Investigation Into The Effect of Varying L-leucine Concentration on the product characteristics of Spray-dried Liposome Powders


Published in:
Journal of Pharmacy and Pharmacology

Document Version:
Peer reviewed version

Queen's University Belfast - Research Portal:
Link to publication record in Queen's University Belfast Research Portal

General rights
Copyright for the publications made accessible via the Queen's University Belfast Research Portal is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The Research Portal is Queen's institutional repository that provides access to Queen's research output. Every effort has been made to ensure that content in the Research Portal does not infringe any person's rights, or applicable UK laws. If you discover content in the Research Portal that you believe breaches copyright or violates any law, please contact openaccess@qub.ac.uk.
Title: Investigation Into The Effect of Varying L-leucine Concentration on the product characteristics of Spray-dried Liposome Powders

K-H Chen\textsuperscript{a}, W. Mueanoom\textsuperscript{b}, S. Gaisford\textsuperscript{b}, V.L. Kett\textsuperscript{a*}

\textsuperscript{a} School of Pharmacy, Queen’s University of Belfast, Belfast BT9 7BL UK
\textsuperscript{b} The School of Pharmacy, University of London, 29/39 Brunswick Square, London WC1N 1AX

*corresponding author email address v.kett@qub.ac.uk

Telephone +44 (0)2890 972008
Fax +44 (0)2890 247794
Abstract

Spray-dried formulations offer an attractive delivery system for administration of drug encapsulated into liposomes to the lung, but can suffer from low encapsulation efficiency and poor aerodynamic properties. In this paper the effect of the concentration of the anti-adherent L-leucine was investigated in tandem with the protectants sucrose and trehalose. Two manufacturing methods were compared in terms of their ability to offer small liposomal size, low polydispersity and high encapsulation of the drug indometacin. Unexpectedly sucrose offered the best protection to the liposomes during the spray drying process, although formulations containing trehalose formed products with the best powder characteristics for pulmonary delivery; high glass transition (Tg) values, fine powder fraction (FPF) and yield. It was also found that L-leucine contributed positively to the characteristics of the powders, but that it should be used with care as above the optimum concentration of 0.5% (w/w) the size and polydispersity index increased significantly for both disaccharide formulations. Relating to the method of manufacture it was found that while both the sucrose and trehalose conferred protection on the liposomes produced using either method the ethanol-based proliposome method offered improved drug incorporation and did not suffer from loss of drug caused by dilution effects.

(200 words)

Keywords. liposomes; spray drying; disaccharide; leucine
1. Introduction

Liposomes are suited to encapsulation of a variety of drugs from small molecular weight compounds to macromolecules and including both hydrophilic and lipophilic entities. This is reflected in the range of therapeutics that have been tested in liposomal formulations including cytotoxic agents [3]; bronchodilators and anti-asthmatics [4; 5]; antibiotics [6] as well as photosensitizing agents [7] and genetic material [8-10]. The use of spray drying to produce stable powder formulations for pulmonary administration is attractive since it offers several advantages over the parenteral route [1]. Aerodynamic diameter is a crucial factor in determining deposition of particles in the different sites of the respiratory tract. Particles in the range 1-6 µm are best suited to deposition in the lower airway following inhalation [2]; those with diameters > 6 µm are deposited in the oropharynx, whereas smaller particles (< 1 µm) are exhaled during normal tidal breathing. In addition, fine particle fraction (FPF, the fraction of powder emitted from the inhaler with a particle size ≤5 µm) is a critical parameter to predict the proportion of the emitted dose that can deliver deeply into the lower respiratory system. The Use of liposomes as carriers offers benefits including protection of drug from enzymatic degradation; prolonging retention time and reducing side effects.

Many methods are available for the manufacture of liposomes, including thin-film hydration [11], organic solvent injection [12], reverse-phase evaporation [13] and dehydration-rehydration [12; 14]. Both the ethanol injection and proliposome methods of
Liposome preparation offer good potential for scale-up and have been used to encapsulate a wide range of substances [15-25] [26-31]. The ethanol injection method involves the rapid injection of a lipid-ethanol solution into an excess of aqueous medium to spontaneously form large unilamellar vesicles (LUVs) [12]. Advantages of the technique include simplicity and low risk of lipid degradation or oxidation. The ethanol-based proliposome method is based on the preparation of hydrated stacked bilayer sheets in a water-ethanol solution termed proliposomes. Spontaneous formation of liposomal suspensions (multilamellar vesicles, MLVs) is achieved by addition of excess aqueous solution to a lipid mixture [26]. The MLVs produced can be further processed for the preparation of oligolamellar and unilamellar liposomes.

The effect of disaccharide protectants on the stability of spray dried liposomes has been examined previously [32-34], while amino acids have been shown to play an important role in improving the aerosol behaviour of spray-dried powders by reducing moisture sorption and surface tension of dried particles [37; 38]; they can also protect proteins against thermal stresses and denaturation [39]. Leucine, in particular, tends to improve powder aerosol properties, this anti-adherent effect has been attributed to its hydrophobic character and surfactant-like properties that allow it to migrate rapidly to the surfaces of the particles during drying [40; 41] and it has been shown to interact well with lipid membranes [42]. Two studies have used leucine in combination with sugars at very
high fixed concentrations [43; 44]. To the best of our knowledge, the effect of varying leucine concentration on the spray drying of liposomal systems has not been investigated previously. In this paper we demonstrate the effect of varying concentrations of the anti-adherent L-leucine in combination with optimised concentrations of disaccharides and show for the first time that formulation effects can lead to significant differences in the product characteristics for spray dried liposomes, especially in terms of for sucrose formulations. Furthermore, at higher concentrations leucine increases liposome size. Additionally, we investigated whether switching from the ethanol injection to the proliposome method of liposome preparation affected liposome properties or drug release. Indometacin was chosen as it has been used previously as a model drug in liposomal studies [45; 25].

2. Materials and methods

2.1 Materials

Cholesterol (PhEur grade), sucrose (>99%), L-Leucine (PhEur grade), NaCl (≥99%), Trifluoroacetic acid (99%), Iron (III) chloride hexahydrate (≥99%), Ammonium thiocyanate (≥99%), Sephadex G-50 (20-80 μm), Indometacin (≥99%) phosphotungstic acid hydrate and ethanol (≥99.5%) were purchased from Sigma Chemicals. α,α-Trehalose Dihydrate (high purity, low endotoxin) was purchased from Ferro Pfanstiehl. Soy phosphatidylcholine (LIPOID S 75, 80%) and soy phosphatidylcholine (Lipoid S PC, >98%) were purchased
from Lipoid. Methanol (HPLC grade, >99.8%) and chloroform (99.2%) were purchased from VWR. Phosphate buffered saline tablets was purchased from Invitrogen Corporation. All aqueous solutions were prepared with de-ionized water.

2.2 Methods

2.2.1 Preparation and drying of liposomal dispersions

2.2.1.1 Preparation of liposomes by ethanol injection

Small unilamellar vesicles (SUV’s) were prepared by ethanol injection method followed by sonication in order to reduce liposomal size. Each batch was prepared on a 10g scale. 0.02 g Indometacin was dissolved in 0.7 g ethanol (at 57 °C) together with the lipids composed of 1g SPC and 0.115 g cholesterol. Hydration media were prepared by dissolving varying amounts (2.5, 5, 7.5, 10 and 15% w/w) of disaccharides (sucrose or trehalose) in 0.9% NaCl solution. Once the optimum concentration of these protectants had been determined, liposomal dispersions were prepared using various concentrations (0.25, 0.5 and 1% w/w) of L-Leucine added into the hydration medium also containing the optimum concentration of each protectant. The lipid solution was rapidly injected into 8.165g of hydration medium at room temperature with stirring. After 2 hr hydration, the prepared liposomal suspension was then submitted to a probe sonication process with a sequence of 40 s of sonication and 20 s of rest in an ice bath to the desired size. In all cases, the initial turbid liposomal suspension was translucent after sonication. Then, the
sonicated liposomes were annealed at 4 °C overnight before centrifugation (12,000 rpm, 30 min) and diluted 4-fold to give a final lipid concentration of 25 mg/mL of lipid.

2.2.1.2 Preparation of liposomes by proliposome method

1.5g of the appropriate hydration medium was added to a lipid dispersion containing soya lecithin (750 mg), cholesterol (86.4 mg) and indometacin (30 mg) in ethanol (600 mg) at 60 °C. The dispersion was stirred for 10 min at 60 °C, cooled to room temperature and then converted to a 25 mg/mL liposome suspension by drop-wise addition of the rest of hydration medium with continuous stirring for 2 hr following sonication and centrifugation.

2.2.1.3 Spray drying of liposomes

Spray drying was performed with a Mini Spray-dryer (Buchi 190). Applied spraying parameters were: inlet temperature 100 °C, outlet temperature 70 °C, air-flow 600-650 Nl/h, aspirator setting 20 (100%), pump setting 2.5-3 ml/min. A 0.5-mm nozzle was used.

2.2.2 Liposome size analysis

The average liposome size was determined with a ZetaSizer 3000HS (Malvern Instruments Ltd, Malvern, United Kingdom) at a temperature of 25±0.1 °C. Samples of the dispersion were diluted with hydration medium and the Z-average vesicle size and polydispersity were determined at 25 °C by dynamic light scattering. The values of the
viscosity and refractive index used in the calculation of the liposome size of the light scattering data were modified in terms of protectant concentration. The intensity of the laser light scattered by the samples was detected at an angle of 90° with a photomultiplier. For each specimen 10 autocorrelation functions were analyzed using a Contin analysis. From this analysis, the z-average diameter \((D_z)\) was obtained, which is an approximation of the diameter of the liposomes. The particle size distribution was characterized using the polydispersity index (PI). The spray-dried liposomes were reconstituted with de-ionized water to attain the original lipid content according to the method of Bligh and Dyer [46] and then performing the Stewart assay [47]. Samples were further diluted with hydration medium for liposome size analysis.

2.2.3 HPLC analysis of indometacin

HPLC was carried out using a Waters system (Waters 1525 Binary HPLC Pump, Waters IN-Line Degasser AF, Waters 2487 Dual λ Absorbance Detector, Waters 717 plus Autosampler). Luna C-18 column (100Å, 150 X 4.6 mm 5µm, Phenomenex) was used. The detector wavelength was set at 260 nm. The mobile phase consisted of a gradient of methanol and 0.1% v/v trifluoroacetic acid. The gradient schedule was: (a) 0-4.5 min, 85% methanol, flow rate of 1ml/min; (b) 4.5-5.5 min, 85 → 100% methanol, flow rate of 1ml/min; (c) 5.5-7 min, 100% methanol, flow rate of 1.2 ml/min; (d) 7-9 min, 100% methanol, flow rate of 1.5 ml/min; (e) 9-15 min, 100% methanol, flow rate of 1.5 ml/min; (f) 15-16 min,
100% methanol, flow rate of 1 ml/min (g) 16-17 min, 100→85% methanol, flow rate of 1.0 ml/min; (h) 17-23 min, 85% methanol, flow rate of 1.0 ml/min. In order to determine encapsulation efficiency (EE %) 50 µL of separate liposome solutions and reconstituted dispersions were applied to a Sephadex G-50 column and eluted with hydration medium. The fractions were diluted with methanol and the concentrations of indometacin were determined by HPLC. Loading efficiency was determined by dividing the encapsulated drug content (µg) by the lipid content (mg). The encapsulation efficiency (EE (%)) of indometacin was determined from the ratio of encapsulated to total drug concentration.

2.2.4 Water content and thermal analysis of the powders.

Thermogravimetric studies were carried out to measure the water content of the spray-dried liposomes using a TGA (Q500, TA instruments). Samples were heated from 20 to 25 °C at a scan rate of 10 °C /min. Modulated differential scanning calorimetry (MTDSC) measurements of the dried products were performed on TA Q100 Differential Scanning Calorimeter (Q100, TA Instruments, which had been calibrated for temperature, enthalpy and heat capacity). The product was sealed into a hermetic aluminium pan and after equilibration at 0 °C, was heated at 2 °C /min to 200 °C with a modulation of ± 0.4 °C/40 sec. Tg values are recorded as onset values from the reversing heat flow signal and reported as mean values (n=4-6) with standard deviation.

2.2.5 Scanning electron microscopy (SEM)
The spray-dried powders were coated with gold in a sputter coater and their surface morphology was observed using a scanning electron microscope (JEOL 6500F field emission scanning electron microscope).

### 2.2.6 Powder particle size analysis

The volume mean diameter of spray-dried liposomes was measured by Laser Light Diffraction Analyzer (HELOS/BR, Sympatec, Clausthal-Zellerfeld, Germany). Approximately 5 mg of powder were suspended in chloroform in a 50 ml glass cuvette and stirred with a magnetic bar at 1000 rpm. A short period of sonication (60 s) at a power of 60W (CUVETTE, Sympatec; 8.5 mm diameter ultrasound tip) was applied before sizing [48; 49]. A R4 lens was used allowing measurements in the range of 0.45-875 µm.

### 2.2.7 in vitro release of indometacin from liposomes

Free drug was removed from the reconstituted liposome suspensions by use of centrifugal filter tubes (Amicon Ultra 15 MW Cut-off 10 KDa, Millipore). 1 ml portions were sealed into dialysis tube (MW cut-off 7 KDa, Thermo Scientific), and added to 50 mL of pH 7.4 PBS release medium in a shaking incubator (37° ±0.5 °C, 60 rpm, 25 mm throw; Unitron, Infors HT, Switzerland). 2-ml samples were drawn periodically and the amount of drug release determined using the HPLC method. The release volume was kept constant throughout.

### 2.2.8 Aerodynamic study

A twin-stage liquid impinger was used to determine the emitted dose and fine particle
fraction (FPF) of the spray-dried powders. The dried liposome powders were filled into number 3 gelatin capsules. A dry powder inhaler (Cyclohaler, Teva, UK) was attached to the mouthpiece of emitted dose apparatus. Ten capsules were pierced and the dried liposome powders were emptied at 60 L/min for 10 seconds. The powders deposited at each stage were washed out and recovered. The powders deposited in the inhaler and capsules were also collected. After being diluted with methanol to a suitable concentration, each indometacin solution was assayed by HPLC. The FPF values were defined as the powder mass recovered at stage 2. The results are expressed as the percentage of drug dose emitted to the capsule content (loaded dose).

2.2.9 Statistical analysis:

Kruskal-Wallis analysis with Dunn’s all pairwise multiple comparisons or Mann-Whitney U test were used to calculate the p values using SigmaPlot 8 software. Differences were deemed significant if p values were <0.05.

3. Results and discussion

3.1 Initial Determination of Optimum Disaccharide and L-leucine concentrations

3.1.1 Effect of disaccharide concentration on liposomal size and PI

The size change of any liposomes prior to and after spray drying is a critical parameter in the assessment of liposomal stability so this was used as an initial screening parameter in choosing which formulations to take forward to the next step of formulation optimisation.
Initially the optimum concentration of either sucrose or trehalose as protectants during the spray drying process was determined.

As shown in Figure 1a, the addition of 2.5% (w/w) sucrose to the hydration medium did not prevent the aggregation and fusion of reconstituted liposomes after spray drying, but increasing the concentration to 5% (w/w) inhibited liposomal size increase prior to spray drying and after reconstitution more effectively. A further increase to 7.5% (w/w) showed no significant change in the PI values. 10% (w/w) Sucrose was more protective still; this was the only formulation containing disaccharide alone that showed no statistical difference in liposomal size after drying. A further increase in concentration to 15% (w/w) resulted in an increase in liposome size. This effect can also be seen by comparing the $S_F/S_I$ ratios, where $S_F$ is the final liposomal size after rehydration and $S_I$ is the initial liposomal size [50]. Previous research into freeze-drying of liposomes demonstrated that solute incorporated by liposome in the presence of lyoprotectant in a mass ratio of sugar: lipid of 2:1 could be effectively retained after freeze-drying [51], in the 5% (w/w) sucrose dispersions the mass ratio of sucrose to lipid is close to this ratio. A similar effect was observed when trehalose was used as the protectant (Figure 1b). However, the protective effect of trehalose was not so good; the addition of 15% (w/w) trehalose dihydrate to the hydration medium did not prevent the liposome size increasing after reconstitution ($p < 0.05$).
3.1.2 Effect of disaccharide concentration on recovery rate and water content of spray-dried liposome powders

The recovery rate of the spray-dried powders significantly improved when increasing the concentration of protectants from 2.5% (w/w) to 5% (w/w) for both sugars (Figure 2), although further addition of either disaccharide showed little additional effect. Further, the spray-dried liposomes with trehalose had a much higher recovery rate than those with sucrose, which is ascribed to its higher Tg (101 °C compared with 64 °C for sucrose [36]).

The sticky point (Ts) of an amorphous powder is generally considered to lie 10-20 °C above Tg; if the outlet temperature of the dryer surpasses Ts then particle cohesion increases sharply and there may also be increased adhesion to the dryer walls [35]. Because the powder temperature would have been greater than the Tg for the sucrose formulation, the particles would have been more prone to adherence to the walls of the spray-dryer. Increasing the concentration of either protectant reduced the water content of the spray-dried powders (Figure 2). Since Tg data are inversely related to water content this would be expected. Based on the results in 3.1.1 and 3.1.2 the formulations containing 10% sucrose and 15% trehalose were selected for further optimisation by addition of the anti-adherent L-leucine.

3.2 Effect of varying L-leucine concentration
3.2.1 Effect of L-leucine concentration on liposomal size

Figure 3a indicates that the inclusion of L-Leucine at a concentration of 0.5% (w/w) was the most effective in preventing size changes for both the 10% (w/w) sucrose and the 15% (w/w) trehalose formulations, with no statistical difference seen in the sizes prior to spray drying compared with the reconstituted dispersions. Increasing the concentration to 1% (w/w) L-Leucine had the effect of markedly increasing the liposomal size in the reconstituted dispersions. This might be caused by partitioning of the hydrophobic amino acid into the lipid membrane during drying, causing vesicle fusion. While this phenomenon has not been investigated for spray dried liposomes it has been observed previously during freezing and freeze-drying of liposomes; Anchordoguy et al [52] found that the amino acids with hydrocarbon side chains increased membrane damage during freeze/thaw trials. Popova et al [53] observed that the amphiphilic aromatic amino acids tryptophan and phenylalanine induced solute leakage and membrane fusion during freezing studies of liposomes, while Mohammed [54] et al demonstrated that basic, polar amino acids stabilized liposomes during lyophilisation but that at higher concentrations these amino acids promoted vesicle fusion.

<Figure 4>

3.2.2 Effect of L-leucine concentration on recovery rate and water content

Addition of 0.5% (w/w) L-Leucine markedly improved the recovery rate of the 10% (w/w) sucrose formulations (Figure 4) but had little effect on the liposomes formulated with 15%
(w/w) trehalose, which exhibited good yields already. L-Leucine did not have a significant effect on the water contents of the powders (Figure 4) but a marked improvement in the appearance of the powders was apparent to the naked eye. Formulations without L-leucine were clumped into aggregates in the mm size range while increasing L-leucine reduced the size. This effect was especially apparent for the sucrose formulations.

Taking all data from 3.1 and 3.2 into account the two formulations selected for further testing were those containing 0.5% (w/w) leucine and either 10% (w/w) sucrose or 15% (w/w) trehalose.

### 3.3 Effect of Liposome manufacturing method on liposomal size and drug content

Once the optimal levels of disaccharide and anti-adherent had been determined, the effects of manufacturing process variables were investigated. Table 1 shows that liposomal size was clearly larger for the formulations prepared by the proliposome method. It has been suggested that the proliposome-prepared liposomes produce multilamellar vesicles [31], while those prepared by the ethanol-injection method are primarily unilamellar, which are more amenable to size reduction under the same sonication conditions. Based on encapsulation efficiency and loading efficiency, the two methods have the same capability to incorporate indometacin. However, lipid loss during the alcohol injection method could not be avoided owing to this process involving injection of lipid solution into hydration medium. It has previously been reported that ethanol concentration is a decisive factor in
liposome size reduction using a high-pressure homogenizer, whereby the liposomal size
and range decreased with increasing concentration of ethanol [55].
Statistically significant changes were seen between freshly prepared and reconstituted liposomes prepared by the ethanol injection in terms of PI value, encapsulated content and loading efficiency. The small increases in encapsulated content and loading efficiency were attributed to unentrapped drug being reincorporated into the liposomes during reconstitution, as has previously been demonstrated for liposomes prepared using the DRV (dried-rehydrated vesicle) method [56]. Even though the formulation was optimised using the ethanol injection method, excellent encapsulation efficiency in liposomes prepared by the proliposome method was obtained. Dispersions produced using the proliposome method showed small statistically significant reductions in PI values for both formulations, while for the trehalose formulation there was also a small statistically significant reduction in liposomal size after drying. However, the liposomal indometacin content and its loading efficiency did not significantly change during spray drying. In short, while sucrose enabled a better loading efficiency when comparing samples produced by the ethanol injection method; the combination of either 10% (w/w) sucrose and 0.5% (w/w) leucine or 15% (w/w) trehalose and 0.5% (w/w) leucine in the formulations could effectively protect liposomes prepared by either method against spray drying stress. In terms of drug loading and efficiency liposomes prepared by the proliposome method incorporated drug more effectively than those produced using the ethanol-injection method (Table 1). In addition, a
dilution effect was observed when comparing loading efficiency of liposomal stock solutions and the final dispersions prepared by the ethanol-injection method, which was attributed to encapsulated drug leaking out of the liposome stock solutions upon dilution as observed by Foldvari et al [57]. TEM images (data not shown) indicate that the vesicular structure of liposome in the presence of the optimised disaccharide and anti-adherent formulations could be preserved very well through spray drying.

<Figure 5>

3.4 Imaging, size analysis and aerodynamic properties of the spray-dried powders

SEM analysis showed that particles of the optimally formulated sucrose powder exhibited a smooth surface (Figure 5a and b), while the powders containing trehalose were wrinkled (Figure 5d, e and f), which potentially prevents particles from adhering tightly to each other, thus preventing aggregation and lowering the energy required to disperse them [58]. This may explain why the sucrose formulation appeared to contain more aggregates.

Furthermore, the fine particle fraction was higher for the trehalose/L-leucine formulation than for that containing sucrose/L-Leucine (Figure 6).

<Figure 6>

Bosquillon et al [59] had reported that the type of sugar incorporated did not affect morphology of the spray-dried powders. The wrinkled surface perhaps can be attributed to the inclusion of additives that alter the surface tension that controls droplet shape during
drying as Adler et al [60] demonstrated that surfactant could change the interface viscosity and that consequently, the dried particle morphology also changed. By increasing the ratio of surfactant to other additives, more spherical, smooth particles were obtained. It is proposed that L-leucine at a concentration of 0.5% (w/w) functions as a surfactant. Hence, it is suggested that the ratio of 0.5% (w/w) leucine to 15% (w/w) trehalose is below the critical point of powder morphology conversion between roughness and smoothness and so it tends to exhibit a wrinkled surface. The formulation containing 10% (w/w) sucrose and 0.5% (w/w) exhibited a smoother morphology. To investigate whether this was a concentration effect rather than being specific for trehalose, spray dried liposomes were prepared with a sucrose concentration of 15% (w/w), but these still had smooth surfaces (Figure 5c and d), indicating that the wrinkling is not caused by surface tension effects alone. The SEM images also show that the diameters of all the spray dried powders were less than 10 µm (Figure 5). Accurate size distribution data are given in Table 2, which show that all formulations the volume mean diameters were measured to be 3~4 µm with the exception of the formulation containing 10% (w/w) sucrose and 0.5% (w/w) L-leucine, prepared by ethanol injection, which gave a larger diameter of 5.40 µm. This size increase cannot be attributed to hygroscopicity of the sucrose formulations [34] as the water content values for all formulations were similar. Although the sucrose formulations exhibited lower Tg values than the trehalose formulations, as might be expected, there was no significant difference between those obtained for the liposomes prepared by the ethanol injection.
rather than the proliposome method. The emitted dose and fine particle fraction of
spray-dried liposome formulated with trehalose and L-leucine were higher than those
formulated with 10% (w/w) sucrose and 0.5% (w/w) L-leucine. (Figure 6). Therefore, spray
dried liposomes with 15% (w/w) trehalose and 0.5% (w/w) leucine exhibited better aerosol
powder performance than those in the presence of 10% (w/w) sucrose and 0.5% (w/w)
leucine in terms of emitted dose, aerodynamic diameter and fine particle fraction.

<Figure 7>

3.5 Release of indometacin from reconstituted liposomes

Release data for Indometacin from reconstituted liposomes for both optimised
formulations using both methods of manufacture are shown in Figure 7. Diffusion of free
indometacin through the dialysis membrane was measured as a control. The release profile
of free drug shows over 80% diffusion in 4 hr. Over the same interval, the reconstituted
liposomes prepared by the ethanol-injection method released 15.1 % and 16.9% drug
(optimised formulations containing sucrose & trehalose respectively) and released ~45% of
the encapsulated drug over 24 hr. Those prepared by the proliposome method in the
presence of disaccharide (sucrose or trehalose) and leucine exhibited drug release of
21.8% and 24.3% in 4 hr and in total released 54.5% and 61.5% over 24 hours respectively
(Figure 7). These differences are not significant suggesting that the mode of release is the
same for all formulations. The mechanism responsible for the release of drug from the
liposomes may be due to diffusion phenomena, degradation effects, or a combination of
both processes. To examine the drug release kinetics and mechanism, the release data were fitted to models representing zero-order, first-order, and Higuchi’s square root of time and the Korsmeyer–Peppas models (Table 3). All systems showed best correlation with the Higuchi model and anomalous (non-Fickian) diffusion \((n > 0.5)\). It is reasonable to propose that this is because the indometacin is located within the phospholipid membrane of the liposomes and must diffuse through in order to be released.

4. Conclusions

The inclusion of either 10% (w/w) sucrose or 15% (w/w) trehalose dihydrate and 0.5% (w/w) L-leucine protected liposomes prepared by ethanol injection or proliposome method against spray drying stress in terms of size change, polydispersity index, encapsulated drug content and loading efficiency of the reconstituted liposomes. Formulation design was of more importance than the method of liposome manufacture. The method for preparing the liposomes had no effect on the stability or encapsulation efficiency of spray-dried liposomes with optimal protectant and anti-adherent.

This paper has demonstrated for the first time that L-leucine should be used with care as an additive for spray dried liposomes in combination with disaccharide. While there was a clear advantage in using this material at an optimum level; at higher concentrations it caused an increase in liposomal size upon rehydration of the spray dried powders that might be attributed to it partitioning into the lipid membrane during drying, causing vesicle
fusion. This is an area for future research.
References


Table 1. The characterization of liposomes prepared by ethanol injection and by proliposome. The hydration buffers were in either a combination of 10% (w/w) sucrose and 0.5% (w/w) L-Leucine or 15% (w/w) trehalose dihydrate and 0.5% (w/w) L-Leucine. * denotes P<0.05 (Mann-Whitney U test) in comparison with the corresponding samples prior to spray-drying. + P<0.05 (Mann-Whitney U test) compared to formulations with the inclusion of trehalose/L-Leucine. Each value represents the mean ± SD (three different batches) and n denotes replicate measurements of each batch.

<table>
<thead>
<tr>
<th>Preparative methods</th>
<th>Ethanol injection</th>
<th>Proliposome</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before spray-drying</td>
<td>After reconstitution</td>
</tr>
<tr>
<td>Liposome with 10% (w/w) sucrose and 0.5% (w/w) L-Leucine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liposome size (nm) &amp; (Pl)</td>
<td>107.4 ± 13.8 (0.20 ± 0.02)</td>
<td>115.1 ± 11.5* (0.25 ± 0.04*)</td>
</tr>
<tr>
<td>Total drug content (µg/mL)</td>
<td>407.5 ± 7.9</td>
<td>384.8 ± 7.3*</td>
</tr>
<tr>
<td>Encapsulation efficiency (%)</td>
<td>33.3 ± 6.1</td>
<td>53.2 ± 15.1*</td>
</tr>
<tr>
<td>Encapsulated drug content (µg/mL)</td>
<td>135.6 ± 24.9</td>
<td>204.8 ± 58.1*</td>
</tr>
<tr>
<td>Lipid content (mg/mL)</td>
<td>23.57 ± 0.8</td>
<td>21.60 ± 1.17*</td>
</tr>
<tr>
<td>Loading efficiency (µg drug/ mg lipid)</td>
<td>5.7 ± 1.0</td>
<td>9.5 ± 2.9*</td>
</tr>
</tbody>
</table>

| Liposome with 15% (w/w) trehalose dihydrate and 0.5% (w/w) L-Leucine |
| Liposome size (nm) & (Pl) | 130.7 ± 2.7 (0.31 ± 0.03) | 132.7 ± 4.2 (0.36 ± 0.02*) | 138.5 ± 4.8 (0.52 ± 0.04) | 127.3 ± 3.6* (0.40 ± 0.03*) |
| Total drug content (µg/mL) | 442.1 ± 7.4 | 428.2 ± 10.9* | 899.9 ± 8.1 | 868.6 ± 16.7* |
| Encapsulation efficiency (%) | 37.4 ± 6.4 | 42.5 ± 3.8 | 49.7 ± 7.1 | 52.0 ± 7.0 |
| Encapsulated drug content (µg/mL) | 165.3 ± 27.4 | 181.8 ± 16.5 | 447.5 ± 67.7 | 449.1 ± 66.0 |
| Lipid content (mg/mL) | 26.21 ± 0.70 | 25.51 ± 1.80 | 29.60 ± 0.10 | 27.85 ± 0.39 |
| Loading efficiency (µg drug/ mg lipid) | 6.3 ± 0.9 | 7.2 ± 0.70 | 15.1 ± 2.2 | 15.7 ± 2.0 |