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The preparation of liposomes using compressed carbon dioxide: strategies, important considerations and comparison with conventional techniques

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Abstract

Numerous strategies are currently available for preparing liposomes, although no single method is ideal in every respect. Two methods for producing liposomes using compressed carbon dioxide in either its liquid or supercritical state were therefore investigated as possible alternatives to the conventional techniques currently used. The first technique used modified compressed carbon dioxide as a solvent system. The way in which changes in pressure, temperature, apparatus geometry and solvent flow rate affected the size distributions of the formulations was examined. In general, liposomes in the nano-size range with an average diameter of 200 nm could be produced, although some micron-sized vesicles were also present. Liposomes were characterized according to their hydrophobic drug-loading capacity and encapsulated aqueous volumes. The latter were found to be higher than in conventional techniques such as high-pressure homogenization. The second method used compressed carbon dioxide as an antisolvent to promote uniform precipitation of phospholipids from concentrated ethanolic solutions. Finely divided solvent-free phospholipid powders of saturated lipids could be prepared that were subsequently hydrated to produce liposomes with mean volume diameters of around 5 μm.

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Introduction

Since their discovery (Bangham et al 1965), many studies have been published describing the use of liposomes for a wide range of drug and vaccine delivery applications. Despite this considerable success at the research level, relatively few liposomal products have so far made it to the market place. One factor contributing to this situation is that industrial-scale manufacturing of high-quality formulations is difficult because of the limitations of existing production techniques.

Numerous methods currently exist for preparing liposomes. Many are suitable primarily for small-scale production, for example the preparation of millilitre quantities for individual research activities. Such techniques include those of Bangham et al (1965), Huang (1969), Johnson & Bangham (1969), Deamer & Bangham (1976), Szoka & Papahadjopoulos (1978), Brunner et al (1976) and Kirby & Gregoriadis (1984).

With respect to larger-scale production, detailed information regarding specific manufacturing strategies in the pharmaceutical industry is not freely available in the public domain. However, it is possible to speculate as to which liposomal production methods would be best suited to industrial-scale manufacturing under the conditions of current good manufacturing practice (cGMP). For example, high-pressure homogenization techniques, methods based on the original ethanol injection technique of Batzri & Korn (1973) and extrusion processes stand out as being particularly useful. Despite the merits of these techniques, they are not without their drawbacks. For example, homogenization is known to produce vesicles with poor encapsulating capacities (Mayhew et al 1984; Brandl et al 1998) and large temperature rises occurring in the interaction chamber may cause partial lipid or drug degradation (Martin 1990). An industrially viable advancement of the ethanol injection technique (Wagner et al 2002)

produces liposomes with a good size distribution, but the liposomes reportedly produced have average diameters of between 270 and 290 nm, which may make them too large for many intravenous (i.v.) drug delivery applications, especially where extravasation into diseased tissue or malignant tumours is essential. In these instances, liposomal diameters should not exceed 200 nm (Forssen 1997; Ishida et al 1999). When using extrusion through polycarbonate membranes to produce large batches of liposomes (e.g. Amselem et al 1993) there is a reliance on other liposomal production methods to firstly produce crude multilamellar vesicle batches for processing, and these techniques come with their own disadvantages. It is therefore desirable to develop alternative strategies so that the choice of available methods for pharmaceutical manufacture is extended. One such alternative is to develop processes that use compressed carbon dioxide (supercritical fluid or subcritical liquid) as a solvent or antisolvent.

Compressed gas solvents and antisolvents

In both the liquid and supercritical state compressed gases can display considerable density-dependent solvent power. This solvent capability is reversed on returning the solvent to its gaseous state at ambient pressure. In the supercritical state, substances also possess additional interesting properties, including gas-like viscosities and self-diffusivities that are higher than in liquid solvents (Eckert et al 1996).

Currently, carbon dioxide is the most widely used compressed gas solvent and is used as an alternative to conventional organic solvents in many commercial-scale extraction (King & Bott 1993; McHugh & Krukonis 1994), chromatographic (Berger 1997) and crystallization/particle production techniques. The last of these is of particular interest here and includes technologies that use compressed carbon dioxide as either a solvent or an antisolvent. Solvent techniques include the rapid expansion of supercritical solutions (RESS) method (Matson et al 1987), while antisolvent techniques include gas antisolvent recrystallization (Gallagher et al 1992), solution enhanced dispersion with supercritical fluids (SEDS) (Hanna & York 1994) and precipitation with compressed antisolvent (Dixon et al 1993). Given the fact that many drugs and pharmaceutical excipients have been successfully processed using these methods (e.g. Debenedetti et al 1993; Subramaniam et al 1997; Edwards et al 2001; Elvassore et al 2001; Tservistas et al 2001; Reverchon et al 2002), it is worth exploring whether their utility can be extended to liposomal production. Indeed, over recent years a handful of groups have begun to investigate the possibility of using supercritical carbon dioxide to prepare liposomes. Frederiksen et al (1997) described a method in which lipids were first dissolved in supercritical carbon dioxide modified with a co-solvent, ethanol. Small quantities of ethanol were required to increase the polarity of the non-polar carbon dioxide, which would not otherwise dissolve polar phospholipids. The fluid/lipid mixture was then rapidly expanded to atmospheric pressure in a manner similar to that described in RESS processing. During the expansion process, an aqueous solution containing the solute to be entrapped was simultaneously added to produce liposomes. Otake et al (2001) and Imura et al (2002, 2003) prepared large unilamellar liposomes of between 0.1 and 1.2 μm in size using a technique that they described as the 'supercritical reverse phase evaporation method'. Similar methodology is also discussed in the patent of Castor (1998). In an area related to liposomal production, Magnan et al (2000) and Badens et al (2001) micronized various grades of soya phosphatidylcholine using supercritical carbon dioxide as an antisolvent, although no vesicle preparation was reported.

In this contribution, two contrasting strategies for liposomal production are considered. The first employs compressed carbon dioxide modified with ethanol as a solvent, and is similar in nature to that discussed by Frederiksen et al (1997). This method was chosen because the previously described work reported liposomal sizes that would be useful for i.v. drug delivery and subsequent extravasation from the circulation. This application is currently one of the most successful from a commercial point of view. A key aim of this part of the study was to increase understanding of how process parameters affected the characteristics of the resultant liposomes, particularly their size and size distributions. In addition, the entrapped aqueous volumes within the vesicles were quantified and their ability to hold a model hydrophobic drug assessed. Overall, the information obtained allowed comparisons with conventional techniques to be made. The second method involved the production of phospholipid powders using compressed carbon dioxide as an antisolvent. The aims were to determine which phospholipids could be successfully processed using this method and to examine the possibility of preparing liposomes directly from the powders. From a process point of view, the key advantages of such a technique are that: (i) it lends itself well to (semi)continuous operation, (ii) the low solubility of phospholipids in carbon dioxide can be exploited, (iii) the technique is potentially applicable to a large range of both natural and synthetic phospholipids and (iv) removal of trace organic solvent from the powders as an integral part of the process produces a product requiring little further processing.

Materials and Methods

Materials

Lipids

Soya phosphatidylcholine (SPC) and hydrogenated soya phosphatidylcholine (HSPC) (>96% pure) were purchased from Lipoid GmbH (Ludwigshafen, Germany). Distearoylphosphatidylcholine (DSPC) and dimyristoylphosphatidylcholine (DMPC) (>98% pure) were obtained from Northern Lipids (Vancouver, Canada).

Cholesterol (99% pure) was purchased from Sigma-Aldrich (Poole, UK). All lipids were stored at −20°C and used as received.

Carbon dioxide and ethanol
Carbon dioxide (CP grade, liquid withdrawal) was supplied by BOC (Guildford, UK). Absolute ethanol (99.9% pure) was supplied by Fisher Scientific (Loughborough, UK) and was of Analar grade.

Other chemicals and reagents
The water used in experimental procedures was distilled and filtered through a 0.22 μm membrane prior to use. Phosphate buffered saline (PBS) tablets were purchased from Sigma-Aldrich (Poole, UK) and reconstituted in water as per the manufacturer's instructions. All solvents for chromatographic procedures were purchased from Fisher Scientific (Loughborough, UK) and were of Analar or HPLC grade.

Model drugs
Ciprofloxacin HCl was purchased from Promochem Limited (Welwyn Garden City, UK) and was of USP reference grade. α-Tocopherol acetate (vitamin E acetate) was purchased from Sigma-Aldrich (Poole, UK) and was > 99% pure. Ciprofloxacin HCl was protected from light and stored at room temperature. α-Tocopherol acetate was stored at 4°C in a refrigerator.

Experimental rigs

Schematic representations of the experimental rigs used for preparing liposomal suspensions and phospholipid powders for hydration are shown in Figures 1 and 2, respectively. All high-pressure tubing used in the construction of the rig was seamless ¼″ or ⅛″ outer diameter 316 stainless steel rated to 750 bar, connected by stainless steel Swagelok fittings (Manchester Fluid Systems Technologies Ltd, UK).

Preparation of liposomal suspensions using a modified compressed carbon dioxide solvent system

General method and preparation of empty liposomes
The experimental conditions employed in each liposomal production run are summarized in Table 1. The following general method was employed, using the apparatus shown in Figure 1.

Two hundred milligrams of SPC with or without 44 mg of cholesterol were packed within layers of glass wool within the pressure vessel. When cholesterol was included, these lipid ratios were chosen following investigations into solubility behaviour (not reported here) that showed such quantities produced near-equimolar mixtures of the two lipids in the compressed gas solvent systems. Carbon dioxide was then

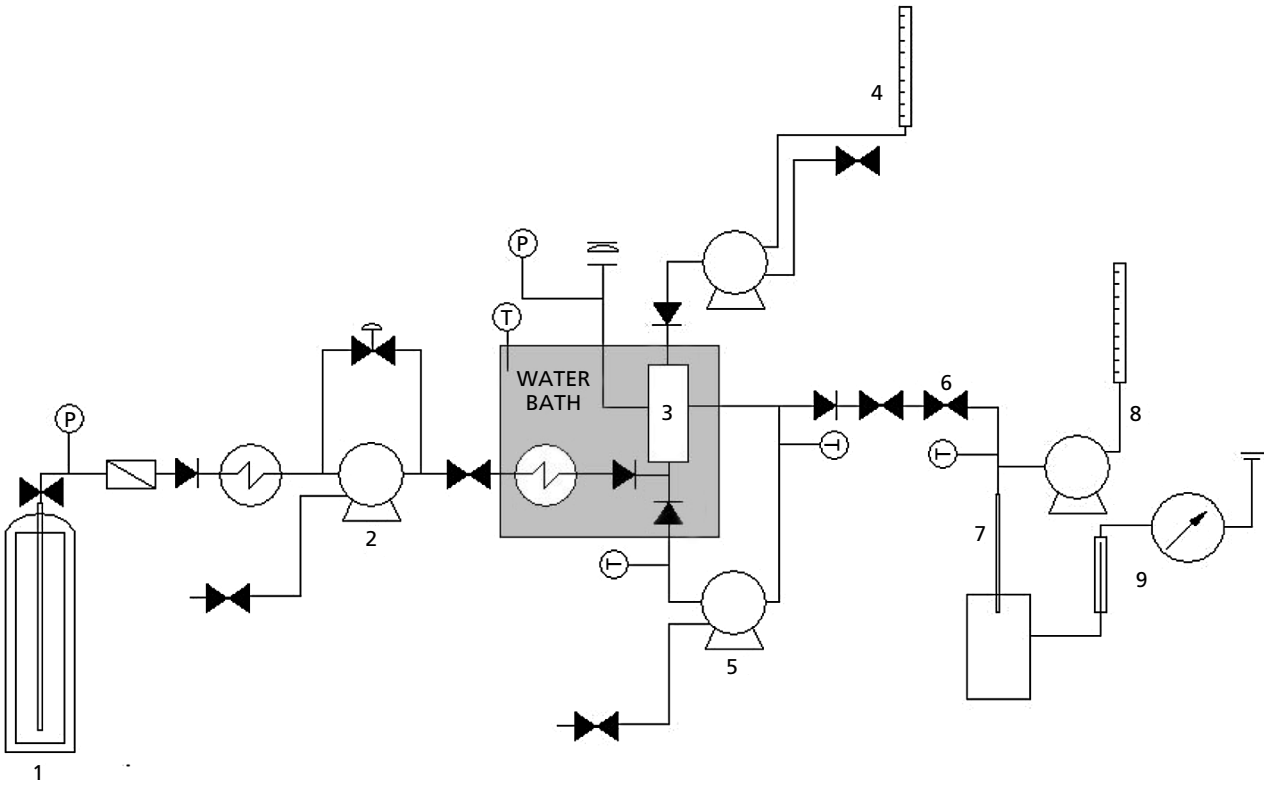

Figure 1 Schematic representation of apparatus used for the production of aqueous liposomal suspensions. 1, Carbon dioxide supply; 2, carbon dioxide pump; 3, pressure vessel; 4, co-solvent reservoir and delivery system; 5, recycling pump; 6, micrometering valve; 7, expansion capillary; 8, hydration medium reservoir and delivery system; 9, gas rotameter and wet test gas flowmeter.

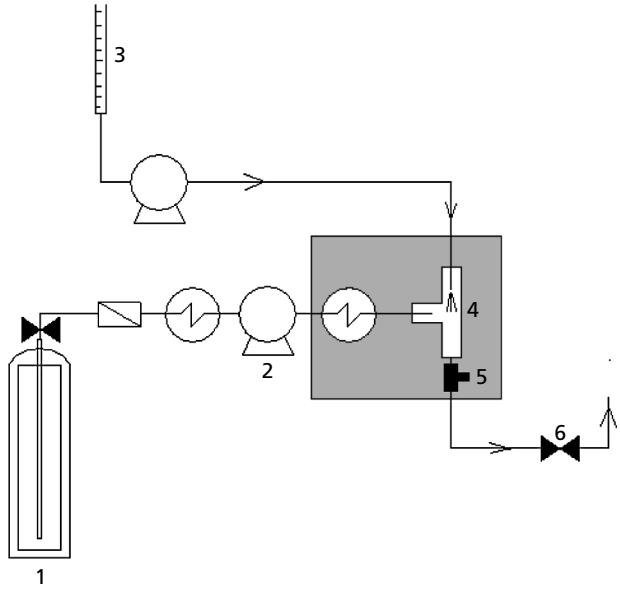

Figure 2 Schematic representation of apparatus used for phospholipid powder production. 1, Carbon dioxide supply; 2, carbon dioxide pump; 3, ethanolic phospholipid solution and delivery pump; 4, T-piece housed within an oven; 5, powder collection filter; 6, back pressure regulator.

introduced into the rig, the pressure and temperature raised to the required levels and 7 mol% ethanol added to the pressure vessel. The critical temperature of carbon dioxide modified with this quantity of ethanol is 52°C (Gurdial et al 1993), so above this temperature the solvent system was supercritical and below it it was subcritical. The recycling pump was then switched on to facilitate lipid dissolution. After 30 min of recycling, a sample of the equilibrium mixture (approximately 9 L carbon dioxide at standard pressure and temperature) was expanded to atmosphere via a fine capillary. To maintain the pressure of the system during expansion, fresh carbon dioxide was introduced. The extent to which this 'diluted' the equilibrium mixture was minimized in this study by sampling from the top of the vessel while carbon dioxide entered from the bottom and limiting the size of sample withdrawn from the vessel. The latter of these restricts the applicability of the method for the production of larger batches and a means of overcoming this is discussed in a later section.

The mass flow rate of carbon dioxide through the expansion capillary was controlled either entirely by the dimensions of the capillary or by adjusting a micrometering valve situated just upstream of it. During the expansion process, aqueous hydration medium (water or PBS) was simultaneously added at a rate of 5 mL min^{-1} to form liposomes that were collected in a glass vessel. The rig was then depressurized and the pressure vessel emptied prior to cleaning. The size distributions of all liposomal formulations were assessed by photon correlation spectroscopy (PCS) using a Malvern Zetasizer 3000 HS (Malvern Instruments Ltd, UK). The effects of the experiment type on the PCS-derived intensity diameters and polydispersity indices (PIs) were statistically examined. The Kruskal–Wallis non-parametric statistical hypothesis test was employed to test for differences between mean intensity diameters and polydispersity indices when the results of three or more experiments were under simultaneous scrutiny. Post-hoc analysis with Dunn's test was subsequently used where necessary. The Mann–Whitney U-test was used to examine for differences where experiments were paired for comparison (Jones 2002). For clarity of interpretation, experiments subject to statistical analysis were grouped or paired so that only one variable was under investigation at any one time. The chosen level of significance, α, for all tests was 0.05. Size distributions for the formulations were also assessed by laser light diffraction using a Malvern Mastersizer E (Malvern Instruments Ltd, UK). Selected samples were visualized using

Table 1 Details of experimental conditions used to prepare empty liposomes and PCS-derived size characteristics of the resulting formulations

Experiment	Aqueous phase	P (bar)	T (°C)	M (g min^{-1})	C: L (cm) × i.d. (μm)	Lipids	Mean intensity diameter (nm)	Mean PI
1 (n = 5)	Water	250	55	22	10 × 127	SPC + Chol	202 ± 13	0.68 ± 0.04
2	Water	250	40	22	10 × 127	SPC + Chol	225 ± 33	0.75 ± 0.03
3	Water	250	20	22	10 × 127	SPC + Chol	428 ± 84	1 ± 0
4	Water	200	55	18	10 × 127	SPC + Chol	392 ± 59	1 ± 0
5	Water	150	55	13	10 × 127	SPC + Chol	1943 ± 362	1 ± 0
6	Water	250	55	22	23 × 178	SPC + Chol	209 ± 9	0.63 ± 0.10
7	Water	250	55	43	10 × 178	SPC + Chol	192 ± 11	0.73 ± 0.02
8	Water	250	55	9	10 × 127	SPC + Chol	439 ± 58	1 ± 0
9	PBS	250	55	22	10 × 127	SPC + Chol	181 ± 10	0.51 ± 0.03
10	PBS	250	40	22	10 × 127	SPC + Chol	195 ± 7	0.53 ± 0.03
11	PBS	250	55	22	10 × 127	SPC alone	115 ± 15	0.52 ± 0.03
12	Water	250	55	22	10 × 127	SPC alone	105 ± 7	0.55 ± 0.04

For all experiments compressed carbon dioxide was modified with 7 mol% ethanol. Unless otherwise stated, all experiments were conducted in triplicate. For sizing measurements, each value represents the overall mean from at least three batches ± s.e.m. P, pressure; T, pre-expansion temperature; M, mass flow rate of carbon dioxide during expansion; C, capillary dimensions; L, length.

transmission electron microscopy to examine vesicle size and lamellarity (number of phospholipid bilayers).

Preparation of drug-loaded liposomes and quantification of drug entrapment

Ciprofloxacin HCl was used as a water-soluble marker to determine the volume of aqueous space entrapped within the liposomes. To produce liposomes containing this drug, an aqueous solution containing 1 mg mL^{-1} ciprofloxacin HCl was used to hydrate the lipid components of the liposomes during expansion, in the same way as water or PBS was used in the experiments described for producing empty liposomes. Liposomes were produced using both supercritical and subcritical solvent mixtures at 250 bar/55°C and 250 bar/20°C, respectively. All other experimental parameters were as for experiments 1 and 3 (Table 1). Following liposomal production, vesicles were separated from non-encapsulated drug using gel permeation chromatography (Sephadex G-50 M). The liposomes were then solubilized in sufficient absolute ethanol to produce a clear solution. The amount of ethanol used was accurately measured so that it could be taken into account in subsequent calculations to determine drug loading. The quantity of drug that was entrapped within the vesicles was determined using an HPLC assay adapted from Kang (2000) (mobile phase = buffer/acetonitrile/triethylamine, 81:18:1, where the buffer was 0.2% w/v aqueous solution of sodium phosphate adjusted to pH 2.8–3 with phosphoric acid; fluorescence detection at ex = 280 nm and em = 440 nm; flow rate 2 mL min^{-1}; injection volume 80 μL; column reverse phase, C18, 5 μm silica, 150 × 4.6 mm). The quantity of phospholipid and cholesterol from the lysed liposomes was also determined by HPLC using a method adapted from Choudhari et al (1996) (mobile phase methanol/acetonitrile/water, 70:25:5; UV detection at 205 nm; flow rate 2 mL min^{-1}; injection volume 20 μL; column normal phase, 5 μm silica, 300 × 4.6 mm).

α-Tocopherol acetate was used as a model hydrophobic drug to assess the extent to which the liposomal membranes could hold drugs. Quantities of α-tocopherol acetate ranging from 14 to 70 mg were placed in the pressure vessel with 250 mg SPC at the start of the liposomal production experiment. All other experimental conditions for liposomal production were as for experiment 12 (Table 1). Drug-loaded liposomes were separated from free drug using centrifugation and then solubilized in absolute ethanol. Lipid quantification was achieved using the assay described above, while the drug was quantified using a further HPLC method (mobile phase methanol/acetonitrile, 75:25; UV detection at 280 nm; flow rate 1.7 mL min^{-1}; injection volume 100 μL; column reverse phase, C18, 5 μm silica, 150 × 4.6 mm; column temperature 20°C; retention time for α-tocopherol acetate 6.1 min.

Preparation and analysis of dry phospholipid powders for hydration

Compressed carbon dioxide was pumped via a heat exchanger into the side arm of a ¼″ T-piece housed within an HPLC oven. Once the pressure, temperature and flow rate of the carbon dioxide were constant, an ethanolic solution of phospholipid was simultaneously introduced via a capillary (I.D. 75 or 150 μm) into the central cavity of the T-piece using an HPLC pump. As the ethanolic and carbon dioxide streams mixed within the T-piece, the ethanol solution expanded, causing it to become supersaturated. This resulted in the precipitation of phospholipid particles that collected in an in-line filter assembly situated downstream of the T-piece. The carbon dioxide/ethanol mixture then exited to atmosphere via a back pressure regulator that was employed to ensure that the system pressure remained constant throughout the experiment. Once a total of 10 mL of the phospholipid solution had passed through the system, the pump delivering the phospholipid solution was switched off. Before collecting the powder from the filter assembly, it was 'washed' in a stream of pure carbon dioxide for 15 min to remove any residual ethanol. Further experimental details are shown in Table 2.

Table 2 Details of experiments carried out to produce phospholipid powders

Experiment	Lipid	Concentration of lipid in ethanol (mg mL^{-1})	Ethanol flow rate (mL min^{-1})	Antisolvent (CO_2) flow rate (mL min^{-1})	Capillary i.d. (μm)
A	SPC	45	0.5	15	150
B	HSPC	45	0.5	15	150
C	HSPC	45	0.5	20	75
D	HSPC	45	0.5	15	75
E	DMPC	45	0.5	15	150
F	DSPC	45	0.5	15	150
G	DSPC	45	0.25	15	150
H	DSPC	45	0.25	20	150
I	DSPC	45	0.25	20	75
J	DSPC	45	0.50	20	75

All studies were carried out with an antisolvent pressure and temperature of 150 bar and 40°C, and the flow rates shown are at these conditions. Each experiment was conducted in duplicate.

Successfully produced powders were analysed using IR spectroscopy to determine whether or not they contained residual ethanol. The traces produced from the powders were compared to those of the respective unprocessed phospholipid and samples that had been deliberately spiked with 5 μL of absolute ethanol.

Scanning electron microscopy (Jeol, 53-10) was used to assess the primary particle size of the powders. Samples of the phospholipid powders were sprinkled onto carbon pads mounted on a supporting stub and covered with a fine coating of gold prior to visualization.

Powder samples were hydrated to produce liposomes by firstly adding 10 mL of water to 10 mg of sample. The mixture was then agitated by hand within a water bath that was maintained at a temperature exceeding the glass transition temperature of the lipid in question. Vesicle formation was confirmed by examination with phase contrast light microscopy using 100× magnification (Olympus BX50). The particle size distributions of the resulting liposomal suspensions were then determined using laser light diffraction.

Results and Discussion

Characteristics of liposomes prepared using modified compressed carbon dioxide solvent systems

Size distributions of empty liposomes

The results from PCS analysis are shown in Table 1. The size distributions for all formulations were broad, spanning typically from less than 50 nm up to, or just beyond, a micron. The main purpose of the PCS measurements was to determine whether there were any significant differences in size distribution between formulations prepared under different experimental conditions.

Comparison between experiments 1, 2 and 3 indicated that there was no statistically significant difference between experiments 1 and 2, indicating that the differences in physical properties between liquids and supercritical fluids at these conditions did not influence the outcome of the process ($\alpha = 0.05$). Similarly, no statistically significant difference was observed between experiments 9 and 10 ($\alpha = 0.05$). It would therefore appear that there is no particular advantage or disadvantage in working at supercritical conditions. However, although the thermodynamic state of the solvent mixture did not, in itself, have a significant effect on the size distribution of the formulations, decreasing the temperature of the solvent to 20°C was observed to broaden the size distributions, with PCS traces from experiment 3 all displaying significant peaks in the region above 1 μm. Indeed, the differences between the mean intensity diameters and PIs of experiments 1 and 3 were found to be statistically significant ($\alpha = 0.05$). While this was not the case for experiments 2 and 3, an intuitive assessment of the results suggests a similar trend.

Lowering the pre-expansion pressure below 250 bar was found to significantly increase the PIs of formulations (comparison between experiments 1, 4 and 5, $\alpha = 0.05$). Size distribution peaks above 1 μm were seen in the traces for liposomes made at 200 bar (experiment 4). When the pressure was reduced further to 150 bar (experiment 5) there was very little evidence of any submicron particles and the mean intensity diameters of the formulations produced at this pressure were found to be significantly higher than those prepared at 250 bar ($\alpha = 0.05$). Formulations produced in experiment 5 were full of particulate matter that was visible to the naked eye. Analysis using HPLC showed that these formulations comprised mainly cholesterol with very little phospholipid, presumably because the phospholipid displayed reduced solubility in the solvent mixture, which was of lower density at these conditions. As such, it is likely that a predominance of large cholesterol particles was formed alongside a smaller number of liposomes. In addition to affecting the solubility of the lipid components, reducing the pressure also led to a reduction in the maximum obtainable mass flow rate through the capillary. This may also have led to the increase in size distribution (see below).

Comparison of results from experiments 6 and 1 showed that capillary dimensions alone did not have a statistically significant effect on the size distributions ($\alpha = 0.05$). In experiment 7, the mass flow rate of carbon dioxide during expansion was also a variable, but again the size distributions of these formulations appear unaffected. In experiment 8, however, lowering the mass flow rate to 9 g min^{-1} broadened the distributions and an increase in both mean intensity diameter and PI was observed. It would therefore appear that significant reductions in mass flow rate have an adverse effect on the size distributions of the vesicles. Based on the available results for this system, maintaining the maximum mass flow rate obtainable for a particular capillary at 250 bar would seem prudent.

The hydration step is known to be very important in influencing the outcome of liposomal formation. Factors such as the rate of aqueous phase addition, temperature, degree of agitation and ionic conditions can all affect the size, lamellarity and entrapped aqueous volume of liposomes (Martin 1990). In this study, however, changing the ionic strength and pH of the formulations by using PBS instead of water did not have a statistically significant effect on the mean intensity diameters of formulations produced in experiments 9 and 10 when comparing them to the results obtained in experiments 1 and 2, respectively ($\alpha = 0.05$). The PIs of preparations 9 and 10, prepared in PBS, were statistically lower than those prepared in water ($\alpha = 0.05$). However, one must exercise caution when interpreting the practical significance of such differences in PI when dealing with broad intensity distributions. Indeed, this 'buffer vs water' effect on PI was not observed between experiments 11 and 12.

It is also important to note that the original hydration medium will change in composition during the process as a result of its contact with carbon dioxide during expansion, leading to the formation of carbonic acid. As such, in this work, the final pH values of formulations prepared with water or PBS were 4.7 and 5.9, respectively. The

significance of the changes in pH and ionic strength as a result of carbon dioxide dissolution, and indeed strategies for their quantitative in-process monitoring, are certainly worthy of investigation.

Liposomes containing cholesterol have a greater tendency to fuse than their phospholipid-only counterparts (McAllister 1996). Analysis of experiments 11 and 12 against experiments 9 and 1, respectively, showed that the mean intensity diameters for cholesterol-free formulations were significantly lower than for those containing cholesterol ($\alpha = 0.05$). Although the individual liposome sizes may not have actually differed significantly, aggregates or fused groups of liposomes may have been analysed as single, larger constructs in PCS measurements, which may have skewed the size distributions for the cholesterol formulations.

It is always good practice to use more than one technique to investigate the size characteristics of liposomal suspensions. Because of the broad size distributions seen with PCS, laser diffraction was also employed to analyse the samples. It was found that micron-sized populations also existed in the formulations that were more pronounced in those samples displaying the broadest spread in PCS analysis. Although aggregation could not be ruled out as one likely cause of the larger 'particles', TEM analysis showed that some micron-sized vesicles were indeed present. The overall conclusion based on TEM, PCS and laser diffraction analysis is therefore that two populations of liposomes (nano- and micron-sized) exist in most formulations. Whether or not this was the case when the technique was used by Frederiksen et al (1997) is not clear, as they did not perform laser diffraction analysis. However, as their PCS-derived average diameters and polydispersity indices were essentially identical to many of those obtained in this work, it is not unlikely that they too were producing larger vesicles in addition to those reported in the nano size range. On balance, therefore, the introduction of a further processing step to reduce the size distributions would appear to be necessary before certain i.v. applications could be considered.

Lamellarity of liposomes

The representative transmission electron micrographs in Figure 3 show that the majority of the liposomes possessed between three and five bilayers, and could therefore be classified as oligolamellar vesicles. Frederiksen et al (1997) reported that, as visualized using TEM, a similar method in their hands produced mainly small unilamellar vesicles with a mean diameter of between 20 and 50 nm. No images were presented to support this statement.

Encapsulated aqueous volumes

The encapsulated aqueous volumes within the liposomes were calculated from the amount of drug released after solubilizing the vesicles. A standard assumption in such calculations is that the concentration of the hydrating drug solution is identical to that within the final vesicles. For example, if the original concentration of drug stock solution is $1\,\mathrm{mg\,mL^{-1}}$ then each milligram of drug entrapped within the liposomes must also 'occupy' a volume of 1 mL. However, this assumption does not take into account the fact that the concentration of the hydrating drug solution may actually change over time as it is diluted with ethanol from the solvent mixture during the expansion stage. It is therefore impossible to accurately state the exact drug concentration at the point during expansion when the liposomes actually formed and trapped the solution. Therefore, a range of possible

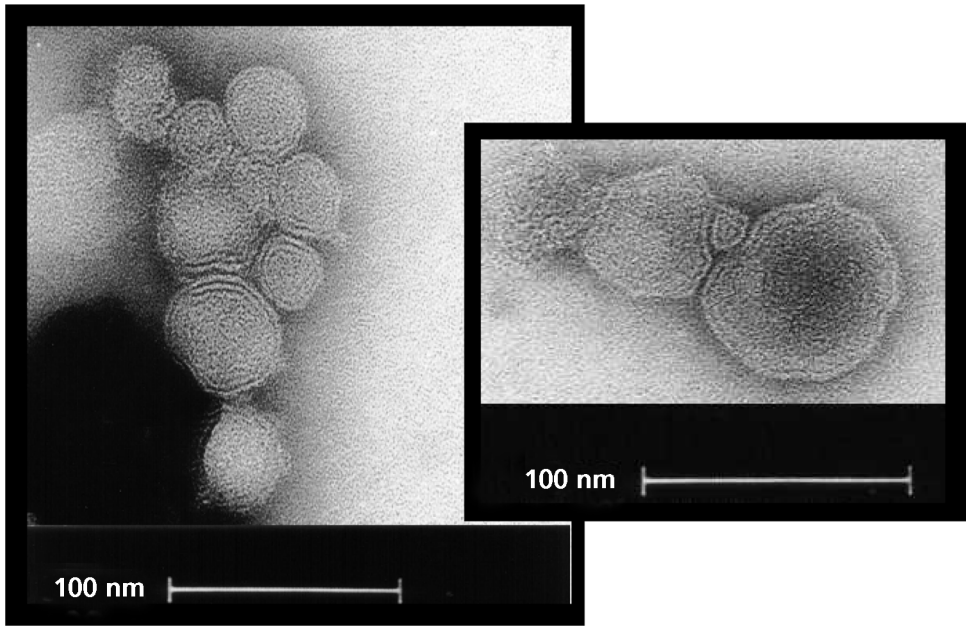

Figure 3 Transmission electron micrographs of liposomes prepared using a modified compressed carbon dioxide solvent system (scale bar = 100 nm).

entrapped aqueous volumes was calculated for each experiment. The *minimum* values stated below are calculated on the assumption that no dilution of the stock solution took place on expansion, while the *maximum* values are those derived from calculations in which full mixing between the solution and ethanol had occurred.

For liposomes produced using a subcritical solvent mixture, the encapsulated aqueous volume was calculated to be between a minimum of 1.18 ± 0.21 L mol^{-1} and a maximum of 1.54 ± 0.27 L mol^{-1}. For the supercritical solvent mixture, the minimum and maximum values were 1.27 ± 0.25 L mol^{-1} and 1.66 ± 0.32 L mol^{-1}, respectively. As a comparison, the theoretical calculated encapsulated aqueous volume for 200 nm diameter liposomes with five bilayers and similar composition is 1.63 L mol^{-1} (calculation methodology as for Perkins et al (1993) using the closest available structural parameters from Lis et al (1982) for egg phosphatidylcholine/cholesterol 1:1 liposomes, where the limiting values for bilayer thickness, interbilayer spacing and area occupied by the phospholipid headgroups are 40.3 Å, 25.2 Å and 96.0 Å^2, respectively). Despite the broad size distributions, the calculated and experimentally derived volumes relate well, indicating that the captured volumes obtained here are typical of those liposomes with similar size and lamellarity.

A comparison between the values presented here and those measured by Frederiksen et al (1997) is shown in Table 3, together with the entrapped aqueous volumes quoted for other liposomal methods that are suitable for commercial-scale industrial use. It can be seen that the encapsulated aqueous volumes seen in this method are generally greater than those seen with the conventional techniques, which is an advantage when considering hydrophilic drug-loading capacity.

Hydrophobic drug-loading efficiency

The encapsulation efficiency of α-tocopherol acetate (μmol drug μmol phospholipid^{-1}) was initially seen to increase with the total amount of available drug substance (i.e. the amount placed in the pressure vessel at the start of the experiment). However, once the drug-loading efficiency had reached about 0.28, no further loading could be seen and the profile plateaued. It is proposed that the liposomal membranes became saturated with drug at this point and were unable to hold any more.

α-Tocopherol acetate was chosen for these drug-loading studies because of its known solubility in compressed carbon dioxide (Chen et al 2000, Škerget et al 2003). However, one would expect drug incorporation by this method to be difficult if the solubility of an agent was poor in the compressed gas solvent. It is therefore important that the formulator establishes the degree of drug solubility in the solvent before attempting to load hydrophobic drugs in this way.

Table 3 Entrapped aqueous volumes within liposomes prepared by various methods

Production method	Entrapped volume within liposomes (L mol^{-1})	References
Compressed carbon dioxide solvent method	0.75–2.19	Frederiksen et al (1997)
	1.2–1.7	This work
Ethanol injection	0.5	Batzri & Korn (1973)
Homogenization	0.7–1.0	Perkins et al (1993)
Extrusion through polycarbonate membranes	0.9–1.2	Perkins et al (1993)

Characteristics of phospholipid powders prepared using supercritical carbon dioxide as an antisolvent

It was found that phospholipid powders could be produced from the saturated phospholipids DSPC and HSPC (experiments B, C, D and F–J). In contrast, oily, lumpy residues were produced from SPC and DMPC (experiments A and E). Magnan et al (2000) and Badens et al (2001) also found that antisolvent processing of soy lecithin produced highly agglomerated particles that further coalesced on contact with air. There are two possible reasons why DSPC and HSPC were processed most successfully: (i) their solubility in ethanol is lower than for the other two lipids therefore at the concentrations employed supersaturation will have occurred more rapidly, leading to the production of smaller particles, and (ii) because of their higher melting and phase transition temperatures there would have been less tendency for particles to become 'sticky' and agglomerate at the experimental temperature employed.

Examination of the processed powders by SEM showed that the primary particles within the finest powders were typically 1–3 μm in diameter (Figure 4).

Neither solvent nor antisolvent flow rate were seen to have a discernible effect on the results. In contrast, Magnan et al (2000) found that increasing the ethanol flow rate decreased particle size, although the smallest primary particle sizes they achieved in their systems with soya lecithin were still larger (10–15 μm) than those observed here with the saturated lipids.

The diameter of the capillary was found to have an effect. It was expected that decreasing the diameter of the capillary would result in the production of finer powders, as finer droplets would have a larger surface area. This would increase the rate of mixing with the carbon dioxide, leading to more rapid expansion and faster supersaturation. However, those powders produced using a capillary with an internal diameter (i.d.) of 75 μm (C, D, I and J) were considerably more coarse than those prepared with a capillary with an i.d. of 150 μm. Examination of these coarser powders using scanning electron microscopy showed that they lacked clearly defined primary particles. It is possible that the finer capillaries became blocked

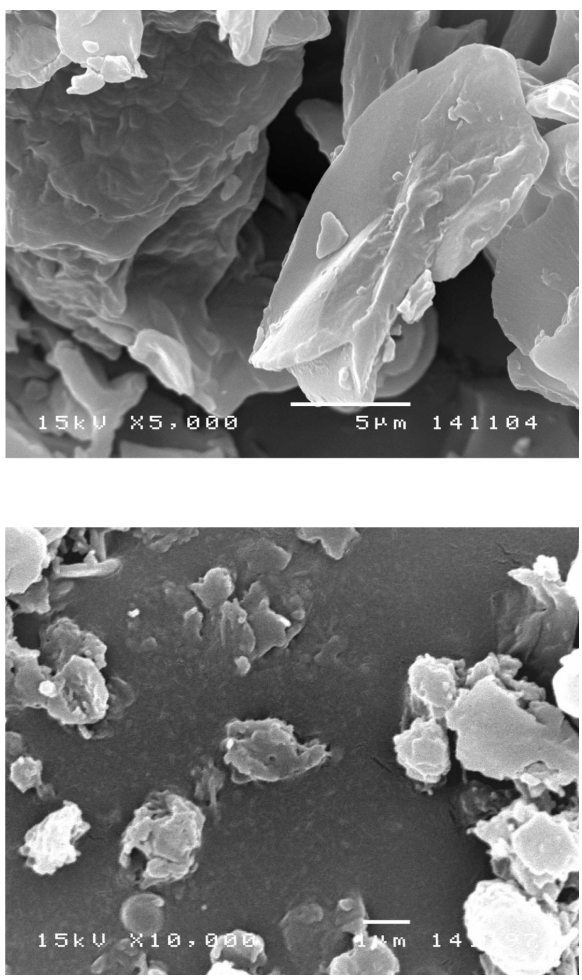

Figure 4 Scanning electron micrographs of (A) unprocessed HSPC (scale bar = 5 μm) and (B) fine HSPC powder produced in experiment B (scale bar = 1 μm).

more readily, which made the flow of the ethanolic phospholipid solution uneven and pulsatile.

IR analysis of successfully produced powders showed them to be free from residual ethanol.

Size distributions of liposomes prepared from the phospholipid powders

The finer powders were hydrated with water to produce liposomes and the presence of vesicles was confirmed using optical microscopy. As the powders from which the formulations were prepared were ethanol free, the resulting liposomal suspensions would also have been free from residual solvent – a key advantage in pharmaceutical formulation. Typical size distribution traces for the vesicles were seen to span from 1 to ~10 μm, with mean volume diameters around 5 μm. Although such micron-sized vesicles are too large for parenteral applications, a significant proportion of the vesicle population could have utility in pulmonary delivery, where particles of ~2–5 μm aerodynamic diameter are desirable. Additionally, the powders may have applications in oral delivery, for increasing the availability of poorly water-soluble actives. The size distributions of these formulations were considered to be relatively narrow for liposomes formed spontaneously, i.e. not subjected to the very high shear of homogenization, high energy of prolonged sonication or forced size reduction through membranes. Production of smaller liposomes is dependent on producing finer powders, which may be possible through manipulation of parameters such as the solvent:antisolvent ratio, solution concentration and temperature. It is also important to note that these liposomes were composed from a single phospholipid, and it is not yet known whether production of multicomponent formulations is possible using this method.

Adaptation to meet cGMP requirements

For any liposomal production process to be industrially useful, the possibility of adapting it to comply with cGMP requirements must exist. It has already been demonstrated that this is a possibility for supercritical particle production technologies, e.g. the antisolvent SEDS process has now been successfully scaled-up for industrial-scale pharmaceutical processing under GMP conditions (Palakodaty et al 2000). Some of the key considerations involved in the adaptation of the set-ups and procedures described in this work include the following (many of which are common to any pharmaceutical manufacturing strategy): (i) material management (e.g. ensuring all lipids, solvents and gases are of the correct quality and from GMP-compliant sources), (ii) use of appropriate quality water (e.g. water for injection) so that bioburden is controlled, (iii) labelling of all rig components and development of full standard operating procedures, (iv) ensuring all equipment finishes are smooth and easily cleaned, e.g. stainless steel components should be highly polished, (v) in-process monitoring and validation of critical steps and necessary documentation procedures should be adopted and (vi) defining cleaning protocols which could include flushing the system with absolute ethanol between batches or steam sterilization.

In the case of the first technique, a strategy to overcome the problems associated with dilution by fresh carbon dioxide during batch sampling would also need to be adopted. One such strategy is to introduce the lipid in a continuous stream of ethanol that is then contacted and well-mixed with compressed carbon dioxide prior to expansion. Once armed with quantitative knowledge of lipid solubilities in modified carbon dioxide, one can ensure that the total quantities of lipid, carbon dioxide and ethanol in the resultant mixture are identical to those that leave the pressure vessel at the start of the expansion stage in the equivalent batch process. In contrast to an antisolvent process, the original concentration of lipid in the ethanol is such that all components remain in solution after mixing so that premature lipid precipitation does not occur.

Conclusions

All conventional methods for liposomal preparation have their individual advantages and disadvantages, and this also applies to the two techniques described here.

The first technique, involving the use of modified compressed carbon dioxide as a solvent, was able to produce nano-sized liposomes without the need for any *toxic* organic solvent. Model drugs could also be incorporated with relative efficiency. However, the size distributions were broad and some micron-sized liposomes were also present in the formulations. If narrow size distributions are essential for the performance of a formulation, other liposomal production strategies such as high-pressure homogenization may be preferable. Alternatively, a further processing step such a filtration or extrusion through polycarbonate membranes could be employed to reduce the size distributions.

Supercritical carbon dioxide was successfully used as an antisolvent to produce phospholipid powders from DSPC and HSPC. The key advantage of this technique was that liposomal formulations that were apparently free from residual solvent could be produced. In addition, it is likely that lipids that would have minimal or no solubility in modified carbon dioxide, e.g. some cationic phospholipids, could be processed using this method. To investigate the potential utility of this technique further, the possibility of preparing multi-component preparations and finer powders would need to be explored.

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