebulization of liposomes generated using an ethanol-based proliposome technology. J Liposome Res 21, 173-180.

Gala RP, Khan I, Elhissi AM, Alhnan MA., 2015. A comprehensive production method of self-cryoprotected nano-liposome powders. Int J Pharm 486, 153-158.

Gregoriadis G. 1980. Tailoring liposome structure. Nature 283, 814-815.

Guan P, Lu Y, Qi J, Niu M, Lian R, Hu F, Wu W. 2011. Enhanced oral bioavailability of cyclosporine A by liposomes containing a bile salt. Int J Nanomedicine 6, 965-74.

Hochhaus G. 2004. New developments in corticosteroids. Proc Am Thorac Soc 1, 269-274.

Hunt CA, Tsang S. 1981. α-Tocopherol retards autoxidation and prolongs the shelf-life of liposomes. Int J Pharm 8, 101-110.

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

Kumar R, Gupta RB, Betageri GV. 2001. Formulation, characterization, and in vitro release of glyburide from proliposomal beads. Drug Deliv 8, 25-27.

Kushner DJ, Baker A, Dunstall TG. 1999. Pharmacological uses and perspectives of heavy water and deuterated compounds. Can J Physiol Pharmacol 77, 79-88.

Lawaczeck R, Kainosho M, Chan SI, 1976. The formation and annealing of structural defects in lipid bilayer vesicles. Biochim Biophys Acta 443, 313-330.

Loomis CR, Shipley GG, Small DM. 1979. The phase behavior of hydrated cholesterol. J Lipid Res 20, 525-535.

Ma L, Ramachandran C, Weiner ND. 1991. Partitioning of an homologous series of alkyl p-aminobenzoates into multilamellar liposomes: effect of liposome composition. Int J Pharm 70, 209-218.

McCallion ONM, Taylor KMG, Thomas M, Taylor AJ, 1996. Nebulisation of monodisperse latex sphere suspensions in air-jet and ultrasonic nebulisers. Int J Pharm 133, 203-214.

Meisner D, Pringle J, Mezei M. 1989. Liposomal pulmonary drug delivery I. In vivo disposition of atropine base in solution and liposomal form following endotracheal instillation to the rabbit lung. J Microencapsul 6, 379-387.

Momin MN, Hedayati A, Nokhodchi A. 2011. Investigation into alternative sugars as potential carriers for dry powder formulation of budesonide. Bioimpacts 1, 105-111.

Najlah M, Parveen I, Alhnan MA, Ahmed W, Faheem A, Phoenix DA, Taylor KM, Elhissi A, 2014. The effects of suspension particle size on the performance of air-jet, ultrasonic and vibrating-mesh nebulisers. Int J Pharm 461, 234-241.

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

Payne NI, Timmins P, Ambrose CV, Ward MD, Ridgway F. 1986a. Proliposomes: a novel solution to an old problem. J Pharm Sci 75, 325-329.

Payne NI, Browning I, Hynes CA. 1986b. Characterization of proliposomes. J Pharm Sci 75, 330-333.

Perrett S, Golding M, Williams WP. 1991. A simple method for the preparation of liposomes for pharmaceutical applications: characterization of the liposomes. J Pharm Pharmacol 43, 154-61.

Radhakrishnan R. 1990. Novel liposome composition for sustained release of steroidal drugs in lungs: US Patent 4906476 A.

Radhakrishnan R. 1991. Novel liposome composition for the treatment of interstitial lung diseases: US Patent 5049389 A.

Saari M, Vidgren MT, Koskinen MO, Turjanmaa VMH, Nieminen MM, 1999. Pulmonary distribution and clearance of two beclomethasone formulations in healthy volunteers. Int J Pharm 181, 1-9.

Safinya CR, Ewert KK. 2012. Materials chemistry: liposomes derived from molecular vases. Nature 489: 372-374.

Simon SA, McIntosh TJ, 1984. Interdigitated hydrocarbon chain packing causes the biphasic transition behaviour in lipid/alcohol suspensions. Biochim Biophys Acta 773, 169-172.

Stewart JCM. 1980. Colorimetric determination of phospholipids with ammonium ferrothiocyanate. Anal Biochem 104, 10-14.

Taylor KMG, Taylor G, Kellaway IW, Stevens J. 1990. The stability of liposomes to nebulisation. Int J Pharm 58, 57-61.

Tripathi G, Chaurasiya K, Katare P. 2013. Liposomal current status, evaluation and current advances. Int J Curr Pharm Res 5, 4-14.

Weast RC. 1988. Handbook of Chemistry and Physics. Boca Raton, Florida: CRC Press, Inc.

Wong M, Thompson TE. 1982. Aggregation of dipalmitoylphosphatidylcholine vesicles. Biochemistry 21, 4133-4139.

Zeng XM, Pandhal KH, Martin GP. 2000. The influence of lactose carrier on the content homogeneity and dispersibility of beclomethasone dipropionate from dry powder aerosols. Int J Pharm 197, 41-52.

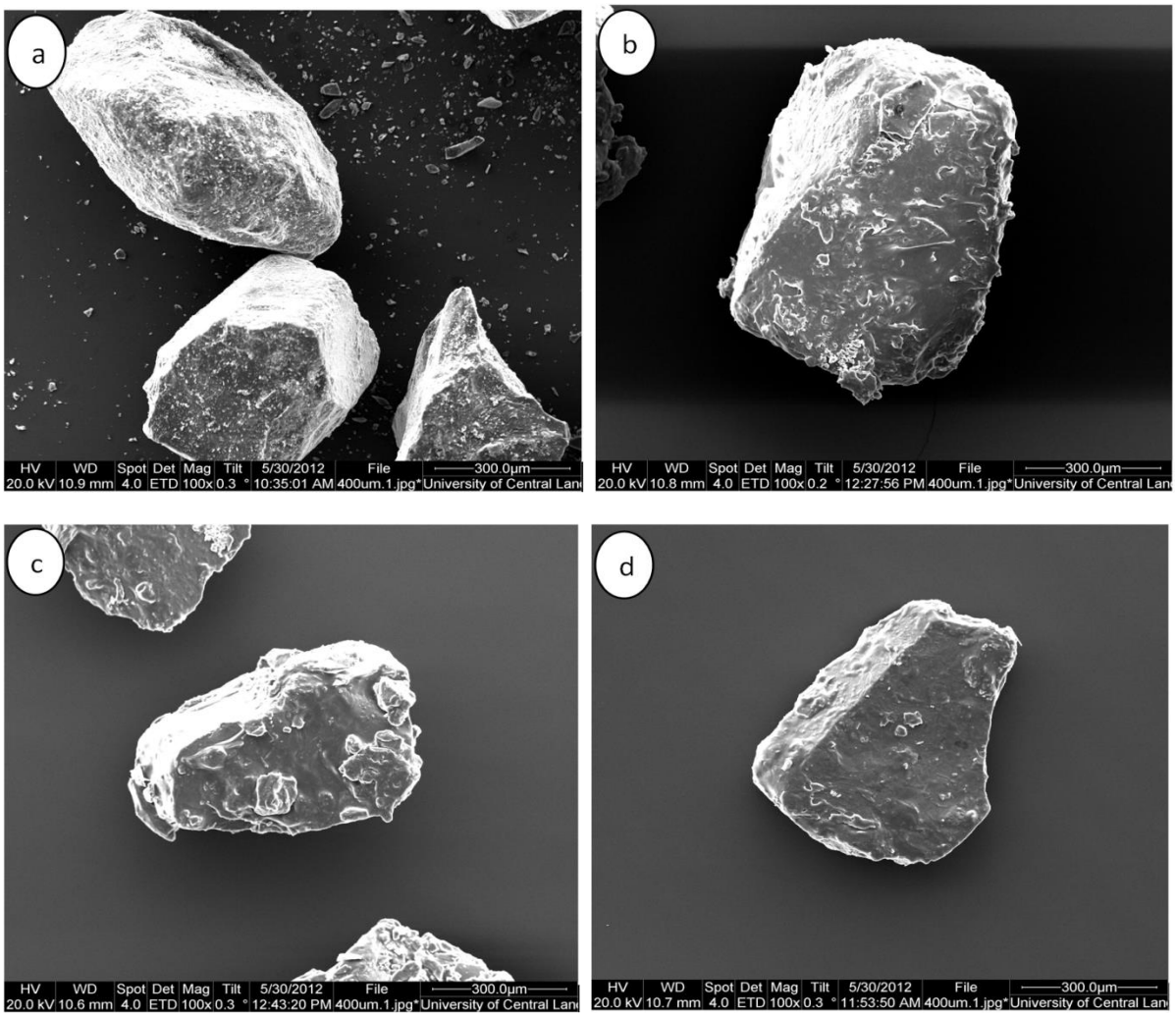

Figure 1: SEM images of sucrose-based proliposomes formulated via the slurry method with a range of lipid to carrier ratios; coarse sucrose prior to loading with lipid (a) proliposomes made using sucrose carriers formulated in a 1:5 w/w lipid to carrier ratio (b), proliposomes made using 1:10 w/w lipid to carrier ratio (c) and proliposomes made using 1:15 w/w lipid to carrier ratio (d)

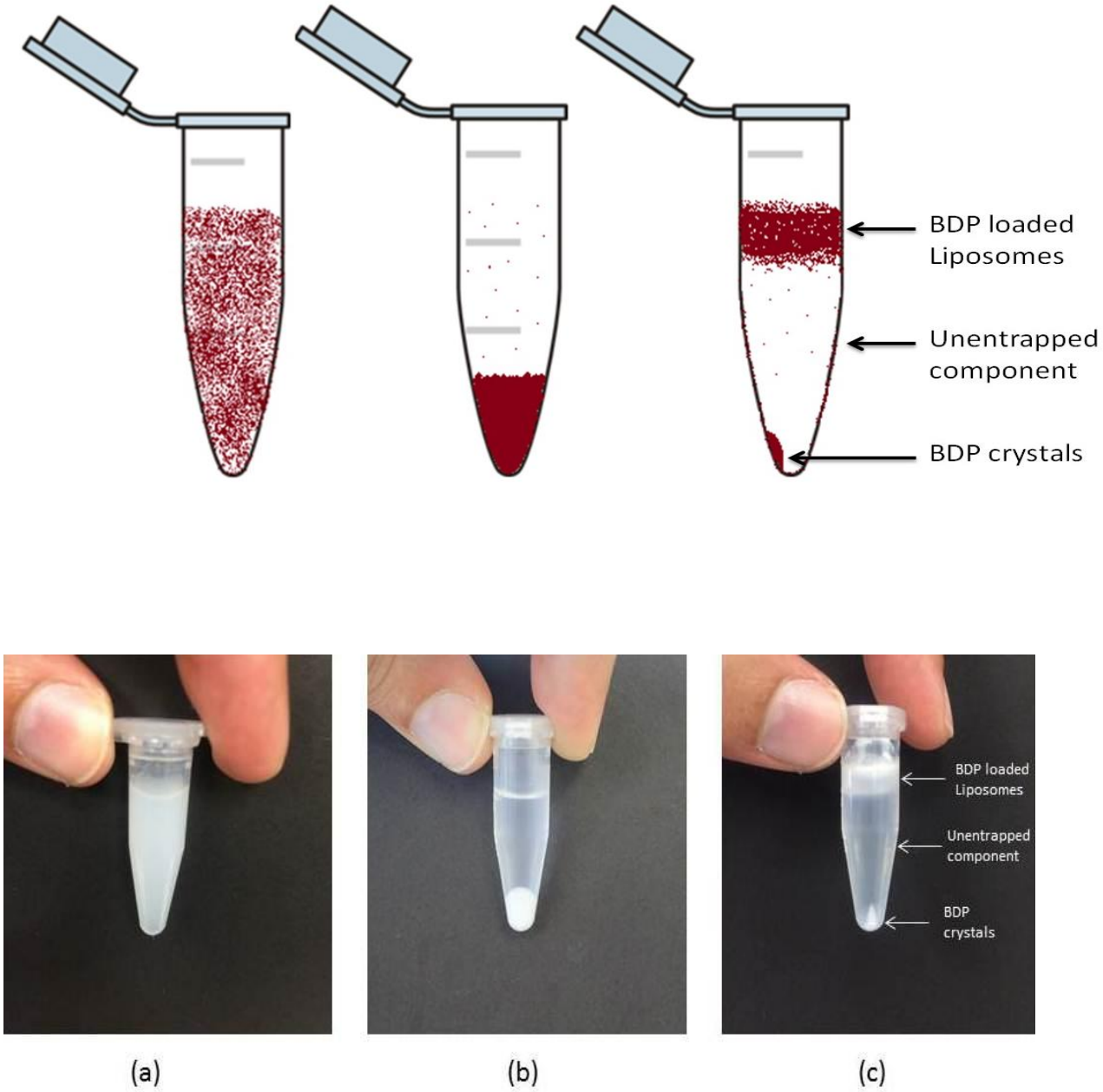

Figure 2: Liposome separation using DW or D_2O as dispersion media. The dispersion prior to centrifugation when liposomes were uniformly distributed in the dispersion medium (a), whilst following centrifugation in DW, both liposomes and BDP (entrapped and free) were sedimented at the bottom of the tube (b). By contrast, when D_2O was used as dispersion medium, liposomes floated on the surface (with the entrapped BDP) whilst drug crystals (i.e. part of the unentrapped drug) was sedimented, and some drug was associated with the clear aqueous phase in the middle region (c). The aqueous phase (in between the floating

lipid layer and the drug sediment) may add to the unentrapped fraction of the drug (in the soluble form or as crystals that failed to sediment)

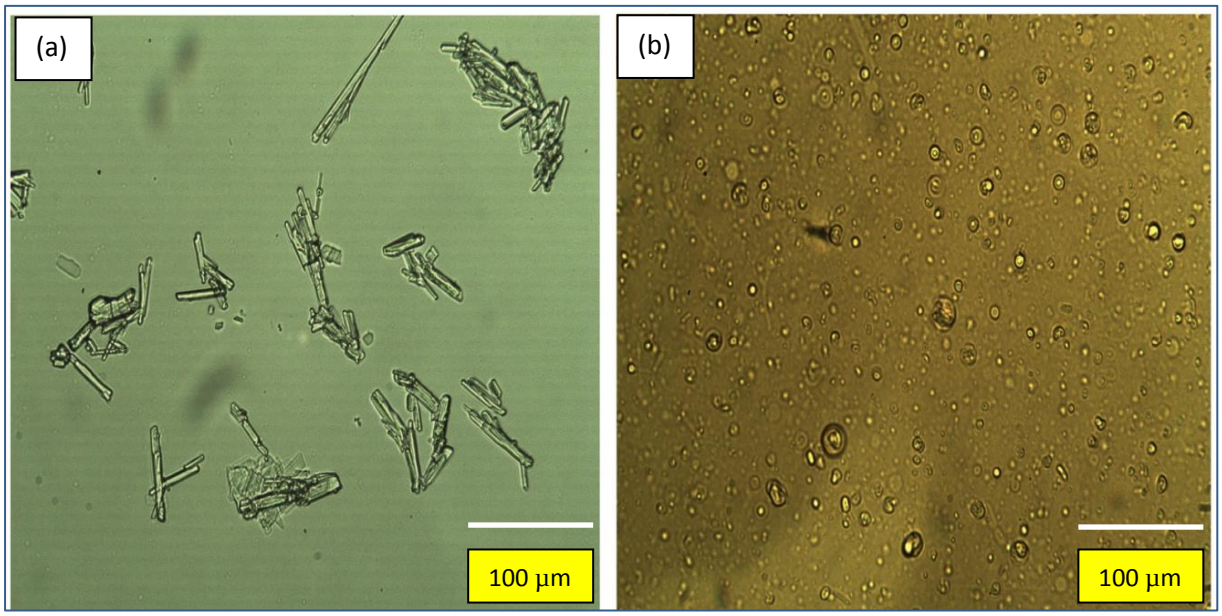

Figure 3: Following centrifugation in D_2O at 15,500 g for 90 min, light microscopy images showed the presence of BDP crystals in the sedimented pellet (a), while no crystals were seen in the floating "creamy" supernatant which was rich with liposome vesicles (b).

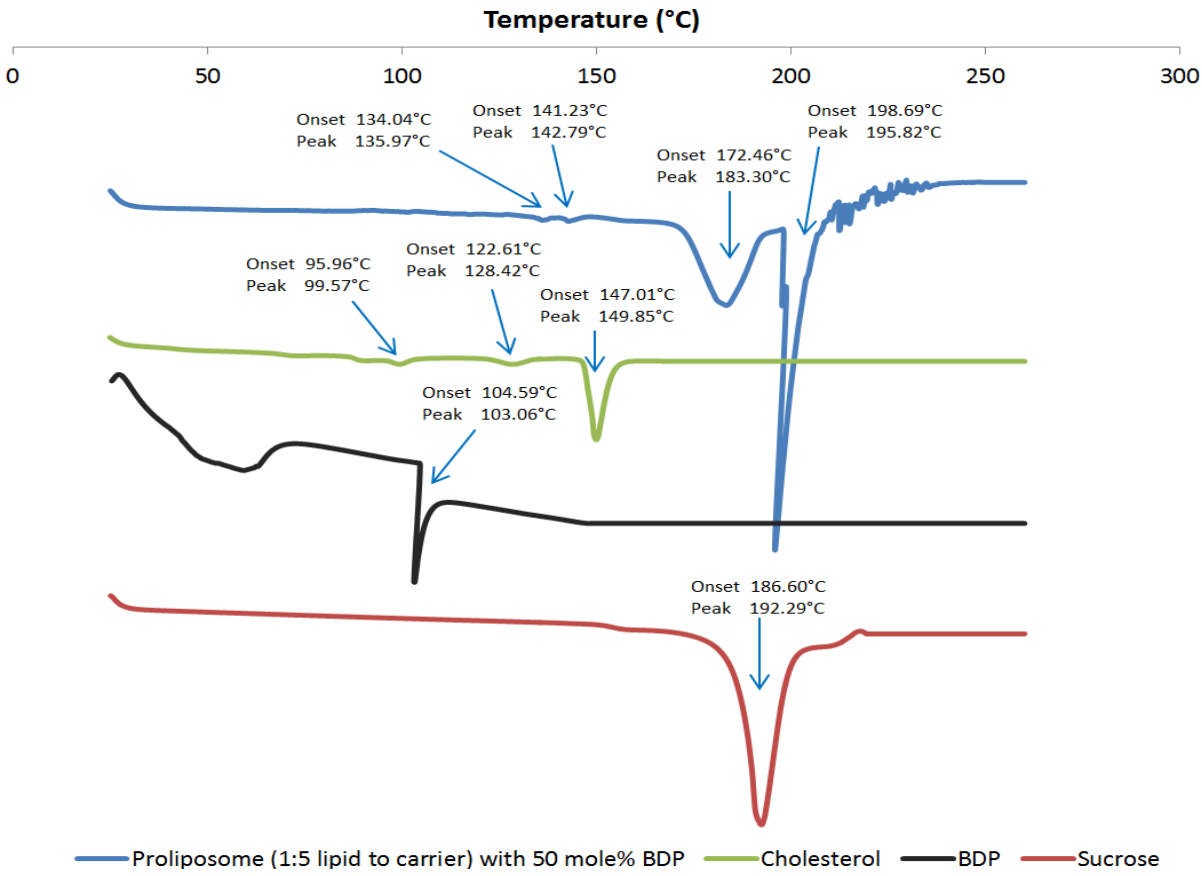

Figure 4: Superimposed DSC thermographs of BDP, cholesterol and sucrose compared with proliposome with 50 mole% BDP, showing the absence of an endothermic peak of the drug in the proliposome formulation.

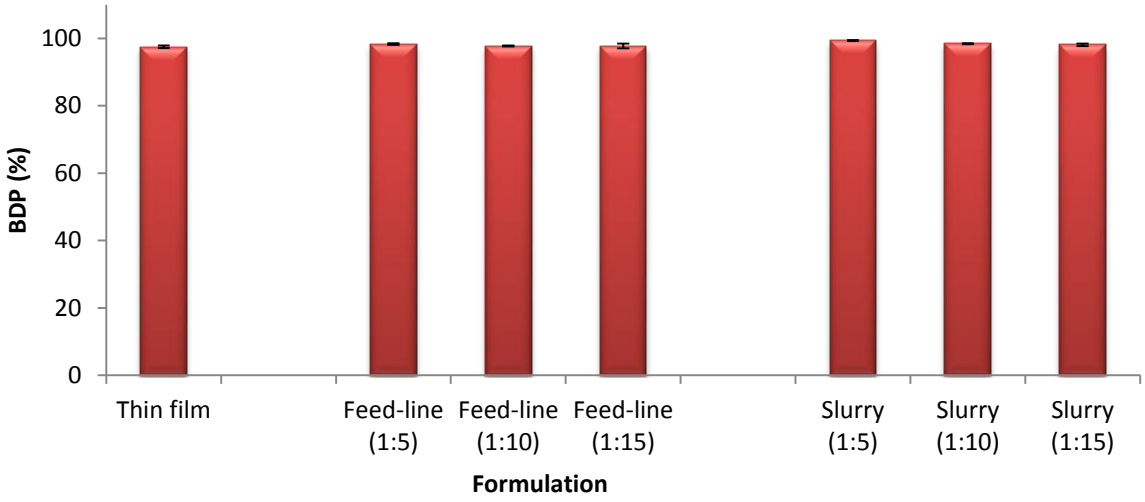

Figure 5: Apparent entrapment efficiency of BDP in liposomes post-centrifugation in DW of multiple formulations prepared using the thin-film method, and from proliposomes prepared by feed-line and slurry methods. The proliposome formulations were made using the following lipid to carrier ratios: 1:5, 1:10 and 1:15 w/w. Data are expressed as mean values (n=3± sd)

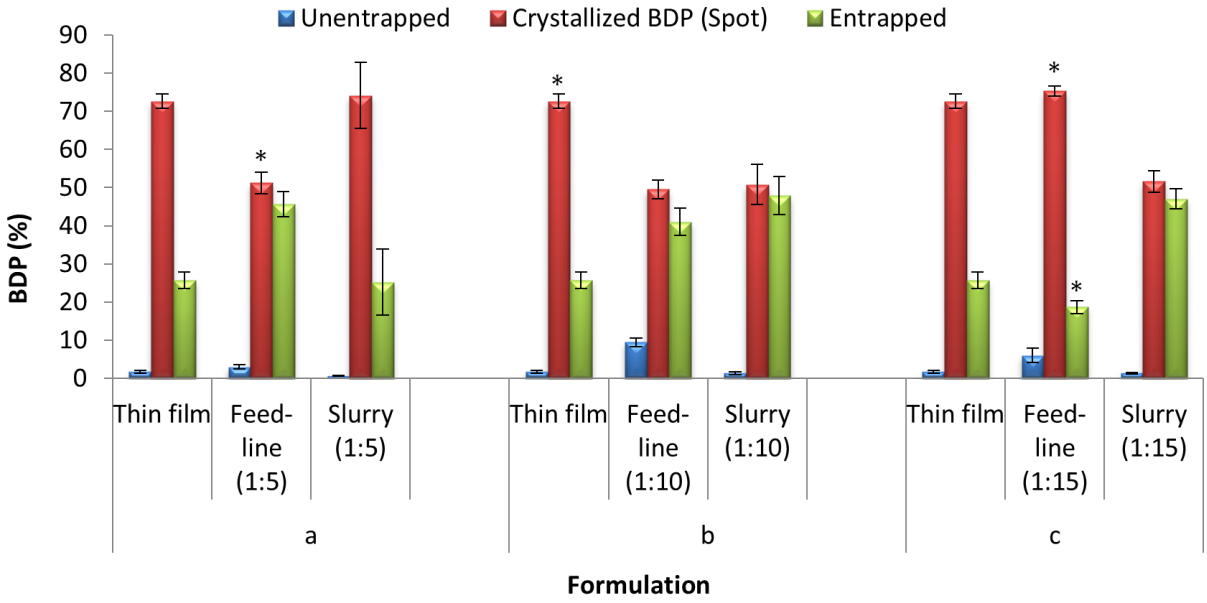

Figure 6: Entrapment efficiency of BDP in liposomes prepared using the thin-film method, and from proliposomes prepared by feed-line and slurry methods. Formulations were made with the following lipid to carrier ratios: 1:5 w/w (a), 1:10 w/w (b) and 1:15 w/w (c) for the proliposome methods (feed-line and slurry-based). Data are mean values (n = 3 $\pm$ sd)