The application of liposomes for solubilisation of hydrophobic drugs

Mathew Louis Steven Leigh

A thesis submitted to the University of London for the degree of Doctor of Philosophy.

Department of Pharmaceutics
School of Pharmacy
University of London

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Abstract

The application of liposomes for the solubilisation of lipophilic hydrophobes for intravenous administration has been investigated.

The lipid composition of four commercially available phospholipids extracted from soya bean and egg sources were studied. The particle size of liposome dispersions generated from pro-liposomes made from these four phospholipids was determined using a variety of sizing techniques. Various factors potentially affecting the particle size such as the degree of agitation, lipid composition and hydration of the liposome were studied. The size distribution of the dispersions without drug were compared to a commercially available intravenous emulsion.

The stability of three placebo anhydrous lipid formulations was examined under accelerated conditions. The harsh conditions induced extensive browning in the soya phospholipid and egg phospholipid samples. Oxidative changes were most pronounced in the soya phosphatidylcholine formulations. Long term stability studies at 40 °C, 20 °C and 4 °C were also carried out on a specific soya phospholipid blend pro-liposome formulation. In this particular study, the oxidative status of the lipid remained essentially unchanged throughout the test period.

The model hydrophobe selected for these studies was the immunosuppressant cyclosporin A (cyA). Association of cyA was found to be dependent upon a careful balance of the pro-liposome components. Furthermore, association was further improved by hydrating the pro-liposome with an appropriate amount of water.

Freeze drying was investigated as a possible alternative presentation of cyA liposomes. Studies on drug-free dispersions revealed that successful freeze drying was reliant upon a variety of factors such as lipid composition, careful selection of stabilisers and freezing. The particle size of all liposome dispersions examined was larger than the original dispersion prior to lyophilisation. However, the association of cyA was unaffected by freeze drying. After rehydration, cyA remained associated with all the liposome dispersions tested- even those which had fused/aggregated to a large extent.
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Dedication

For my wonderful family.
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<th>Description</th>
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<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>Aq.</td>
<td>Aqueous</td>
</tr>
<tr>
<td>BHT</td>
<td>Butylhydroxytoluene</td>
</tr>
<tr>
<td>BP</td>
<td>British Pharmacopoeia</td>
</tr>
<tr>
<td>B.N.</td>
<td>Batch number</td>
</tr>
<tr>
<td>CMAH</td>
<td>Polar alkali TLC solvent system</td>
</tr>
<tr>
<td>CMC</td>
<td>Critical micelle concentration</td>
</tr>
<tr>
<td>CMH</td>
<td>Non-polar TLC solvent system</td>
</tr>
<tr>
<td>cyA</td>
<td>Cyclosporin A</td>
</tr>
<tr>
<td>D-90</td>
<td>90 % undersize value</td>
</tr>
<tr>
<td>DRV</td>
<td>Dried-reconstituted vesicles</td>
</tr>
<tr>
<td>DSC</td>
<td>Differential scanning calorimetry</td>
</tr>
<tr>
<td>EE</td>
<td>Entrapment efficiency</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>EPG</td>
<td>Egg phosphatidylglycerol</td>
</tr>
<tr>
<td>FD</td>
<td>Freeze drying</td>
</tr>
<tr>
<td>FM</td>
<td>Freezer frozen and gently inverted for one minute</td>
</tr>
<tr>
<td>FS</td>
<td>Freezer frozen and left to hydrate without shaking</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>GPR</td>
<td>General purpose reagent</td>
</tr>
<tr>
<td>GPRA</td>
<td>Glycerol pro-liposome used in stability study</td>
</tr>
<tr>
<td>GRAS</td>
<td>Generally regarded as safe</td>
</tr>
<tr>
<td>HII</td>
<td>HexagonalII phase</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>LA</td>
<td>Ethanol pro-liposome formulation used in stability study</td>
</tr>
<tr>
<td>LD</td>
<td>Laser diffraction</td>
</tr>
<tr>
<td>LD50</td>
<td>Median lethal dose</td>
</tr>
<tr>
<td>LIP</td>
<td>Solid lipid formulation used in stability study</td>
</tr>
<tr>
<td>LM</td>
<td>Light microscopy</td>
</tr>
<tr>
<td>LUV</td>
<td>Large unilamellar vesicle</td>
</tr>
<tr>
<td>LPC</td>
<td>Lyso-phosphatidylcholine</td>
</tr>
<tr>
<td>MCT</td>
<td>Medium chain triglyceride</td>
</tr>
<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
</tr>
<tr>
<td>MLV</td>
<td>Multilamellar vesicle</td>
</tr>
<tr>
<td>MPS</td>
<td>Mononuclearphagocyte system</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NA</td>
<td>Not applicable</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NDE</td>
<td>New drug entity</td>
</tr>
<tr>
<td>NM</td>
<td>Nitrogen frozen and gently inverted for one minute</td>
</tr>
<tr>
<td>NS</td>
<td>Nitrogen frozen and left to hydrate without shaking</td>
</tr>
<tr>
<td>O.D.</td>
<td>Optical density</td>
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<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PA</td>
<td>Phosphatidic acid</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PCS</td>
<td>Photon correlation spectroscopy</td>
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<td>PE</td>
<td>Phosphatidylethanolamine</td>
</tr>
<tr>
<td>PG</td>
<td>Phosphatidylglycerol</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>P.I.</td>
<td>Polydispersity index</td>
</tr>
<tr>
<td>PL</td>
<td>Phospholipid</td>
</tr>
<tr>
<td>^{31}P-NMR</td>
<td>Phosphorus nuclear magnetic resonance</td>
</tr>
<tr>
<td>PRO</td>
<td>Glycerol pro-liposome used in particle size study</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>PVC</td>
<td>Polyvinylchloride</td>
</tr>
<tr>
<td>PVP</td>
<td>Polyvinylpyrrolidone</td>
</tr>
<tr>
<td>RES</td>
<td>Reticuloendothelial system</td>
</tr>
<tr>
<td>REV</td>
<td>Reverse phase evaporation</td>
</tr>
<tr>
<td>R_f</td>
<td>Rate of flow value</td>
</tr>
<tr>
<td>s.d.</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
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<tr>
<td>SPLV</td>
<td>Stable plurilamellar vesicle</td>
</tr>
<tr>
<td>SUV</td>
<td>Small unilamellar vesicle</td>
</tr>
<tr>
<td>TBA</td>
<td>Thiobarbituric acid</td>
</tr>
<tr>
<td>TBARS</td>
<td>Thiobarbituric acid reactive substances</td>
</tr>
<tr>
<td>TBARS(F)</td>
<td>TBARS in the presence of iron</td>
</tr>
<tr>
<td>T_c</td>
<td>Transition temperature</td>
</tr>
<tr>
<td>T_g</td>
<td>Glass transition temperature</td>
</tr>
<tr>
<td>TEP</td>
<td>1,1,3,3-Tetraethoxypropane</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>TPN</td>
<td>Total parenteral nutrition</td>
</tr>
<tr>
<td>USP</td>
<td>United States Pharmacopoeia</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra-violet</td>
</tr>
<tr>
<td>w/o</td>
<td>Water in oil emulsion</td>
</tr>
<tr>
<td>w/o/w</td>
<td>Water in oil in water system</td>
</tr>
<tr>
<td>w/w</td>
<td>Weight for weight</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight for volume</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume for volume</td>
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Chapter one

Introduction
1 Introduction

The following introduction is divided into three sections. The first part provides a general introduction to liposomes in the context of intravenous applications. This is followed by a general section relating to the formulation of water insoluble drugs for intravenous administration. These two sections are united in the third section by introducing liposomes as solubilisers for hydrophobic drugs.

1.1 An introduction to liposomes

1.1.1 Liposomes

Liposomes are organised assemblies of phospholipids, which are amphiphilic molecules possessing a hydrophilic headgroup and two hydrophobic hydrocarbon tails (Fig. 1.1). These amphiphiles have the ability to self aggregate into discrete vesicles, which usually have concentric bilayers when equilibrated with excess water (Bangham et al., 1965). These phospholipid bilayers are usually arranged as smectic liquid crystals, i.e. the phospholipids are arranged into distinct layers with their long axis parallel to one another (Florence and Attwood, 198J).

As a consequence of this closed structure, liposomes have the ability to entrap/interact with a diverse variety of drugs with differing properties. Molecules at both ends of the solubility spectrum can be entrapped, as well as drugs with intermediate aqueous solubility. Water soluble compounds can be entrapped within the aqueous core and channels of the liposome. In contrast, hydrophobic materials can be sequestered between the fatty acids of the phospholipid bilayer, where a hydrophobic hydrocarbon domain exists. Lipophiles with a hydrophilic moiety may span these two regions (Juliano and Stamp, 1979a). Charged species can be electrostatically attached to the surfaces of some types of liposomes (Lee and Schreier, 1993), further illustrating the versatility of liposomes to interact with biologically active materials.

Structurally, liposomes are arbitrarily classified by their size and the number bilayers: if the average diameter of the liposomes exceeds 100 nm they are conveniently classed as large, whilst if the diameter is 100 nm or less, they are referred to as small. A liposome which possesses a single bilayer is known as a unilamellar vesicle, a liposome with a few bilayers (2-3) is classified as oligolamellar and a lipid vesicle with many/several bilayers is classed as multilamellar. As will become evident later, the size and
lamellarity are important to control, because these factors may influence the behaviour of liposome \textit{in vivo}.

<table>
<thead>
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<th>Lipid class</th>
<th>Chemical structure</th>
</tr>
</thead>
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| Glycerophospholipid | \[
H_2C-O-R_1 \\
HC-O-R_2 \\
H_2C-O-P-O_{-\text{base}}
\]

\begin{align*}
\text{diacyl: } & R_1, R_2 = \text{CO-R} \\
\text{dialkyl: } & R_1, R_2 = -\text{R} \\
\text{lyso: } & R_3 = \text{H}
\end{align*}

Figure 1.1 Generalised structure of a glycerophospholipid molecule

1.1.2 General applications of liposomes

Due to their structural similarities to cell membranes, liposomes were originally employed in biochemistry as models of cells. However, since the early 1970s, various liposome applications have been investigated in fields diverse as pharmaceutics, vaccination, imaging, agriculture, aquaculture, cosmetics and food technology.

One of the main areas of intense research has been for pharmaceutical applications. Most administration routes have been studied: pulmonary (Schreier, 1994), intranasal (Vyas et al., 1995), topical (Schmid and Korting, 1996), follicular, oral (Ueno et al., 1987) ocular (Singh and Mezei, 1983) and parenteral (Gregoriadis and Florence, 1993). Preparations have been employed to treat a variety of disease states from the life threatening, e.g. cancer (Kim, 1993), to the cosmetic, e.g. male pattern baldness (Li and Hoffman, 1995). The aim of almost all of these studies has been to alter the biodisposition of the active component, by entrapment or association with the liposome.

Some of the early promises and benefits of liposomal formulations have been recently realised in the form of a few commercially available products for clinical use. However, the anticipated widespread and diverse use of this delivery system has not occurred. The products which have been and are being currently commercialised are limited to highly specialised, expensive applications.
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The pharmaceutical industry has viewed liposomes with a degree of scepticism over the years for a variety of reasons. One reason has been that the pharmaceutical characterisation/development lagged behind the intensive animal testing of the early 1980s. This meant that even if preclinical liposome formulations appeared successful in animal models, to scale up the manufacturing procedure to reliably produce sufficient quantities for preliminary human investigations was often problematic. The importance of scaling up has often been underestimated, even though the production of large quantities is a prerequisite for most industrial/commercial applications (Ostro, 1988). To appreciate why scaling up has been problematic, one need only reflect on most of the techniques currently used to generate liposomes (section 1.1.3). Secondly, economic considerations may also have contributed to the slow development of liposomal formulations: the raw material phosphatidylcholine, which is the backbone of most liposome formulations, is expensive. This is presumably the main reason why most commercial liposomal applications have focused upon life threatening disease states, particularly systemic microbial infections and cancer, where a higher cost of the product can be justified. Thirdly, the performance of liposomes particularly for systemic targeting may have been overestimated (Poste, 1986). Physical factors such as avoidance of the mononuclear phagocyte system (MPS), extravasation and access to the target site, particularly to solid tumours may limit the efficacy of even the most optimised long circulating liposome systems. The MPS, formerly known as the reticuloendothelial system (RES), is a collection of cells and tissues which play a key role in immunity by engulfing foreign bodies such as bacteria. Some tissues rich in these cells include the liver's Kupffer cells, spleen, bone marrow and lymphoid tissue. Methods preventing the uptake of liposomes by this tissue have been studied to prolong the circulation time with the intent of targeting non-MPS tissues.

1.1.3 Liposome production techniques

Many of the techniques which have evolved for manufacturing liposomes are limited to laboratory scale or small scale production. Hence difficulties may be encountered when these processes are scaled up. The majority of these techniques, although ideal for laboratory scale, are unsuitable for pharmaceutical production. This may be because of toxic residues of solvents/intermediates in the final dispersion or simply the physical impracticalities of the process, when scaling up is attempted.
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The main methods employed to form liposomes are outlined below and have been evaluated based on three criterion:

1) Ease of large scale production.
2) Suitability for parenteral production: the presence of residual solvents and/or contaminants in the final dispersion.
3) Particle size of the liposomes generated.

Sterility has not been listed here, because aseptic filtration of the final dispersion can be used to prepare a sterile product, if appropriately sized liposomes are produced. Additionally, association of lipophilic hydrophobes has not been included, because it is difficult to assess the association of hydrophobic agents in general. The degree of association seems to be highly dependant upon the individual molecule and its interaction with the bilayer (Cullis et al., 1987). This contrasts with aqueous entrapment, which can be assessed by determining the capture volume of the liposome dispersion (Perrett et al., 1991; Lidgate et al., 1993).

In order to produce a liposome dispersion suitable for intravenous administration, generally a two stage procedure is needed: the first stage involves forming a coarse liposome dispersion, which is usually followed by a modification technique reducing the size and size distribution of the dispersion.

Forming a liposome dispersion is a relatively simple procedure: in the presence of excess water, most phospholipids reorganise themselves to form liposomes. However, to form liposomes with particular characteristics, such as appropriate size, good aqueous entrapment and satisfactory stability, may be rather more challenging.

The main methods for producing liposomes broadly fall into the following categories:

1) Film hydration
2) Emulsion
3) Freeze drying
4) Detergent based methods
5) Solvent injection
6) Pro-liposome
1.1.3.1 Film hydration
This was the first technique used to form liposomes. It involves the creation of a large surface area for the hydration of phospholipid by formation of a thin film (Bangham et al., 1965). This film is formed by drying down the phospholipid from a volatile organic solvent onto the inner wall of a glass round bottomed flask using a rotating evaporator. The resultant film can easily be hydrated at the appropriate temperature (section 2.1.3) and subsequently dispersed by handshaking or mechanical agitation. The amount of water and degree of shaking added at this stage can influence the type of liposome generated. Generally, without the addition of charged species and agitation, the liposomes are multilamellar in character.

A modification of the film technique was developed by Payne et al. (1986). It involves depositing a phospholipid film onto a selected water soluble substrate such as a saccharide or sodium chloride. Typically this is achieved by evaporating the volatile solvent from an organic solution of phospholipids in the presence of water soluble substrate. As the volatile solvent is removed, the water soluble substrate becomes coated with a film of phospholipid. This coating improves the hydration of the phospholipid by creating a larger surface area. The particle size of the liposomes hydrated in this manner is reported to be somewhat smaller than those generated by the original film method.

In its original form, the film hydration technique is simple, and the most trouble free for laboratory production. However, these techniques are difficult to scale up and control of particle size, particularly for parenterals, is difficult. Hence a post production size reduction technique would normally be required. The type of liposome can also be influenced to some degree by the addition of charged species, which tends to favour fewer number of bilayers (Cowley et al., 1978).

1.1.3.2 Emulsion techniques
i) Water in oil method (W/O)
A small amount of water containing the aqueous material to be encapsulated is added to phospholipid dissolved in an immiscible organic solvent, e.g. chloroform. The aqueous phase is dispersed into fine droplets by the input of mechanical energy, e.g. sonication. These small water droplets are emulsified at the solvent/water interface by the phospholipid monolayer, which forms the core of the inner leaflet of the bilayer (New, 1990a). To add the outer leaflet of the bilayer, the double emulsion technique is
employed. This is achieved by introducing the emulsified water droplets in the
immiscible organic solvent into an aqueous environment. Upon a more gentle
mechanical agitation, a w/o/w system is generated. Removal of the organic phase by
evaporation results in the formation of a liposome dispersion, which is predominantly
unilamellar in character. The size of the liposomes is largely dependant upon the degree
of mechanical input when generating the emulsions, but liposome diameters down to
100 nm have been reported.

ii) Reverse phase evaporation (REV)
This technique involves dissolving the lipid in a water immiscible organic solvent, to
which a small volume of the water is added (Szoka and Papahadjopoulos, 1978). This
mixture is sonicated and the resultant w/o type emulsion is partially evaporated until a
gel-like lamellar structure is obtained. This semi-solid gel is vortexed vigorously until
structural collapse occurs and a liposome dispersion is generated. The resultant
liposome is about 500 nm in diameter, with improved aqueous entrapment. The
liposomes are predominantly unilamellar, though some oligolamellar liposomes are also
present.

iii) Stable plurilamellar vesicles (SPLV)
This technique is similar to the w/o method but the drying down is carried out under
nitrogen and continuous bath sonication (Gruner et al., 1985). The resultant vesicles,
known as stable plurilamellar vesicles (SPLV), are reported to be osmotically stable,
because no osmotic gradient exists between the bilayers of the vesicles compared to
MLVs. However, in practice this difference between SPLVs and MLVs may not matter
if post-production techniques, such as extrusion, microfluidisation or homogenisation
are employed. The processing is likely to result in osmotically homogeneous liposomes,
because the liposomes are being disrupted during size reduction.

All of the emulsion techniques described above are unacceptable pharmaceutically,
because it is difficult to lower organic solvent residue levels to acceptable limits,
particularly in the presence of water.

1.1.3.3 Freeze drying: Dried-reconstituted vesicles (DRV)
Dried-reconstituted vesicles are created by firstly generating a dispersion of SUVs,
which have poor aqueous entrapment. This dispersion is freeze dried without any
stabilising agents (Gregoriadis et al., 1990). During the drying process of lyophilisation,
the SUVs fuse to form MLVs, which have improved aqueous entrapment. Although it is a satisfactory technique for entrapping water soluble materials, it is probably inappropriate for hydrophobic materials for parenteral purposes. It would be illogical to produce SUVs in order to generate larger liposomes which would have to be reduced in size again.

1.1.3.4 Detergent technique

The principle behind this technique is to form mixed micelles from phospholipid and detergent. For parenteral applications, the detergent employed is typically an anionic bile salt, though for non-pharmaceutical applications a variety of other surfactants, e.g. β-alkyl glycoside, can also be used. This mix can be converted to unilamellar vesicles by lowering the detergent concentration in the micelles by dialysis (Kagawa and Racker, 1971), column chromatography or dilution (Son and Alkan, 1989). Once the detergent level has been lowered sufficiently, the mixed micelles vesiculate into liposomes.

One advantage with this approach is the absence of solvent residues in the final dispersion, although for intravenous applications residual detergent could be toxic if the detergent is not carefully selected. Appropriate selection of the conditions enables control of the average size of the liposomes within a narrow size distribution. Homogeneous liposomes of diameter around 100 nm or less can be produced, the exact size depends upon the lipid concentration and detergent employed. It is reported that 100% association of hydrophobes can be achieved, if the material, e.g. membrane spanning proteins, is intrinsically suited to the hydrophobic region within the membrane. However, if the material is lipophilic but not anchored to the membrane, this technique may not be suitable for this type of drug, because it may disassociate from the membrane during dialysis. Large scale production of liposomes using the detergent technique has been made feasible using cross flow filtration techniques (Schubert, 1996).

1.1.3.5 Solvent injection

The are two main methods employing solvent injection. The first technique carefully introduces a weak ethanolic solution of phosphatidylcholine into water (Batzri and Korn, 1973). The resultant liposome dispersion is composed entirely of SUVs. The main disadvantage is the dilute concentration of lipid, which can not be increased to above about 2 mg/ml without additional processing.
The second solvent injection technique employs ether as the organic solvent (Deamer and Bangham, 1976). Unlike ethanol, ether is a water immiscible solvent. The liposomes are produced in a similar way to the ethanol method in terms of injection technique. However, instead of mixing the ether solution with the aqueous phase at room temperature, the ether is vaporised as the ether solution of lipid is injected into a warm aqueous phase heated to 60 °C. The liposomes generated are mostly unilamellar and have a particle size of approximately 100 nm.

The advantage of these procedures is that the liposome dispersion probably does not require any further size reduction and can be filter sterilised. However, these techniques are difficult to scale up, slow to manufacture and produce very dilute liposome dispersions. In the case of the ethanol injection technique, the dispersion may require solvent removal and a procedure to concentrate the liposomes.

1.1.3.6 Pro-liposome

This technique is based upon the aqueous dilution of an ethanolic solution of phospholipid to form liposomes (Perrett et al., 1991). The liposomes generated are characteristically MLV in type, though if the blend of lipid and pro-liposome composition are carefully selected, either oligolamellar liposomes or large unilamellar liposomes can be generated. The liposomes are simple to manufacture, do not require the employment of any pharmaceutically unacceptable solvent at any stage and the method is eminently suited for large scale production. The main disadvantage for parenteral applications is the large liposome size.

1.1.4 Size reduction techniques

Many of the techniques described above may require a further processing step after manufacture to reduce liposome size or enhance the aqueous entrapment. In the context of using liposomes as carriers for parenterals, one of the main concerns is to reduce the size of the liposomes. Therefore, most manufacturing protocols employ two stage procedures to generate liposomes suitable for intravenous administration.

The basis of all the following size reduction techniques is to generate energy to fragment the liposome bilayer. This induces the break up of the liposome and subsequently reduces the liposome size after reformation. For efficient size reduction, the processing temperature has to be above the phase transition of the phospholipid (section 2.1.3), i.e. the phospholipid molecules in the liposomes have to be in a relatively fluid state. The
various methods by which this liposome size reduction can be achieved are outlined below:

1.1.4.1 **French press**

French presses are used to disrupt cells under high pressure. In the case of liposomes, the mechanical ram of the press forces the liposome dispersion through a narrow orifice, which ruptures the vesicles by shearing. The resultant translucent dispersion consists predominantly of SUVs (Barenholz et al., 1979; Hamilton et al., 1980).

1.1.4.2 **High pressure homogenisation**

This technology was originally developed for reducing the size of emulsion droplets in the food industry to prevent creaming in dairy products. It was later employed in pharmaceutics in an allied technology to produce fat emulsions for parenteral nutrition. This technology is the current commercial technique of choice to reduce coarse liposome dispersions to small unilamellar liposomes. The homogeniser reduces the size of the liposomes via cavitation and shearing during laminar and turbulent flow (Brandl et al., 1990; Bachmann et al., 1993). This is achieved by passing the dispersion through a small knife edged gap under high pressure. After optimising the process, it is possible to generate homogeneous liposomes with average diameters of less than 50 nm within a very narrow size band. These translucent dispersions are eminently suited to aseptic filtration. This direct method of producing liposomes is amenable to large scale processing. Furthermore, scaling up the production is easy: once the parameters for small scale production have been established and optimised, the information can usually be directly transferred to larger scale manufacturing equipment for larger batches. The main disadvantage is that during use, particulates may be shed from the interaction chamber. Additionally, there is little control over the size of the liposome, only small unilamellar vesicles can be generated.

1.1.4.3 **Microfluidisation**

This process is similar to high pressure homogenisation, the main difference being the interaction chamber, which relies upon head on collision of the liquid at right angles against a plate of the interaction chamber (Mayhew et al., 1984). The advantage is that high amounts of lipid (up to 20% w/v) can be handled. The disadvantages are the same as high pressure homogenisation.
1.1.4.4 Membrane extrusion

Originally described by Olson et al. (1979) and Szoka et al. (1980), the liposome dispersion is physically extruded through a filter with cylindrical channels of defined diameter. These channelled pores are created by laser etching through a polycarbonate filter. After repeated extrusions, the upper size limit of the dispersion approaches the diameter of the pores. This method is useful for producing small batches of liposomes with a defined size, ranging from 30 nm up to several microns in diameter. If the liposome diameters are 200 nm or less, the dispersions can be readily sterilised by filtration. Although this technique can be scaled up (Schneider et al., 1995), without arranging several filters in parallel, the batch volume is usually restricted to approximately 100 litres (O’Hara, personal communication). This is due to the filter diameter, which can not easily be increased above 47 mm without the filter integrity being compromised.

1.1.4.5 Sonication

There are two types of sonication: probe and bath. Probe ultrasonication employing a titanium probe can be used to produce SUVs between the lowest theoretical limit of about 25 nm up to 80 nm (Huang, 1969). For size reproducibility between batches, it is important to maintain the exact position of the probe. The main disadvantages are possible oxidative and hydrolytic lipid damage, which can occur if the process is not controlled. This is due to the highly energy intensive nature of the process. Secondly, the probe sheds titanium into the dispersion, resulting in fine grey deposits which have to be removed from the dispersion by centrifugation. Bath sonication is less intense than probe sonication and, therefore, the rate of the size reduction is inferior and final liposome diameter is generally larger.

1.1.4.6 Freeze thaw sonication

This technique involves alternating cycles of sonication and freezing of a liposome dispersion (Pick, 1981). For large scale production this technique is impractical and is normally applied to enhance the aqueous entrapment of water soluble materials by creating larger unilamellar vesicles (LUVs).

The adoption of the mechanical disruption techniques, particularly microfluidisation and high pressure homogenisation, have greatly aided the commercialisation of liposome products. However, these techniques are expensive and may require large capital
investment. The size reduction step should be appreciated in the context of the manufacturing process as a whole. It is just a part of the manufacturing process, further processing steps, such as sterilisation and stabilisation, may also be required.

1.1.5 Liposome formulations for intravenous applications

Many liposome formulations for parenteral applications are intended to alter the bio-disposition of biologically active compounds. The parenteral route has been concentrated upon, because this route bypasses the natural barriers of the body. Within parenterals, although many different routes have been examined, most effort has been devoted to the intravenous route (Ostro and Cullis, 1989). From the very beginning, liposomes have been advocated as “magic bullets”, in order to target biologically active materials. Hence, work has focused upon extending the circulation time of liposomes \textit{in vivo} with the aqueous drug stably entrapped and targeting these vesicles to specific sites.

In the early 1980s extensive work was carried out employing liposomes as a targeted carrier system. It was believed that once injected, they would circulate around the bloodstream unnoticed, targeting and delivering the encapsulated drug to the desired site. However, it was discovered that if unsaturated phosphatidylcholine vesicles are employed, the liposomes not only immediately leaked their entrapped material but also rapidly disintegrated \textit{in vivo} (Gregoriadis, 1988). In order to decrease this leakage and disintegration, two difficulties had to be overcome. Firstly, the stability and circulation time of liposomes in the bloodstream had to be improved. Secondly, suitable biologically active materials had to be selected which could be effectively entrapped and retained inside the liposome.

Bilayer integrity was greatly aided by the addition of membrane stabilising components, such as the sterol cholesterol (Guo et al., 1980; Allen et al., 1981). Further improvements based upon the same principle, i.e. reducing the membrane mobility and increasing the rigidity, were made by employing saturated phosphatidylcholines. These types of liposomes with hydrogenated phospholipids and cholesterol are being used to entrap daunorubicin, a toxic cytotoxic agent, in a commercially available product “Daunoxome®” (NeXstar, USA). Although such modifications greatly aid the integrity of the liposome \textit{in vivo}, they do not confer the liposome with the ability to evade the host’s defence system. The liposome is still viewed as foreign by the immune system: once in the bloodstream, most liposomes are rapidly recognised and engulfed by the

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MPS. This efficient clearance system may severely limit the half life of the liposome and the drug entrapped within the liposome.

In order to extend the circulation time of the liposome it was realised the size had to be carefully controlled. It was believed that a liposome diameter of 100 nm or less maximised the circulation time of the vesicles (Hwang et al., 1980; Allen and Everest, 1983; Proffitt et al., 1983). Further improvements in circulation time of the liposome were made as different surface properties of liposomes were explored. Work in the 1980s employed phosphatidylinositol as a means of altering the properties of the liposome (Kao and Loo, 1980; Gabizon and Papahadjopoulos, 1988). This negatively charged phospholipid was incorporated into the bilayer, thereby projecting inositol groups at the surface of the bilayer, which significantly prolonged the retention of the liposome in the circulation. The mechanism of this prolongation has been attributed to steric stabilisation. By reducing liposome opsonisation by the plasma proteins, the liposome is not as quickly detected by the MPS. However, due to the high cost and potential toxicity, this approach was not adopted for commercial applications. Nevertheless, it demonstrated the principle that it was possible to increase the half life of liposomes in the bloodstream. The next major advance in circulation prolongation was the development of liposomes incorporating lipids covalently attached to polymers (Blume and Cevc, 1990; Woodle and Lasic, 1992). Although a variety of different polymers can be employed, the liposomes incorporating PEG-ylated phosphatidylethanolamine were selected for further development. Selection of a specific PEG size and incorporation of PEG-ylated phospholipid between 5-10% greatly improved the circulation time of these liposomes.

In terms of selecting the appropriate drug, due to the relative ease of entrapping water soluble compounds stably, almost all liposome systems have employed hydrophilic compounds. Lipophilic hydrophobes, as explained in section 1.3.4.1, have a tendency to leak from the bilayer and therefore have been sidelined for targeting purposes. Originally, aqueous materials were entrapped passively. This meant the entrapment of non-membrane interacting hydrophilic drugs was directly proportional to the volume of aqueous spaces inside the liposomes. Hence entrapping more than 30-40% of the material was generally considered difficult. In order to achieve 100% entrapment, i.e. all of the drug inside the liposome, it was necessary to separate out the liposomes from
unentrapped material by column chromatography or centrifugation. This was an inefficient and a wasteful means of entrapping material. Various techniques, such as pH gradient active loading and remote loading (Bally et al., 1985; Hope et al., 1985; Mayer et al., 1990), have greatly improved the loading efficiency of liposomes. These methods exploited the properties of weak bases: by creating a pH gradient between the liposome interior and exterior, it was possible to direct some drugs towards the aqueous interior of the liposome. The uncharged molecule diffused across the membrane, but once inside the aqueous channels of the liposome, the molecule became charged as a result of the change in pH. The charge prevented diffusion of the molecule out of the liposome, thereby entrapping the charged material within the liposome. Further refinements were made using an elegant ammonium sulphate gradient for amphiphiles, such as doxorubicin. This enabled up to 90% of the active material to be entrapped inside the liposome without further purification (Cohen, 1991; Haran et al., 1993).

Although these systems have developed considerably since the first description of the classical liposome in 1965, the challenge, however, of true site specific delivery still remains. Currently the most advanced system being evaluated clinically are the liposomes incorporating PEG-ylated phosphatidylethanolamine with ammonium gradient loaded doxorubicin (Doxil®, Sequus, USA). These liposomes are highly stable in vivo, and have prolonged circulation times. However, they still do not offer true targeting, even if tumour sites may be more permeable than healthy tissue and enable slightly higher amounts of cytotoxic to be delivered. The true specificity required for “targeting” has yet to be achieved. An added intelligent step, which dictates where and when systems should release their load is required, before the term “targeted” can be genuinely applied. Many ideas have been considered and proposed for this programming, e.g. magnetic, fusogenic, pH dependant release, thermo release and antibodies (Straubinger et al., 1988 and Matthay et al., 1989). However, to date there has been little success with these approaches.

1.2 Parenteral administration

1.2.1 Parenteral route

The parenteral route is a general term used to describe the breaching of any dermal/mucosal barrier by injection to administer drugs into the body. The term parenteral is derived from the Greek para and enteron meaning “beyond the intestine”.
Parenteral administration includes the intravenous, intramuscular, subcutaneous, intradermal, intra-arterial, intracardiac, intraspinal, intra-articular, intracerebral, intraocular and intraperitoneal routes (Pharmaceutical Codex, 1994a). The most common route for parenteral administration is intravenous, indeed the term parenteral is often used synonymously with the intravenous route.

Parenteral formulations are often difficult to develop, primarily because the product has to be free from microbial contamination, i.e. sterile, throughout its shelf life (Akers, 1985). The reason for the necessity of sterility is that after the product has been injected, the natural barriers, e.g. skin/membrane, which usually keep foreign contaminants out of the body have been breached: this enables foreign bodies to be introduced along with the injection, which can lead to infection and/or inflammatory responses. Ideally a form of terminal sterilisation, such as γ-irradiation or heat treatment, should be used to sterilise the formulation in its final container. However, if the drug or system is not amenable to these forms of sterilisation (British Pharmacopoeia, 1993c), aseptic filtration may be employed as an alternative.

Although the formulation principles for most parenterals are similar, some aspects differ considerably. The site and the volume of injection to be administered can influence the types of components that can be used. In particular, intrathecal products and intravenous products (with a volume greater or equal to 15 ml) can not be formulated with any bactericide, due to potential toxicity (British Pharmacopoeia, 1993a). Another restriction on systemically administered solutions is the presence of particulate contamination. For large volume infusions, i.e. greater than 100 ml, the preparation must comply with the limit test for particulate matter in the preparation. In this text, unless otherwise stated, all aspects of parenteral formulation will refer exclusively to the intravenous route for large volume injectables.

40% of pharmaceutical preparations administered in hospitals are parenteral products (Akers, 1987). This route of administration is one of the most important, since patients who are unable to take medications orally, as a result of surgery or medical reasons, are often given their medication intravenously. Additionally, since intravenous injection introduces the drug into the systemic circulation directly, the desired blood levels for therapeutic efficacy can usually be reached rapidly. This is particularly important when an immediate pharmacological response is urgently required for acute conditions, e.g.
thrombolytics after myocardial infarction. Intravenous administration also enables the
dose of the medicament to be accurately titrated, so that the drug level in the
bloodstream can be tightly controlled. This may be particularly important when narrow
therapeutic index drugs are initially administered, e.g. digoxin. Finally, some drugs can
only be administered intravenously because of their intrinsically poor oral
bioavailability. This may be due to either their inactivation if administered perorally,
e.g. polypeptides such as immunoglobulin, or their poor absorption when administered
orally, e.g. amphotericin B.

1.2.2 Drugs with poor aqueous solubility

Most drugs which are commercially available are sufficiently water soluble to formulate
as injectable solutions. However, there is an increasingly significant proportion of drugs
which have poor aqueous solubility. In order to formulate such compounds, it is
necessary to understand the fundamental chemistry of the molecule. If the compound
possesses an ionisable group, e.g. an acidic or basic group, it may be possible to control
the pH with a buffer such that the compound is soluble at a particular pH
(Pharmaceutical Codex, 1994b). This is well illustrated with the broad spectrum
antibacterial ciprofloxacin (Yu et al., 1994), which is used to treat a variety of
respiratory, urinary and gastro-intestinal infections. This antimicrobial has pKa values of
6.15 and 8.66, and above an acidic pH of about 3.5, its solubility is decreased and it can
precipitate out of solution (Hope and Wong, 1985). However, if the pH is carefully
controlled using the hydrochloride or lactate salt, a low pH can be maintained which
keeps the quinolone in solution.

However, the challenge for the formulator increases further when the poorly water
soluble drug can not be formulated by controlling the pH, i.e. it has no ionisable side
group. Poorly water soluble drugs which have an aqueous solubility of less than 1 g in
10,000 ml are defined as insoluble by the Pharmacopoeias (British Pharmacopoeia,
1993b). Other terms which are sometimes used to describe a water insoluble compound
are the terms lipophile and hydrophobe. The former term is derived from the Greek lipos
and philus reflecting a substances “affinity for lipids”. It is useful to refer to compounds
as lipophilic, because it distinguishes lipophiles from inorganic substances. However,
this loose term may be somewhat misleading, because not all lipophiles are water
insoluble. Hence the term hydrophobe, meaning “water hating”, is often used in preference to lipophile, because it encompasses molecules which are water insoluble.

1.2.3 Hydrophobic drugs

Although the number of hydrophobic agents is increasing, in comparison to hydrophilic compounds, the number of water insoluble drugs reaching commercialisation is still small. Nevertheless, the new drug entities entering clinical trials are increasingly more water insoluble. There are numerous hydrophobes in various phases of development ranging from preclinical, through phase I/II up to phase III. Potent, low dose hydrophobic drugs such as the topoisomerase I inhibitors (InPharma, 1996), bryostatin (Waugh and Smith, 1993) and the immunosuppressant tacrolimus (Prograf® data sheet, 1996) reflect the increasing trend towards highly potent hydrophobic agents. For example, in the case of bryostatin, the dose of this experimental drug is a few milligrams, and its aqueous solubility is 0.6 µg/ml. For initial studies, solvent systems such as the PEG 400: ethanol: polysorbate 80 (for bryostatin) may be satisfactory for small scale phase I/II studies, but may be unacceptable for widespread clinical application. Indeed some trials involving hydrophobes have been delayed and even discontinued, due to the adverse reactions induced by the vehicle. The polyethylene glycol vehicle for the antifungal LY-121019 caused marked metabolic acidosis, which resulted in withdrawal of this intravenous formulation from clinical trials (Lyman and Walsh, 1992). Similarly, initiation of the paclitaxel clinical trials in the early 1980s were delayed due to the problems with the drug’s low solubility (Gelmon, 1994) and toxicity concerns over the solubiliser.

Interestingly many of the clinically used hydrophobes can be conveniently classified into three main categories:

1) Immunosuppressants, e.g. tacrolimus, cyclosporin A.
2) Cytotoxics, e.g. paclitaxel, docetaxel, teniposide, etoposide.
3) Antifungals, e.g. miconazole, amphotericin B.

It is unclear whether the activity of water insoluble compounds is in some way related to the lipophilicity of the compounds and their interaction with membranes (T’Ang and Lien, 1981; Canaves et al., 1991; Sun et al., 1994).
1.2.4 Difficulty of dissolving hydrophobes in water prior to administration

In practical terms, if a drug is insoluble, it is hugely impractical to dissolve the solid drug in water prior to administration for three reasons. Firstly, if the dose is relatively high, say ≥ 100 mg, a large volume of fluid, at least one litre, will be required to dissolve the drug. This presents practical difficulties in the clinic, because it would make handling of the formulation difficult. Secondly, a large infusion volume could overloaded the patient with fluid (Akers, 1987). The third reason is the time required for the hydrophobe to dissolve would be excessive. Even if the amount of hydrophobic drug to be dissolved was low, dissolution in water may be problematic: in the presence of excess water, it may still take an unacceptably long time for the drug to dissolve and reach equilibrium with the aqueous media. The drug's hydrophobic surface may prevent wetting and hence slow the dissolution of the powder. Therefore, ideally it is desirable to concentrate the dose of the hydrophobe in the smallest acceptable volume of fluid, which is easy to handle.

1.2.5 Potential dangers of injecting lipophilic hydrophobes intravenously

The majority of oral preparations are administered as powders, which are usually compacted in the form of a tablet, hard capsule or pellets. However, such powders can never be administered intravenously. After oral administration of powders, the active principal dissolves in the gastrointestinal fluid and is absorbed through the membrane of the intestinal tract into the systemic circulation. Therefore, when the drug is absorbed perorally, it usually enters the systemic circulation in a dispersed molecular state, i.e. in solution. For a product to be administered directly into the systemic circulation in the safest possible manner, the same rule should be obeyed: the drug should be molecularly dispersed. If there are solid particles greater than the size of the capillary there is potential danger of blocking up the microcirculation of the patient (Dempsey and Webber, 1983). The size of the capillary depends upon the organ, but the average capillary diameter is about 5 μm. If blockage due to particulate contamination does occur, it usually primarily occurs in the microcirculation of the lungs. This is because the lung capillary bed receives the venous flow, before any other organ, and hence filters off the larger particles. If this event occurs clinically it is called a pulmonary embolism. Occlusion of other microcirculatory vessels could lead to damage to the brain, kidney, liver and eyes. Emboli are clearly of concern in pharmaceutics, and this is why the
presence of particulates in intravenous solutions (Groves, 1988) and colloidal systems have to be tightly controlled.

Although there are parenteral products in clinical use which introduce the drug into the body as a suspension, these formulations are solely for intramuscular, subcutaneous or intra-articular administration. These injection sites can tolerate suspensions, because they avoid direct access to the capillaries: after injection, the drug dissolves and is absorbed from the site of injection, e.g. steroids and crystalline slow release insulin. However, recently the possibility of generating an ultrafine suspension for intravenous administration using high pressure homogenisation has been investigated (Müller et al., 1994). The concept behind these “nanosuspensions” is simple; the insoluble drug solid is sufficiently reduced in size so that the comminuted particles are small enough to be suitable for intravenous administration. The advantage of adopting such an approach is that it may enable very high concentrations of drug to be administered in a small volume of fluid. Although technically feasible, the main difficulty could be the pharmacokinetic distribution of the solids: unlike a molecular dispersion, because the solid is slow to dissolve, the drug may passively target the cells and organs of the MPS. Accumulation of the drug in these tissue may be undesirable particularly when cytotoxics are concerned, because the MPS activity could be impaired after engulfing the solid. It is therefore preferable for the drug to be introduced into the body in a molecular dispersion. Once injected it is therefore free to distribute itself as if it were in solution, thereby circumventing any unpredictable pharmacokinetic disposition.

1.2.6 Formulation restrictions for large volume intravenous products

Factors such as sterility and particle size make formulating large volume parenterals difficult. However, there are many other factors which have to be considered. Firstly, there are certain physical parameters which the product should adhere to, e.g. pH and isotonicity (Pharmaceutical Codex, 1994a). Generally, large deviations outside of the pH range 3-10.5 and the isotonicity range of 300 ± 40 mosmol/litre may result in unacceptable tissue irritation and possible cell damage at the site of injection. This pH range is broader than for other routes, because the blood is an effective buffer and rapidly dilutes and distributes the intravenous product throughout the body. Another constraint is the restriction on components which can be employed for parenteral purposes. There are a variety of components at the disposal of a formulator to enhance
the performance of the topical or oral formulations. In the case of parenterals, however, the components which can be utilised are severely limited, because the formulation is being introduced directly into circulation. The cyclodextrins illustrate this point well. For oral use, this cyclic β-1,4-linked d-glucose oligomer clathrate is a useful formulation tool, which has the ability to molecularly solubilise hydrophobes such as itraconazole (Van De Velde et al., 1996) and piroxicam (Lee and Balfour, 1994). However, when administered intravenously the cyclodextrins can induce toxic fatalities, associated with renal complications. This illustrates how the intestinal route can protect by selective absorption and/or degrading materials (Szejtli, 1990). Another class of useful ingredients, which are greatly restricted for parenteral use, are the surfactants. For oral use a relatively wide range of non-ionic surfactants can be used to form self emulsifying systems, micro-emulsions and micelles. These include the traditional polyethoxylated non-ions and the derivatised triglycerides. These surfactants are mainly non-ionic, and are likely to be less toxic than the charged surfactants (Ross and Silverstein, 1954), because they interact less vigorously with cell membranes. However, for intravenous applications only two types of non-ionic surfactant: polysorbate 80 (commercially known as Tween 80®) and the polyethoxylated castor oil (commercially known as Cremophor EL®, BASF, Germany) can be employed. Finally, the inclusion of preservatives into parenteral formulations may be limited. These antimicrobial preservatives which prevent or hinder microbial proliferation may only be employed for small dose products, where the total volume does not exceed 15 ml. However, for large volume products their employment is prohibited (British Pharmacopoeia, 1993a).

1.2.7 Terminology used to describe excipients which increase hydrophobe solubility

The term carrier is used generally in pharmaceutics to describe an excipient which accompanies the active therapeutic agent in a formulation. The rationale of including a carrier, is to improve the performance of the formulation by enhancing properties such as drug solubility, flow of the formulation or to reduce inter particulate adhesion. In the context of injectables, the term carrier refers to a system which safely carries the drug in molecular dispersion into the systemic circulation. Other terms, such as clathrate and solubiliser, are often used to describe the carrying of insoluble drugs: a clathrate is more specifically an inclusion complex, where the drug is located within the interior of the
carrier, e.g. cyclodextrin. Solubiliser is a general term describing a system which increases the solubility of the drug.

1.2.8 General properties of an ideal intravenous solubiliser

The solubiliser must have a proven and acceptable safety profile, and it should be used in existing parenteral formulations (Presant et al., 1992). Ideally the solubiliser should not have any detrimental pharmacological activity, indeed it would be beneficial if the therapeutic index of drug could be improved. The solubiliser itself should not accumulate in regions of the body, which could result in any detrimental effect. Therefore, the structure of the solubiliser should either immediately disassemble after injection or be sufficiently fragile to be rapidly destroyed. This would reduce the accumulation of the solubiliser in the MPS. To ensure the remnants of the solubiliser are easily cleared from the bloodstream with no adverse effect, the solubiliser should be biodegradable and readily excreted/metabolised.

As with all formulations the ease of handling should be considered. If the formulation is a powder which requires reconstitution, for example freeze dried or spray dried material, the rehydration should be convenient and rapid. The solubiliser should be completely molecularly dispersed to avoid the separation of any unassociated drug from solubilised drug. This association should be maintained throughout the product's shelf life, so that the drug does not crystallise out from the carrier. As mentioned earlier, the solubiliser should not adversely affect the pharmacokinetics of the drug. The drug should behave in vivo as if it is in molecular dispersion, which means it must be released from the solubiliser immediately.

Ideally, a minimal quantity of solubiliser should be employed to solubilise the hydrophobe. This enables the drug and solubiliser to be formulated in the smallest possible volume of fluid, thereby facilitating the handling of the formulation. Finally, the cost of the solubiliser and overall formulation should be as low as possible.

1.2.9 Solubilising lipophilic hydrophobes

1.2.9.1 Systems currently used to enhance solubility

1.2.9.1.1 Cosolvent systems: This technique involves dissolving the hydrophobe in a mixture of water miscible organic solvents so that upon dilution in water the drug remains in solution. The main disadvantage of this approach is the irritancy of the formulation upon injection. This approach is rarely used nowadays, and when it is
employed it is often only in phase I-II studies. The other disadvantage is the limited number of organic solvents that can be used. The main advantage is its relative ease of sterilisation. Examples of drugs formulated in cosolvent systems in current use include; digoxin in ethanol/propylene glycol and nimodipine in ethanol/PEG 400 (Nimotop®, Bayer, Germany).

1.2.9.1.2 Oil in water emulsions: These colloidal systems are being increasingly used to carry hydrophobes (Singh and Ravin, 1986; Levy and Benita, 1989; Prankerd and Stella, 1990), the oil most commonly used is the triglyceride oil from soya bean, which is suitable for human administration. Examples of commercially available drugs which are incorporated into the emulsions include diazepam (Diazemuls®, Dumex, Denmark) and profolol (Diprivan®, Zeneca, UK). The advantage of this method is that the vehicle is innocuous and easy to mass produce if the drug is soluble within the system. The disadvantage is that heat sensitive materials are usually unsuitable for incorporation into this carrier due to heat processing, which is required to sterilise the emulsion.

1.2.9.1.3 Bile salt micelles and mixed micelles: Bile salts are the main natural surfactants used in these solubilising systems. Despite the natural occurrence of these amphiphiles in the systemic circulation, they can be surprisingly toxic (Martin et al., 1992). The most common example of an intravenously administered drug solubilised by a bile salt is the antifungal amphotericin B (Fungizone® IV data sheet, 1996). The negatively charged salt of the bile acid sodium desoxycholate forms an ion pair with this antifungal triene, which is lyophilised to a cake and is reconstituted with water just prior to use. Another formulation employing a bile salt, in this case glycocholic acid, is in the form of a mixed micelle association with phospholipid for solubilising vitamin K (Konakion® MM, Roche, Switzerland). If a drug can be stably incorporated into these micelles, an elegant formulation can be created which is both easy to sterilise by aseptic filtration and easy to handle.

1.2.9.1.4 Non-ionic micelles: Polyethoxylated castor oil is probably the most commonly employed intravenously administered surfactant. It is widely used to carry a variety of cytotoxics, immunosuppressants and antifungals. Although this surfactant is not the only surfactant employed in intravenous formulations, it is currently the surfactant of choice for most ethanol soluble hydrophobes. New products, particularly for life threatening diseases, are still being registered using this solubiliser. This is despite the fact that
many of the older products for less severe conditions/disease states have been removed from the market, due to adverse reactions (Vanezis, 1979; Dye and Watkins, 1980).

Structurally, this non-ionic surfactant is a heterogeneous mix of castor oil esterified with polyethoxylene chains. Although purified, it is viewed as heterogeneous because it composes approximately 30 molecular components (Desai, 1996). A prerequisite for formulating a hydrophobic drug with this surfactant is that the drug has to be ethanol soluble. The amount of surfactant needed for solubilisation depends upon the interaction of the hydrophobe with the polyethoxylated castor oil. Some drugs, e.g. cyclosporin A, require only about 6 fold w/w of the surfactant whereas other drugs, e.g. tacrolimus and paclitaxel, need 50 fold w/w to solubilise the drug. The advantage of the polyethoxylated castor oil is that it offers a cost effective means of solubilising many water insoluble lipophiles. Moreover, the formulation and manufacture of the anhydrous micelle concentrate is simple and does not require specialist knowledge. Normally, the drug is solubilised by dissolving the drug in an appropriate amount of 50:50 mix of polyethoxylated castor oil: ethanol which is easily sterilised, generally via aseptic filtration. Due to the anhydrous properties of this carrier, it is particularly suitable for hydrophobic drugs which are chemically sensitive to water. The resultant oily mix containing the drug is diluted with infusion fluid prior to administration.

Although this surfactant is an excipient and therefore should be inert, this is not the case. It is pharmacologically active, and in some specific cases, e.g. treating multidrug resistance in cancer chemotherapy, it may confer some improved therapeutic benefit (Dorr, 1994). Being a surfactant, it has the ability to transiently deactivate the p-glycoprotein pump, which is associated with removing cytotoxics from the tumour cells. By blocking this efflux mechanism, the level of co-administered cytotoxics is raised and the destruction of resistant tumour cells may be increased (Woodcock et al., 1990).

The primary disadvantage of employing polyethoxylated castor oil is its toxicity profile. All intravenous products containing this surfactant have an MCA warning (British National Formulary, 1996) stating that it may "induce anaphylaxis and therefore the patient should be observed for at least 30 minutes after the start of the infusion and at frequent intervals thereafter". The incidence of this adverse reaction directly attributable to the polyethoxylated castor oil is unknown, because the solubilised drugs
may also cause anaphylaxis and severe allergic reactions. For cyclosporin A, the incidence of anaphylaxis is extremely rare (Howrie et al., 1985). However for paclitaxel, which requires about 20-25 g of polyethoxylated castor oil for complete solubilisation of the average paclitaxel dose, the incidence of allergy/anaphylaxis is so high that all patients have to be pre-administered with a cocktail of anti-inflammatories before the infusion is commenced (Taxol® data sheet, 1996). Another pharmacological effect which has been associated with this surfactant is nephrotoxicity (Thiel et al., 1986). A practical disadvantage with this solubiliser is its propensity to extract leachable components from PVC containers. Therefore, injections containing this surfactant should not be administered in this type of plastic (Trissel et al., 1994).

1.2.9.2 Systems under investigation to enhance the solubility of hydrophobes

There have been numerous reports in the literature regarding the use of novel carriers/solubilisers for parenteral applications. A few of the main systems currently under investigation will be listed below, for more detailed accounts the reader is referred to the specific references from the literature: liposomes (Thoma and Schmid, 1992), nanoparticles (Müller and Wallis, 1993; Schwarz et al., 1994; Couvreur et al., 1995) and antinucleating polymers (Olson and Faith, 1988; Yonish-Rouach et al., 1990). As discussed earlier, cyclodextrins have been studied but were too toxic for parenteral administration. Chronic administration resulted in irreversible renal toxicity, probably due to the formation of a cholesterol/cyclodextrin complex which crystallised in the kidneys (Frijlink et al., 1991).

1.3 Liposomes as solubilisers

1.3.1 Developing novel carrier systems

The average cost of a New Drug Entity (NDE) is estimated at over $ 200 million (Jones and Platford, 1996). The average time for drug development is 12 years, and only 1 in 3 drugs are profitable. On top of these development pressures, these costs usually have to be recouped within eight years of the launch of the product before patent expiration. Therefore, if a novel colloidal carrier/solubiliser is adopted for a NDE the following factors have to be considered in the context of the above factors: in addition to the difficulties associated with development of the drug entity, one may also be faced with the difficulties associated with the carrier/solubilising system. Indeed, in some cases the development of the carrier/solubilising system may be as problematic as the
development of the drug itself. Despite the fact that in the eyes of a formulator the carrier/solubiliser is viewed as an inactive excipient, often the carrier/solubiliser may be scrutinised by the regulatory authorities as if it were an active component. This may be one important reason why new hydrophobic drug entities for intravenous administration are often rejected in favour of the more simply formulated water soluble compounds. The formulation of a water insoluble drug into a novel system may incur longer development time and greater costs. It is therefore unsurprising that there are still few novel colloidal/solubilising systems being developed for new water insoluble chemical entities.

Additionally, when formulating a carrier system, often the properties/technology required to design the carrier for a specific drug are unique and may not be directly transferable to other chemical entities: with most carrier systems, one must develop and tailor a carrier system to suit the particular drug entity. This again takes time, costs money and may slow the development time of the drug. Perhaps to demonstrate the utility of a novel system it would be necessary to reformulate an old drug which has an established pharmacokinetic and toxicity profile, with the novel carrier system. This may enable an assessment of the carrier to be determined by directly comparing the new carrier against the original formulation. Such an approach has been adopted with the novel lipid formulations of amphotericin B. Although the novel lipid formulations employ relatively expensive excipients and complex manufacturing procedures, their high price is justifiable. Compared to the bile salt colloidal dispersion, these formulations have reduced nephrotoxicity and enable higher amphotericin B doses to be administered without unacceptable toxicity (Gates and Pinney, 1993).

Polyethoxylated castor oil is still widely used in intravenous formulations, despite its inherent toxicity profile. Although this is not an ideal solubiliser, it is extremely useful and is currently the preferred method for solubilising hydrophobes which are ethanol soluble. To replace this surfactant with newer surfactants or alternative formulations, will be difficult for a variety of reasons. Firstly, it is a familiar solubilising aid which has been used in many intravenous formulations for over two decades and has been accepted by regulatory authorities world-wide. Secondly, to introduce a novel excipient would be expensive, and may be as onerous as developing a new chemical entity. Therefore to replace polyethoxylated castor oil as a solubiliser, ideally
surfactants/materials which are currently used in parenteral formulations would have to be employed. Thirdly, although polyethoxylated castor oil has been associated with serious adverse effects, generally such events are rare and are relatively insignificant compared to the toxicity of the solubilised drug and the disease state itself.

There have to be clear benefits in reformulating intravenous formulations containing polyethoxylated castor oil, e.g. a higher dose of the active principal may be administered, the pharmacodynamics may be improved or the toxicity may be markedly reduced. If the advantage is solely based on the grounds of replacing the polyethoxylated castor oil, the novel system is unlikely to be successful. Furthermore, a drug should be selected which when incorporated into the carrier/solubiliser offers genuine advantages over the existing formulations.

1.3.2 Liposomes as solubilisers of IV water insoluble drugs

It is evident from section 1.1.6, that most liposome applications have concentrated upon targeting hydrophilic drugs. Other useful parenteral applications, such as using them as a formulation aid to solubilise water insoluble compounds, have not been as extensively investigated. Indeed, what is being proposed here is almost the antithesis of the targeted systems: with targeted liposomes the drug distribution is altered usually by prolonging the circulation time of the drug encapsulated inside the liposome. What is being proposed in this context, is the complete opposite: the requirement is for drug to be immediately released from the liposome into the systemic circulation, so that the pharmacokinetics are identical to a molecular dispersion of the drug.

Liposomes have been largely overlooked as carriers/solubilisers for hydrophobic drugs by mainstream researchers for a variety of reasons. Firstly, most lipophilic hydrophobes readily and rapidly leak from liposomes in vivo. Without chemical modification of the material, hydrophobes can not be stably entrapped for the purpose of targeting. Secondly, the technical hurdles, such as hydrophobic drug association with the liposomes, stability and ease of manufacture, can be challenging. In comparison to existing systems, such as the polyethoxylated castor oil micelle, liposomes are therefore considerably more difficult to develop. Thirdly, it may not be cost effective to use liposomes as solubilisers, the cost of raw materials and production techniques are expensive. If the only benefit of employing liposome formulations is to avoid the use of surfactants such as polyethoxylated castor oil, it is doubtful whether such preparations
will be commercially viable, since liposome production is too troublesome and often requires specialist equipment and facilities. To be viable, additional benefits such as improved safety and handling are perhaps needed. Finally, although phospholipids are relatively innocuous materials, in the form of aggregated structures, such as some liposome formulations, their toxicity profile may be altered and may limit their potential as solubilisers (Allen et al., 1984; Allen and Smuckler, 1985).

1.3.3 Advantages of using liposomes as solubilisers for IV drugs

First and foremost, phospholipids generally have an excellent toxicity profile (Parnham and Wetzig, 1993); they are safely used in a variety of parenteral products in large doses in excess of >5g, usually in combination with triglycerides. Even larger amounts of phospholipids have been employed in phase one studies of the cytotoxic NSC 251635 (Sculier et al., 1986). In this trial more than 20 g of phospholipid in the form of liposomes was used to carry this hydrophobe. It was suggested by the author that this type of formulation offered a useful means of carrying hydrophobes, despite the fact the lipid was undoubtedly highly oxidised after the γ-irradiation sterilisation and 4 g of the toxic positively charged stearylamine was administered with the phosphatidylcholine. Additionally, not only are phospholipids non-toxic, but in some circumstances they have the ability to improve the therapeutic index of some toxic drugs. This is exemplified specifically by the transfer of the amphotericin B in liposome form to high density lipoproteins (HDL) leading to reduced renal toxicity (Wasan et al., 1994). Due to the reduced toxicity, it may be feasible to administer higher doses of some drugs without the corresponding increase in acute toxicity (Adler-Moore and Proffitt, 1993).

Liposomes are highly versatile structures (Gregoriadis, 1984), which can be formulated with one or more bilayers and vary in size from several micrometers down to the nanometer size range. The size can be tailored to make them small enough for sterilisation by aseptic filtration, which is a requisite for parenteral formulations. Furthermore, a highly flexible, biodegradable, rapidly disintegrating and easily cleared liposome system can be designed by carefully selecting the appropriate phospholipids. Finally, as will be discussed below, the pharmacokinetic profile of most hydrophobes is favourable when incorporated into liposomes.
1.3.4 Pharmacokinetics of the drug and liposome

When formulating novel colloidal carriers/solubilisers for existing drugs, both the pharmacokinetics of the drug and the pharmacokinetic distribution of the carrier itself must be considered. Ideally, the drug pharmacokinetics should be identical to the original product in clinical use. If it differs, it should not adversely affect the efficacy or safety of the product, i.e. it should not accumulate in sites where drug activity could be deleterious. Secondly, the liposome pharmacokinetics have to be cautiously evaluated. It is generally not desirable for the liposomes to accumulate in the tissues such as the MPS. Therefore, phospholipids should be carefully selected to form liposomes which are rapidly destroyed and cleared in vivo. For toxicity reasons, liposomes should not circulate for prolonged periods if they are serving no purpose. Ideally, it is better for them to be destroyed rapidly before they even reach the MPS, where they could potentially cause difficulties. Particulate overload of MPS has been associated with reduced immune activity, which may lead to complications such as infection (Allen et al., 1984). Chronic liposome administration has also been implicated with histological changes occurring in the liver (Allen and Smuckler, 1985).

1.3.4.1 Pharmacokinetic distribution of lipophilic hydrophobes

Elegant work carried out by Fahr et al. (1995) used cyclosporin A (cyA) as a model hydrophobic drug to demonstrate that targeting of most hydrophobes could not be achieved using liposomes. A series of experiments using liposomes with a variety of lipid compositions showed the pharmacokinetic distribution of the hydrophobe to be independant of the liposome fate. Irrespective of the type of liposome employed; saturated, charged, or PEG-ylated, it was demonstrated that in vivo, the drug rapidly equilibrated itself in the body as if it were administered in a micelle formulation. The reason for this is due to the ability of most hydrophobes to leave the liposome bilayer. Although these hydrophobes are defined as water insoluble by the Pharmacopoeias, they possess a degree of aqueous solubility, even if it is to a minimal extent. Therefore, when added to a large volume of water with lipophilic compartments, e.g. the bloodstream, the hydrophobe rapidly partitions out of the liposome via the aqueous environment. It redistributes itself between the liposome and other lipophilic compartments, e.g. fatty tissues, red blood cells and lipophilic plasma proteins, as if it were solubilised in...
micelles. Most hydrophobes will behave in this manner unless the hydrophobe is chemically derivatised to encourage intercalation and anchorage to the liposome bilayer. The findings described above were contrary to the reports of some workers, who reported that the drug pharmacokinetic profile of liposome cyA was altered. Fahr (1995) put forward suggestions to explain these anomalies: although hydrophobes can not be targeted using liposomes, it is possible to modify the half life of cyA by controlling the amount of lipid injected. Drastically increasing the lipid level by introducing large levels of phospholipid into the bloodstream, could possibly alter the pharmacokinetic distribution of the hydrophobe. Injecting large doses of lipid, effectively increases the fat compartment in the bloodstream and the drug partitions into this phospholipid as if it were excess fat. Additionally, differences in blood lipid profiles between animal models may account for the differing pharmacokinetic profiles of the drug between the different animal species. Similar pharmacokinetic behaviour of paclitaxel in animal models has been observed (Sparreboom and Beijnen, 1996). It seemed that the large amount of polyethoxylated castor oil contributed to the non-linear kinetics of the drug by increasing the lipid content of the blood, in the form of the polyethoxylated castor oil.

1.3.4.2 In vivo distribution of classical liposomes

The distribution of the liposome depends largely upon the phospholipid composition. Addition of cholesterol and other membrane stabilising components (Muramatsu et al., 1994 and 1995; Qi et al., 1995) may maintain the integrity of the liposome and direct it towards the MPS. Incorporation of charge is believed to reduce circulation time of liposomes compared to neutral liposomes (Hernandez-Caselles et al., 1993). In this context classical liposomes with lipid compositions using unsaturated PC and unsaturated PL will mainly be considered.

One major concern using liposomes is the passive accumulation of liposomes in the cells of the MPS. High doses of liposomes may lead to saturation of the system and possible reduction in the efficacy of the immune system (Allen, 1988). Originally, this saturation of the MPS was the rationale for pre-administering a high dose of blank liposomes: the objective being to transiently block the MPS activity to enable non-MPS tissues to be imaged/targeted (Proffitt et al., 1983). However, if high levels of cholesterol are omitted from the bilayer and the phospholipids are predominantly unsaturated, the liposomes are likely to be destroyed rapidly, even before they can reach
the MPS. Indeed this destruction seemed to be occurring when the original “targeting” experiments attempted to target liposomes containing hydrophilic materials. These initial experiments yielded disappointing results in animals, because the liposomes did not remain intact in vivo. It was soon realised that classical liposomes composed solely of phosphatidylcholine were both leaky and short lived in vivo. These phosphatidylcholine vesicles were predominantly destroyed by the lipophilic plasma proteins (Scherphof et al., 1978; Scherphof and Morselt, 1984; Bonté and Juliano, 1986). This property is desirable for liposomes if they are to be used as solubilisers.

1.3.5 Studies reporting the use of liposomes as solubilisers

There are relatively few studies reporting the use of liposomes as solubilisers (Lidgate et al., 1988; Thoma and Schmid, 1992). The main classes of drugs which have been studied are the corticosteroids (Arrowsmith et al., 1983), hydrophobic antifungals (Lopez-Berestein and Juliano, 1987) and hydrophobic cytotoxics. Examples of two clinically used cytotoxics include paclitaxel and teniposide, both hydrophobes are currently formulated in polyethoxylated castor oil. Paclitaxel, a natural diterpenoid extracted from yew bark (Wani et al., 1971), was solubilised in PL/ bile salts micelles which formed liposomes upon dilution. This carrier appeared to have reduced toxicity compared to the non-ionic carrier (Alkan-Onyuksul et al., 1994). In mice, the mixed micelle vehicle had a median lethal dose, LD50, which was 1.4 times less toxic than the polyethoxylated castor oil surfactant. A similar study by Sharma and Straubinger (1994) demonstrated that PL liposomes could dramatically reduce the toxicity of the formulation. In this study, paclitaxel could be completely solubilised, if the paclitaxel content was kept ≤ 3 mol% with specific lipid compositions.

Teniposide, a semi-synthetic podophyllotoxin cytotoxic, has been associated with liposomes generated from bile mixed micelles (Alkan-Onyuksul and Son, 1992). However, a large amount of phospholipid relative to teniposide (approximately 50:1 w/w) was required to fully solubilise this hydrophobe.

Examples of novel compounds solubilised in liposomes include some photo dynamic therapy drugs (Richter et al., 1993; Segalla et al., 1994) and tacrolimus (Lee et al., 1995). Photo dynamic therapy drugs are used in cancer therapy, because they have a tendency to selectively accumulate in malignant tissue. Subsequent illumination activates the drug and results in the destruction of the tissue. A specific example of a
photosensitiser incorporated into liposomes is zinc (IV) phthalocyanine, this
hydrophobe was manufactured in a liposome formulation for pilot scale production by
Isele et al. (1994). Liposomes produced by film hydration and extrusion have been used
to study the solubilisation of tacrolimus (Lee et al., 1995), a low solubility
immunosuppressant.

1.3.6 Manufacturing liposome dispersions just prior to IV administration

To date, pre-formed liposome dispersions or freeze dried liposome dispersions are the
only marketed form of liposomes. However, these forms may not be suitable for all
biologically active materials: the limited stability of pre-formed dispersions (section
4.1.6) and the cost of lyophilisation for large dose drugs (section 6.1.1) may prevent the
development of some liposome formulations. In this study, pro-liposomes have been
assessed as a method of generating liposome dispersions just prior to use and also as a
technique of manufacturing dispersions for lyophilisation.

To date, the production of liposome dispersions just prior to their administration has
largely been hindered by the lack of a simple method for generating liposomes of a
suitable size for injection and the difficulty of obtaining liposomes of a definable size
(Talsma and Crommelin, 1993). Therefore, as well as having assessed the stability of
pro-liposomes and the association of liposome dispersions with a model hydrophobic
drug, the particle size of dispersions generated from pro-liposomes was characterised.

1.3.7 General aims of project

The aim of this thesis was to investigate and assess the use of liposomes as solubilisers
of hydrophobic drugs for intravenous administration. The formulations were developed
with pharmaceutical rationality in mind, by employing pharmaceutically acceptable
materials and intermediates at all stages. The liposome dispersions were produced from
pro-liposomes, employing commercially available lipids, which are currently used in a
variety of parenteral applications. The possibility of forming liposomes just prior to
administration from pro-liposome compositions was assessed. Various pharmaceutical
aspects of this system such as stability, particle size and drug association were
investigated. Drug-free formulations were developed first from pro-liposomes without
drug. These formulations were subsequently evaluated in the presence of a model
lipophilic hydrophobe, cyclosporin A. Finally, lyophilised cakes, generated by freeze
drying liposome dispersions, were assessed as alternative presentations.
Chapter Two

Composition of unsaturated phospholipids
2.1 Introduction

2.1.1 Phospholipid structure

Phospholipids are classified into two general groups: the glycerophospholipids and the sphingosylphospholipids (Marsh, 1990). The glycerophospholipids consist of derivatives of the parent compound 1,2-diacyl sn-glycerol-3-phosphate (phosphatidylinositol acid). Only the glycerophospholipids will be discussed because these lipids are the most commonly employed membrane lipids in pharmaceutical applications and were used in this work. In this context, the general term phospholipid will be used to describe these glycerophosphatides. The other group of phosphatides, which have a sphingosine backbone will not be considered, except to mention that the most common lipid in this class is sphingomyelin.

The asymmetry of the glycerol backbone of the phospholipid molecule means that two possible stereoisomers can exist. However, most naturally occurring phospholipids exist in the Laevo (L) form. There are two moieties to the phospholipid structure, the hydrophilic headgroup and the hydrophobic tails. The headgroup esterified to the phosphate on position 3 of the glycerol backbone, determines the net charge of the headgroup. Since this glycerophosphate structure has one negative charge associated with the phosphate, addition of a positively charged base such as choline or ethanolamine neutralises this charge at pH 7. The resulting neutral diacylphospholipids are commonly referred to as phosphatidylcholine (PC) and phosphatidylethanolamine (PE) respectively. In contrast, if the headgroup has no charge or one negative charge the amphipathic molecule possesses a net negative charge of one and two respectively. Examples of the most common negatively charged phospholipids include: phosphatidic acid (PA), phosphatidylserine (PS), phosphatidyglycerol (PG) and phosphatidylinositol (PI).

The hydrophobic moiety of the phospholipid contains two fatty acids which are ester linked to the 1 and 2 hydroxyl positions of the glycerophosphate backbone. These fatty acids may be either identical or mixed. However, the phospholipids extracted from natural sources, usually have heterogeneous fatty acid chains with the longer, more saturated of the two chains usually located in position 2. These fatty acids vary in hydrocarbon chain length, but are typically between 16-20 carbons long and have an even number of carbons in the chains. The degree of fatty acid saturation varies
depending upon the source of the phospholipid. Usually there are between 0 to 4 double bonds on each chain. In natural phospholipids, these double bonds are mostly of the \textit{cis} type. These fatty acids can be designated by the convention- number of carbon atoms: number of double bonds, e.g. an 18 carbon long fatty acid with one double bond may be abbreviated to C18:1. The position of the double bond can be located in two ways: firstly, from the carboxyl group of the fatty acid, the symbol $\Delta$ is used. Alternatively, the double bond can be located from the methyl end using the symbol $\omega$. In this thesis, the latter system describing fatty acid structure will be used along with trivial names of the fatty acid.

2.1.2 Organisation of phospholipids in aqueous environment

Although these amphiphilic phospholipid molecules are practically insoluble in water, above a specific aqueous concentration self assembly of the molecules occurs. This concentration above which monomer molecules self aggregate is called the critical micelle concentration (CMC). Since the CMC of these lipids is several orders of magnitude less than most surfactants (Israelachvili et al., 1980), assembly of phospholipid occurs even at very low concentrations in water. The type of assembly which the phospholipid adopts in water depends upon the shape of the individual phospholipid molecule. The shape is an approximation of the spatial volume occupied by the hydrophilic head and hydrophobic tails of the phospholipid (Cullis and de Kruijff, 1979). Hence, factors which may influence the volume such as pH and temperature can also impact upon the organisation of the assembly. However, in this study, since the predominant lipid component in all formulations was restricted to PC, the structures formed in water were bilayered (Figure 2.1). This organisation is favoured because the cylindrical shape of the PC molecule prefers the packing arrangement of bilayers (Tilcock, 1986).
2.1.3 Transition temperature

Phospholipid bilayers generally exist in one of two thermal states. The thermal state of the bilayer is governed by temperature. In general, phospholipid bilayers undergo a phase transition from a lower temperature solid state (also referred to as the gel state) to a higher temperature fluid liquid crystalline state. In the solid state, the fatty acid chains are tightly packed into a structured lattice, whereas in the more fluid state the phospholipid molecules are in continuous motion within the leaflets of the bilayer. The temperature at which this co-operative phase change occurs is known as the main phase transition temperature ($T_c$). The $T_c$ is strongly influenced by the structure of the phospholipid: longer chain lengths and a greater degree of saturation both raise the $T_c$. This is due to the increased van der Waals attraction forces between the fatty acids of neighbouring molecules. The corollary is also true; a shorter chain length and a higher degree of unsaturation in the fatty acids reduce van der Waals attraction forces and hence lower the $T_c$. The phospholipid headgroup also affects the $T_c$. Charged species reduce the $T_c$ by intermolecular repulsion by spacing the molecules within the bilayer apart. Conversely, the phospholipid phosphatidylethanolamine (PE), which forms hydrogen bonds between the molecules, has a tendency to raise the $T_c$, because molecular attraction is increased. The $T_c$ of phospholipids from natural sources spans a broad range due to the heterogeneity of the fatty acids and phospholipid classes, e.g. the
Chapter Two - Composition of unsaturated phospholipids

Tc of soya PC ranges from -20 °C to -30 °C and the Tc of egg PC ranges from -5 °C to -15 °C (Ladbrooke and Chapman, 1969). In contrast, synthetic homogeneous molecules with identical fatty acid compositions have narrower peaks, e.g. dimyristoyl phosphatidylcholine has a Tc around 23 °C. An understanding of Tc in the context of liposome formation is important. The organised bilayer structures will only form/rearrange if the temperature of the lipids is above their characteristic phase transition temperature. This is of particular relevance for formation of liposomes from pro-liposomes, because the liposomes have to be generated just prior to use at room temperature. Therefore, phospholipids have to be selected which have an appropriately low Tc to enable liposome formation at room temperature.

2.1.4 Importance of lipid composition in liposome formulations

An appreciation of the lipid composition is important for two main reasons: firstly, the lipid composition may directly affect the qualities of the liposome such as lamellarity, entrapment, size and physical stability. In turn, these parameters may influence the in vivo performance of the liposomes. This is exemplified by phospholipid composition influencing the macrophage clearance of the liposomes/colloidal carriers (Davis and Hansrani, 1985). Secondly, though phospholipids have been granted GRAS status (Generally Regarded As Safe), some phospholipid compositions may be unsuitable for intravenous administration (Parnham and Wetzig, 1993). Compositions which contain high levels of one or more of the following lipids may be a cause for concern if administered intravenously:

1) Lyso-phosphatidylcholine (LPC).
2) Heavily oxidised phospholipids.
3) Negatively charged species.

LPC and oxidised phospholipids will be dealt with in chapter four, because these components may develop during the long term storage of lipids. Only charged phospholipids are discussed here, because they may be present in the lipid compositions either as impurities or deliberately incorporated to enhance the performance of the liposomes. Positively charged species are generally precluded from systemic use, because of their adverse interactions with cell membranes (Yoshihara and Nakae, 1986). Charged lipid species from natural sources are negatively charged. There are a variety of reasons for including negatively charged lipids in formulations: the lamellarity may be
controlled, fusion/aggregation of liposomes may be lowered during storage and the interaction of certain drugs with the bilayer may be improved (Proffit et al., 1989). Although most charged phospholipids are suitable for oral administration, for parenteral administration the use of some negatively charged phospholipids may be restricted. Direct introduction into the systemic circulation of certain negatively charged lipids with pharmacological activity may give rise to undesirable toxicities. This is illustrated by PA (Zbinden et al., 1989), which can induce platelet aggregation. The charged lipid with the safest profile seems to be PG. This phospholipid is employed in licensed liposome and lipid complex products containing amphotericin B for parenteral administration. Therefore, PG has been used in this study to supply negative charge to phospholipid bilayers when required.

2.1.5 Analysis of phospholipids
Phospholipids are usually analysed according to their headgroup, because of their relative ease of separation based on headgroups. Three main techniques used to assess phospholipids are thin layer chromatography (TLC), high performance liquid chromatography (HPLC) (Gunstone et al., 1994a) and phosphorus nuclear magnetic resonance ($^{31}$P-NMR).

The fatty acid profile of the phospholipid batches under examination were supplied by the manufacturers of the lipid.

2.1.6 Selecting phospholipids suitable for solubilising intravenous hydrophobes
Most commercialised liposome products for intravenous administration employ highly refined saturated PC. This saturated PC is produced semi-synthetically by exchanging the unsaturated fatty acids from natural PC for saturated fatty acids or by hydrogenating the refined unsaturated PC. The reason for the selection of high $T_c$ PC is two fold. Firstly, compared to unsaturated PC, the aqueous retention characteristics of liposomes generated from high phase transition temperature saturated PC are superior. Secondly, saturated fatty acids avoid many of the oxidative stability problems associated with unsaturated lipids. Throughout this study unsaturated phospholipids have been employed because liposomes are formed, which are more readily destroyed in vivo compared to the more saturated liposomes. Additionally, the unsaturated phospholipids with low $T_c$ (below 0 °C) are ideal for the formation of liposomes prior to administration, because addition of water is always above the $T_c$ of the lipid. Hence
liposome formation can take place at room temperature. This contrasts with the saturated PLs, which usually require addition of water above the $T_c$, which is greater than approximately 40 °C for chain lengths of 16 and above (Ladbrooke and Chapman, 1969).

2.1.7 Sources and applications of unsaturated phospholipids
Although the natural sources of phospholipids are diverse, only egg yolk (animal source) and soya bean (plant source) are used pharmaceutically.

The main application of these phospholipids is as an emulsifier for parenteral emulsions. This application is particularly relevant to this study for two reasons. Firstly, the type of phospholipids used in these emulsions are similar to the lipids under investigation in this study. Secondly, as will be discussed in more detail in section 3.1.4, some of the colloidal structures found in these emulsion preparations are not dissimilar to liposome structures.

2.1.8 Aims of composition study
The purpose of this study was to investigate the phospholipid composition of four natural PC based lipids. Two of the lipids are phospholipids from egg yolk and soya bean, both are used as emulsifiers in parenteral products. The other two lipids are chromatographically purified PC from egg yolk and soya bean sources.

2.2 Materials
2.2.1 Phospholipids under investigation
Epikuron 145 (referred to as soya PL in text), B.N. 1-5-9005, Lucas Mayer, Hamburg, Germany.
Epikuron 200 (referred to as soya PC in text), B.N. 1-4-9018, Lucas Mayer, Hamburg, Germany.
Ovothin 180 (referred to as egg PL in text), B.N. 1-4-9240, Lucas Mayer, Hamburg, Germany.
Ovothin 200 (referred to as egg PC in text), B.N. 1-4-9248, Lucas Mayer, Hamburg, Germany.

2.2.2 Materials used in TLC identification of lipids
Acetone, AnalR, B.N. K20840006 416, BDH Chemicals Ltd., Poole, UK.
Ammonia hydroxide (conc.), AnalAR, B.N. 7198610M, BDH Chemicals Ltd., Poole, UK.
α-napthol, research grade, B.N. 31075362, Fisons scientific equipment, UK.
Chloroform, AnalAR, B.N. K22453741 550, BDH Chemicals Ltd., Poole, UK.
Deionised water, pH approximately 5, from Elgastat, UHQ PS, Elguard.
Iodine, GPR, B.N. 215379413, BDH Chemicals Ltd., Poole, UK.
Methanol, AnalAR, B.N. K20930670 424, BDH Chemicals Ltd., Poole, UK.
Ninhydrin spray, B.N. A86271, BDH Chemicals Ltd., Poole, UK.
Phosphomolybdc acid 20% w/v in ethanol, B.N. 9/30211022, Aldrich Chemical Co., USA.
Potassium permanganate, B.N. 1579800, BDH Chemicals Ltd., Poole, UK.
Silica gel 60 F254 Aluminium backed TLC plates, B.N. 540020711, BDH Chemicals Ltd., Poole, UK.
Silver nitrate solution, B.N. X, BDH Chemicals Ltd., Poole, UK.

2.2.3 Materials used in 31P-NMR analysis
Chloroform-d, B.N. 00423MF, Aldrich Chemical Co., USA.
Triethylphosphate, B.N. 123H2623, Sigma Chemical Co., USA.

2.2.4 Materials for zeta potential
Lipofundin® 10% MCT, Braun, Germany.

2.3 Methods
The phospholipid composition of commercially available egg yolk PL, egg yolk PC, soya bean PL and soya bean PC was assessed quantitatively and qualitatively. Two techniques were employed to determine the phospholipid compositions: 31P-NMR and TLC.

2.3.1 31P-NMR methodology
In the context of phospholipid analysis, 31P-NMR is usually employed to establish the structural organisation of phospholipids. Specifically, it can be used to determine whether the phospholipids are arranged in either a micelle or a bilayer organisation. However, in this context, 31P-NMR was employed to quantitatively determine the phospholipids present in the four lipid samples. It provided both qualitative and quantitative information on the lipids containing a phosphorus headgroup (Henderson et
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al., 1974). The main disadvantage with this technique was that no data on non-phosphorus containing components was generated, e.g. glycolipids.

The lipid sample (100 mg) was dissolved in 3 ml of deuterated chloroform: methanol (2:1 v/v) containing approximately 5 mg of an internal standard triethylphosphate. The $^{31}$P-NMR parameters as described by Sotirhos et al. (1986) were employed. The $^{31}$P-NMR was performed at 25 °C on a Brüker 250 MHZ NMR (Karlsruhe, Germany) at 161.7 MHZ. A pulse angle of 45 ° and a ten second pulse were utilised. Two hundred accumulations were obtained before Fourier transformation of the free induction decay. A $^{31}$P-NMR spectrum of a commercially available egg PC sample is provided in Figure 2.2.

The phospholipids are identified by their chemical shifts at 25 °C ($δ$-25 °C) relative to the internal standard triethylphosphate. In the above example the three peaks at 0.66 (Peak III), 1.40 (Peak II) and 1.60 (Peak I) correspond to phosphatidylcholine, lyso-phosphatidylcholine and sphingomyelin respectively. The quantity of each PL is directly proportional to the area under each peak. Therefore, the mol% of each lipid can be calculated by comparing the integrated areas under each peaks. The mol% for each phosphorus containing lipid in this egg PC sample is shown in Table 2.6.

2.3.2 Thin Layer Chromatography (TLC)

TLC was used to qualitatively assess the lipid composition of the four phospholipid samples. A variety of solvent systems can be used to separate the various lipid
components (Fried, 1991; Hammond, 1993). However, for these investigations, two solvent systems were found to be particularly suitable:

1) Non-polar solvent system. (CMH) Chloroform: Methanol: Water (65:25:4 v/v)

Both these solvent systems were made up fresh when required. Each solvent system was poured into a glass tank to a depth of approximately 1.0 cm. Evaporation of the solvent from the tank was reduced by placing a glass lid on top of the tank. To reduce drifting of the lipid spots, filter paper was placed in the tank to uniformly distribute the solvent vapour within the tanks. Commercially available silica gel 60 with binder on aluminium backing (Merck, Germany) was used as the stationary phase. Approximately 0.2 g of lipid was dissolved in 1 ml of chloroform. Approximately 1.2 µl (equivalent to 0.2 mg of lipid) of this solution was carefully spotted from a glass capillary pipette 2 cm above the bottom edge of the plate. After the spot had dried, the plates were put in an equilibrated glass tank and the lid was immediately placed over the tank. Care was taken to position the plate horizontally and to ensure the edges of the plate were not damaged. After the solvent front had progressed 10 cm from the original position of the lipid spot, the plate was removed from the solvent system. The plate was subsequently dried in a vacuum oven at 50 °C for 10 minutes before subjecting the plate to the reagent.

2.3.2.1 TLC separation of PC samples
To separate the different lipids of the PC samples, the one dimensional TLC with the CMH solvent system, described in section 2.3.2, was found to be satisfactory.

2.3.2.2 TLC separation of PL samples
To separate the individual lipids from the more complex phospholipid mixtures two dimensional TLC was employed. This two dimensional technique enabled greater lipid separation than was possible with one dimensional TLC. The separation was carried out by applying approximately 0.4 mg of lipid in chloroform onto the bottom right hand corner of the plate- 2 cm from the bottom edge and 2 cm in from the right hand edge of the plate. The lipid spot was separated on the plate in the non-polar (CMH) solvent. After the solvent front had travelled 10 cm, the plate was removed from the non-polar solvent and dried in a pre-heated vacuum oven at 50 °C for approximately 10 minutes. The dried plate was then turned 90 ° clockwise and placed in the polar solvent (CMAH).
The solvent front was allowed to travel 10 cm up the plate before removal from the polar solvent. The plates were dried in a vacuum oven at 50 °C prior to spraying with appropriate reagents.

2.3.2.3 Spray/vapour reagents used in TLC identification

A variety of reagents (Table 2.1) were used to locate and in some cases identify the individual lipid components (Kates, 1972; Dawson et al., 1986). The phospholipids were also identified based on their Rf values. The Rf values of the lipids were calculated by measuring the distance travelled by each spot and dividing it by the distance of the solvent front. The Rf values were compared with literature values (Gunstone et al., 1994b). The presence of saccharides after separation was detected after dipping the plate in a silver nitrate solution and spraying with an alkaline spray.

<table>
<thead>
<tr>
<th>Name of spray/vapour</th>
<th>Detects</th>
<th>Appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphomolybdic acid (5%) in ethanol</td>
<td>Phospholipids</td>
<td>Blue-black spots on yellow background</td>
</tr>
<tr>
<td>Sulphuric acid-glacial acetic acid (1:1 vol/vol)</td>
<td>Sterols and sterol esters</td>
<td>Red spots on white background</td>
</tr>
<tr>
<td>Potassium permanganate crystals in closed tank</td>
<td>Any lipid</td>
<td>Black spots on a colourless background</td>
</tr>
<tr>
<td>Ninhydrin spray</td>
<td>PE</td>
<td>Violet spots on white background</td>
</tr>
<tr>
<td>Iodine crystals in closed tank</td>
<td>Unsaturated fatty acids</td>
<td>Brown spots on an off-white background</td>
</tr>
<tr>
<td>a napthol 0.5% in methanol-water (1:1)</td>
<td>Sterol glycosides</td>
<td>Violet spots</td>
</tr>
<tr>
<td>Aqueous sulphuric acid (50%)</td>
<td>All carbon containing material</td>
<td>Dark brown spots on a colourless background</td>
</tr>
<tr>
<td>1) Plate dipped in silver nitrate solution. (Produced by diluting 0.1 ml saturated AgNO₃ to 20 ml in acetone / water)</td>
<td>Saccharides</td>
<td>Silver spots</td>
</tr>
<tr>
<td>2) After drying, spray with 0.5 M NaOH in aq. ethanol.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All plates were placed in a fume cupboard prior to spraying with reagents aerosolised with a hand held chlorofluorocarbon containing propellant canister.

2.3.3 Zeta potential measurements

Each lipid sample was dispersed and diluted in deionised water before measuring the zeta potential of the individual lipid sample using a Malvern Zetasizer IV (Malvern Instruments, UK). As a comparison, Lipofundin® 10% MCT, a commercially available medium chain triglyceride stabilised with soya PL, was also measured in deionised water. Each sample was measured three times. The average and standard deviation of each sample were calculated.

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2.3.4 Gas chromatography (GC) to determine fatty acid profile of phospholipids
The fatty acid profiles of each of the four phospholipids were determined by Lucas Meyer, the manufacturer of the PLs. The technique employed to analyse the fatty acids was capillary gas chromatography after hydrolysis and esterification of the fatty acids. The derivatised fatty acids were separated by GC and detected with flame ionisation (Lucas Meyer, 1995).

2.3.5 Purification of egg PC and soya PC
Approximately 20 g of egg PC and soya PC were refined further by Lipid Products (Nutfield, Redhill, UK) using a general elution protocol developed by Rouser et al. (1967). The purpose of this purification was to reduce the level of non-PC components. These purified PCs were used in some particle size experiments, described in section 3.4.4.5.1. The two purified PCs were analysed by TLC separation using the CMH solvent.

2.4 Results and discussion
2.4.1 Comparison of soya PC and soya PL
2.4.1.1 Identification of lipids in soya PC and soya PL by TLC
The lipids identified in the soya PC and soya PL by TLC are shown in Table 2.2.

<table>
<thead>
<tr>
<th>Lipid source</th>
<th>Soya PC</th>
<th>Soya PL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phosphatidylcholine</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td></td>
<td>Lyso-phosphatidylcholine</td>
<td>Lyso-phosphatidylcholine</td>
</tr>
<tr>
<td></td>
<td>Phosphatidylethanolamine</td>
<td>Phosphatidylethanolamine</td>
</tr>
<tr>
<td></td>
<td>Phosphatidylinositol</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td></td>
<td>Phosphatidylglycerol</td>
<td>Phosphatidylglycerol</td>
</tr>
<tr>
<td></td>
<td>Phosphatidic acid</td>
<td>Phosphatidic acid</td>
</tr>
<tr>
<td></td>
<td>Monogalactodiglyceride</td>
<td>Monogalactodiglyceride</td>
</tr>
<tr>
<td></td>
<td>Digalactodiglyceride</td>
<td>Digalactodiglyceride</td>
</tr>
<tr>
<td></td>
<td>Phytosterols</td>
<td>Phytosterols</td>
</tr>
<tr>
<td></td>
<td>Fatty acids</td>
<td>Fatty acids</td>
</tr>
</tbody>
</table>

2.4.1.2 Results of \(^{31}\text{P-NMR}\) analysis of soya PC and soya PL
The qualitative and quantitative analysis of the phospholipids present in soya PC and soya PL are shown in Table 2.3 and Table 2.4 respectively.
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<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>δ-25°C</th>
<th>mol%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidylcholine</td>
<td>0.62</td>
<td>98.8</td>
</tr>
<tr>
<td>Lyso-phosphatidylcholine</td>
<td>1.38</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Table 2.3 Chemical shifts and relative molar quantities of the phospholipids in soya PC as determined by $^{31}$P-NMR

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>δ-25°C</th>
<th>mol%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidylcholine</td>
<td>0.66</td>
<td>74.6</td>
</tr>
<tr>
<td>Lyso-phosphatidylcholine</td>
<td>1.39</td>
<td>4.2</td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>1.70</td>
<td>4.7</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>1.81</td>
<td>16.5</td>
</tr>
</tbody>
</table>

Table 2.4 Chemical shifts and relative molar quantities of the phospholipids in soya PL as determined by $^{31}$P-NMR

2.4.1.3 Comparing the lipid compositions of soya PC and soya PL

Comparing the results of the $^{31}$P-NMR study in Table 2.3 and Table 2.4 revealed a clear distinction between the composition of the soya PC and the composition of soya PL. The soya PC only contained PC and LPC. In contrast, the lipid components in the soya PL sample were somewhat more complex: in addition to PC and LPC, the $^{31}$P-NMR revealed the presence of PE and the negatively charged phospholipid, phosphatidylinositol. Furthermore, the TLC results (Table 2.2) revealed the presence of many non-phosphorus containing lipids. In total the two dimensional TLC resolved 10 lipid components in the soya PL. This demonstrated the benefit of employing two dimensional TLC. With one dimensional separation using the CMH solvent system some lipid components in the soya PL which had similar/overlapping Rf values could not be clearly separated. However, if two dimensional TLC was adopted, spots which were previously overlapping could be separated. Two dimensional TLC revealed the presence of low levels of two other negatively charged phospholipids: PG and PA. These low levels of charged phospholipids were not detected by $^{31}$P-NMR, possibly because the magnetic strength of the Brüker 250 MHZ NMR (Karlsruhe, Germany) may have been insufficient to resolve low levels of charge. The other main group of lipids detected by TLC in the soya phospholipid were the glycolipids. Three types of glycolipid were located using Rf values and detected with α napthol spray. The glycolipids detected were; digalactodiglyceride, monogalactodiglyceride and a sterol glycoside. A small but detectable level of free fatty acids was present in the soya PL. This was despite the fact that the material had been acetone treated during manufacture, which removed the majority of the free fatty acids. The reason for the incomplete removal of fatty acids may be explained by the fact that this commercially extracted...
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soya PL was not subjected to chromatographic purification. This procedure would have encouraged the elution of the free fatty acids in the solvent front.

2.4.2 Comparison of egg PC and egg PL

2.4.2.1 Identification of lipids in egg PC and egg PL by TLC

The lipids identified in egg PC and egg PL by TLC are shown in Table 2.5.

Table 2.5 Lipids present in egg PC and egg PL as identified by TLC

<table>
<thead>
<tr>
<th>Lipid source</th>
<th>Egg PC</th>
<th>Egg PL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidylcholine</td>
<td></td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>Lyso-phosphatidylcholine</td>
<td></td>
<td>Lyso-phosphatidylcholine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phosphatidylethanolamine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sphingomyelin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cholesterol</td>
</tr>
</tbody>
</table>

2.4.2.2 Results of $^{31}$P-NMR analysis of egg PC and egg PL

The qualitative and quantitative analysis of the phospholipids present in egg PC and egg PL are shown in Table 2.6 and Table 2.7 respectively.

Table 2.6 Chemical shifts and relative molar quantities of the phospholipids in egg PC as determined by $^{31}$P-NMR

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>δ-25°C</th>
<th>mol%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidylcholine</td>
<td>0.66</td>
<td>97.3</td>
</tr>
<tr>
<td>Lyso-phosphatidylcholine</td>
<td>1.40</td>
<td>0.9</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>1.60</td>
<td>1.9</td>
</tr>
</tbody>
</table>

Table 2.7 Chemical shifts and relative molar quantities of the phospholipids in egg PL as determined by $^{31}$P-NMR

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>δ-25°C</th>
<th>mol%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidylcholine</td>
<td>0.66</td>
<td>88.1</td>
</tr>
<tr>
<td>Lyso-phosphatidylcholine</td>
<td>1.43</td>
<td>2.2</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>1.62</td>
<td>2.2</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>1.86</td>
<td>6.9</td>
</tr>
<tr>
<td>Unidentified PL</td>
<td>2.25</td>
<td>0.6</td>
</tr>
</tbody>
</table>

2.4.2.3 Comparing the lipid compositions of egg PC and egg PL

During the manufacture of commercial egg PC and egg PL, both lipids are eluted on aluminium oxide columns to raise the PC content. Therefore neither the egg PC nor egg PL contained detectable levels of charged phospholipids.

$^{31}$P-NMR detected the presence of a low level of sphingomyelin in the commercially available egg PC sample (Table 2.6). Additionally, as with the purified soya PC, a low level of LPC was also present. The reason for the presence of these two components may be attributed to their similar elution patterns to PC on the aluminium oxide column.
in ethanolic solution (Schneider, personal communication). This illustrates the importance of having a starting material with low LPC content. Poor quality starting materials with a high LPC content may have high LPC even after column chromatography.

Both $^{31}$P-NMR and TLC revealed the presence of more components in egg PL than in egg PC. The LPC content in the egg PL was approximately 2 mol%, which was higher than for the egg PC. Additionally, egg PL contained about 7 mol% and 2 mol% of PE and sphingomyelin respectively. This lipid composition was similar to the compositions of the four commercial PLs examined by Sotirhos et al. (1986). This was unsurprising because these commercial samples originated from a similar source (egg yolk). The TLC fingerprint of the egg PL was considerably simpler than the soya PL. No glycolipids nor free fatty acids were detected under the conditions tested. Moreover, no acidic phospholipids were detected.

2.4.3 Fatty acid profile of the four lipids

The fatty acid profiles of the four commercially available phospholipids is shown in Table 2.8.

<table>
<thead>
<tr>
<th>Lipid type</th>
<th>Soya PL</th>
<th>Soya PC</th>
<th>Egg PL</th>
<th>Egg PC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitic (16:0)</td>
<td>14</td>
<td>11</td>
<td>32</td>
<td>38</td>
</tr>
<tr>
<td>Stearic (18:0)</td>
<td>4</td>
<td>3</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>Oleic (18:1 ω: 9)</td>
<td>10</td>
<td>5</td>
<td>25</td>
<td>23</td>
</tr>
<tr>
<td>Linoleic (18:2 ω: 6, 9)</td>
<td>63</td>
<td>69</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Linolenic (18:3 ω: 6, 9, 12)</td>
<td>7</td>
<td>9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Arachidonic (20:4 ω: 6, 9, 12, 15)</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Docosahexaenoic (20:6 ω: 6, 9, 12, 15, 18)</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Minor fatty acids</td>
<td>2</td>
<td>3</td>
<td>7</td>
<td>5</td>
</tr>
</tbody>
</table>

The GC of the hydrolysed lipid samples revealed considerable differences between the fatty acids profiles of the egg sources and soya sources. The details of the fatty acids profiles of the four phospholipids will be discussed in chapter four, in the context of phospholipid stability.

The fatty acid profiles of the lipids differed somewhat compared to the literature values (New, 1990c). The soya PL and egg PL contained non-PC phospholipids, such as PE and sphingomyelin which have slightly different fatty profiles from PC. Therefore, the
profiles can not be directly compared to the PC literature values. However, the soya PC
and egg PC mostly contained PC, therefore direct comparisons of the fatty acid profiles
were valid. The relative abundance of fatty acids in natural sources of soya PC and egg
PC are shown in Table 2.9.

<table>
<thead>
<tr>
<th>Trivial name of fatty acid</th>
<th>Lipid type content mol%</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Number of carbons: no. of double bonds and α positioning of double bond(s))</td>
<td>Soya PC</td>
</tr>
<tr>
<td>Palmitic (16:0)</td>
<td>17.2</td>
</tr>
<tr>
<td>Stearic (18:0)</td>
<td>3.8</td>
</tr>
<tr>
<td>Oleic (18:1 ω: 9)</td>
<td>22.6</td>
</tr>
<tr>
<td>Linoleic (18:2 ω: 6, 9)</td>
<td>47.8</td>
</tr>
<tr>
<td>Linolenic (18:3 ω: 6, 9, 12)</td>
<td>8.6</td>
</tr>
<tr>
<td>Arachidonic (20:4 ω: 6, 9, 12, 15)</td>
<td>0</td>
</tr>
<tr>
<td>Docosahexaenoic (20:6 ω: 6, 9, 12, 15, 18)</td>
<td>0</td>
</tr>
<tr>
<td>Minor fatty acids</td>
<td>-</td>
</tr>
</tbody>
</table>

The main differences between the fatty acid profile of the commercially available soya
PC and the literature value are in the levels of oleic acid and linolenic acid. The level
of linoleic acid in soya PC (63 mol%) seemed considerably higher than the literature
value (47.8 mol%). Conversely, the level of oleic acid in soya PC was lower (5 mol%)
than the literature value (22.6 mol%).

The commercial egg PC sample had a markedly lower level of arachidonic acid
(4 mol%) compared to the literature values (12.6 mol%). Conversely the level of
linoleic acid was higher in egg PC (15 mol%) compared to the literature value of
5.7 mol%.

Possible reasons for these differences may be due to the natural variation of the lipids.
Factors such as dietary intake of the hens may greatly influence the fatty acid
composition of egg phospholipids (Schneider, personal communication). Similarly,
environmental factors such as temperature during the development of soya beans may
affect the fatty acid profile of extracted soya phospholipids.
Chapter Two- Composition of unsaturated phospholipids

2.4.4 Zeta potential measurements

The zeta potential of the four commercially available phospholipids and a medium chain triglyceride (MCT) parenteral emulsion are shown in Table 2.10.

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soya PC</td>
<td>-22.8±0.4</td>
</tr>
<tr>
<td>Soya PL</td>
<td>-36.2±2.1</td>
</tr>
<tr>
<td>Egg PC</td>
<td>-20.1±0.3</td>
</tr>
<tr>
<td>Egg PL</td>
<td>-21.7±0.4</td>
</tr>
<tr>
<td>Lipofundin® MCT</td>
<td>-37.9±2.09</td>
</tr>
</tbody>
</table>

As can be seen in Table 2.9, the zeta potential measurements seemed to broadly reflect the compositions of the phospholipid samples. Both the purified PCs and the egg phospholipid which had no detectable levels of charge had readings of between -20 mV and -24 mV. However, the soya PL had a considerably lower zeta potential of -35 mV, perhaps reflecting the presence of small levels of negative charge in this lipid composition. The parenteral emulsion Lipofundin® had a similar zeta potential reading to the soya PL, which was unsurprising because the soya PL used to emulsify this emulsion was similar in composition to the soya PL examined.

The zeta potential value of Lipofundin® (-37.9 mV) was somewhat lower than the zeta potential value of another parenteral emulsion: Intralipid®, which was reported in the literature to have a value of -31.5 mV (Rubino, 1990). The difference may have been due to differences in charged phospholipid composition or perhaps the presence of hydrolytic breakdown products, such as free fatty acids (Washington and Davis, 1987).

The zeta potentials of commercially available egg PC liposomes (-20.1 mV) and soya PC liposomes (-22.8 mV) were negative. The negative charge on these “neutral” liposomes may have been attributed to the preparation and dilution of the liposomes in deionised water, pH 5 (Mosharraf et al., 1995).

2.4.5 Detection of saccharides in lipid samples using TLC

The presence of saccharides was detected using a silver nitrate dip and sodium hydroxide (0.5 M) ethanol spray. The dark silver spots formed were most prominent in the two phospholipids samples. However, a feint degree of silver colouration was noticed with the egg PC and soya PC after spraying with silver nitrate, indicating that the saccharide level in these two samples was probably lower.
2.4.6 TLC of purified egg PC and soya PC

The lipid components of the purified soya PC and egg PC are shown in Table 2.11.

<table>
<thead>
<tr>
<th>Lipid source</th>
<th>Purified soya PC</th>
<th>Purified egg PC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidylcholine</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.11 Lipid present in purified soya PC and purified egg PC.

Both purified solid phospholipids were off-white in colour. The TLC of the two purified lipids revealed the presence of only one PC spot in each of the samples. The additional elution on aluminium oxide reduced the lyso-phosphatidylcholine content of both purified PCs and further reduced the sphingomyelin level in the purified egg PC.

2.5 Conclusions

The soya PC and egg PC contained approximately 95 mol% phosphatidylcholine based on overall phospholipid content. The minor phospholipid component common to both of these lipids was LPC, which was present at levels between 1-2 mol%. In addition to PC and LPC, the egg PC also contained 2 mol% of sphingomyelin. As expected, the fatty acid profiles revealed the egg PL to be considerably less saturated than the soya phospholipids.

The egg PL and soya PL compositions were qualitatively similar in terms of neutral phospholipid contents, both lipids contained PC and PE. However, the egg PL contained less PE than the soya PL. The lipid profile of soya PL was markedly more complex than egg PL. PI and low levels of PG and PA were detected in soya PL by TLC, which were not detected in egg PL. Furthermore, TLC detected the presence of a variety of non-phosphorus containing lipids in soya PL, including a two types of glycolipid, sterol glycosides and fatty acids, which were all absent in egg PL.
Chapter three

Particle size of liposome dispersions
Chapter three-Particle size of liposome dispersions

3.1 Introduction

3.1.1 Rationale for generating small liposomes

In general, there are three primary reasons for producing small liposomes for intravenous administration. Firstly, a liposome diameter of 200 nm or less enables liposome dispersions to be sterilised via aseptic filtration. Secondly, small liposomes with diameters of 100 nm or less have reduced sequestration by the MPS. The third reason for reducing particle size is perhaps of greater relevance to this study, and concerns the potential harm large liposomes may cause when administered systemically.

3.1.2 Potential dangers of particles in intravenous products

A particle, in the context of an intravenous injection, is defined as a discreet insoluble substance which is present in a fluid. In the context of parenteral products, particles usually relate to unwanted non-viable contaminants such as the sheddings from containers, fragments of rubber tops and fibres from filters (Dempsey and Webber, 1983).

Restrictions on the level of particles exist for large volume injectables. The size and number of particles greater than 2 µm and 5 µm in such preparations have to be limited to 1000 per ml and 100 per ml respectively for injections having a volume greater than 100 ml (British Pharmacopoeia, 1993d). The rationale behind this regulation is to prevent large particles from being injected into the patients circulation. Solid particles larger than 7 µm are believed to be physically trapped within the capillary network of the lung (Illum et al., 1982). However, the potential of non-viable particles to occlude and induce emboli should also perhaps be viewed in the context of there being no recorded fatalities directly associated with the injection of particulate matter (Hargreaves, 1995).

These particulate limits only apply to intravenous solutions with volumes ≥ 100 ml, and are not applicable to the large colloidal particles in dispersions. In fact no pharmacopoeial particle size standards or guidelines currently exist for colloids administered intravenously. This is probably because the potential harm caused by large colloidal particles is highly dependant upon the specific nature of the colloid concerned. A variety of factors other than size could influence the blockage of capillaries, these include the number of particles, the physical and biological properties of the colloid and the location of the colloid within the vascular system.
Although MPS accumulation with large radioactive colloids is deliberately used to image certain organs, for example the liver (Saha et al., 1986), there have been few reports in the literature relating to emboli induced by therapeutic agents entrapped/associated with colloids. Most work has centred around emboli caused by particles. In rats it has been demonstrated that although injection of $4 \times 10^5 \text{ kg}^{-1}$ of 40 $\mu$m particles were well tolerated in rats, rapid death by embolism often resulted if $8 \times 10^6 \text{ kg}^{-1}$ of 40 $\mu$m particles were injected intravenously (Gesler et al., 1973). However, in these studies latex particles were employed, which are insoluble, non-biodegradable and solid. Clearly these results cannot be extrapolated to relatively fluid liposomes, which are hollow, flexible, rapidly bioerodable and biodegradable. Similar experiments in beagle dogs demonstrated that administration of polystyrene divinyl benzene microspheres with diameters ranging from 3.4 $\mu$m to 11.6 $\mu$m appeared to be tolerated (Slack et al., 1981). Another study, this time employing 25 $\mu$m diameter divinyl benzene microspheres found that after administration of doses $2.4 \times 10^9$ of microspheres, no histological tissue damage was found in representative tissue samples. However, this study was only carried out after single doses, and multiple or chronic administration was not investigated (Schroeder et al., 1978a; 1978b).

There have been even fewer published reports examining the potential of large liposomes to induce emboli. One pertinent study involved deliberate localisation of large cholesterol containing liposomes in the lung capillaries for therapeutic purposes (Abra et al., 1984). No gross pathological lesions were observed 24 hours after administration of various liposome dispersions, which had diameters ranging from 1 $\mu$m up to 8 $\mu$m. However, once again these studies were not carried out after multiple or chronic administration. Finally, studies investigating chronic TPN therapy with pulmonary events in infants indicated that there may be potential risks upon chronic administration of large emulsion droplets. In these studies it was suggested that daily administration of only 37,000 particles with diameters between 2 $\mu$m and 100 $\mu$m may have led to serious complications in some infants. However, no direct evidence could be found to correlate the larger sized oil droplets in the emulsion with the pulmonary complications experienced by the infants (Puntis et al., 1992).
3.1.3 Ideal size characteristics for liposomes as solubilisers
In light of the uncertain risks of large particles, conflicting evidence and the absence of pharmacopoeial standards, the prerogative of this study was to generate fine liposome dispersions just prior to their use. Ideally, the size distribution of these dispersions had to be consistent and reproducible.

3.1.4 Total Parenteral Nutrition (TPN) as a standard for the size distribution
TPN has been administered in patients as a high calorie energy substrate for over 30 years (Davis et al., 1983). It is usually prescribed for patients who are either unable to use their gastrointestinal tract or whose oral nutritional intake is insufficient. This type of emulsion has been shown to have a good safety profile even when large volumes are administered chronically. Case reports specifically relating to fat emboli induced by TPN in adults have been exceedingly rare (McCracken, 1991). This suggests that the particle size distribution of this parenteral emulsion is sufficiently fine to avoid complications associated with emboli. Therefore, the particle size data for TPN may perhaps provide size parameters and standards with which liposome dispersions can be compared. Although the *in vivo* behaviour of unsaturated liposomes and parenteral emulsions differ somewhat (Allen and Murray, 1985), these flexible colloids both rapidly interact with lipoproteins in the blood to form hybrid lipid structures (Scherpolf et al., 1978; Westesen and Wehler, 1992). This means the "fluid" liposome structures are readily disassembled *in vivo*, and therefore the presence of a few large liposome particles may not even be problematic because these particles may be short lived.

The actual numbers of large colloidal droplets present in TPN fluids is dependant upon a variety of factors including the manufacturing process, presence of admixtures and emulsifier type (Bock et al., 1994). Hence, companies producing parenteral emulsions set their own in-house standards for the particle size distribution of TPN. Information concerning absolute numbers of larger particles is therefore scarce. In commercially manufactured parenteral emulsions, there are very few large droplets in the emulsion concentrate (Müller and Heineman, 1992; 1993). The number of oil droplets greater than 5 μm in emulsion concentrates is almost undetectable. However, these concentrates are frequently added to solutions containing inorganic electrolytes, amino acids, sugars and/or vitamins. Detecting the number of oil droplets in these emulsion admixtures is probably clinically more relevant than in emulsion concentrates, because most
emulsions are combined with intravenous additives prior to administration. Generally
the proportion of large oil droplets in these emulsion admixtures is considerably higher
than in the concentrates. In some clinically used admixtures 20 million particles per ml
greater than 2 μm have been detected (Mehta et al., 1992). However, the precise number
of large oil droplets will be dictated by the type of admixture regimen (Sayeed at al.,
1987; Müller and Heinemann, 1994a; 1994b).
In this study, the particle size characteristics of oil droplets in 10% Intralipid®, a
commercially available parenteral emulsion, were used as a comparison for the particles
in the liposome dispersions.

3.1.5 Sizing techniques
Frequently dispersions are polydisperse, which means that the particles in the dispersion
are distributed over a broad size range (Barrow and Lentz, 1980). This fact must be
appreciated when sizing dispersions, because sizing techniques may be incapable of
meaningfully characterising the entire size distribution of a highly polydisperse
dispersion. The individual techniques are generally best suited for sizing particles within
a particular size range. Therefore, to overcome the inadequacy of an individual
technique and to obtain a more accurate impression of the size distribution,
complementary sizing techniques may be employed.
There are various methods used to size liposomes. The technique(s) selected will vary
according to the size distribution of the dispersion under examination. Various forms of
microscopy can be employed to observe the dispersions, although electron microscopy
is probably the preferred method of microscopy for sub-micron sized liposomes. Laser
techniques based upon diffraction patterns (Pecora, 1985; Van der Meeren et al., 1992),
photon correlation spectroscopy (Ostrowsky, 1993), differential fractionating
centrifugation methods (Coll and Searles, 1987) and turbidity experiments (Cancellieri
et al., 1974; Barenholz and Amselem, 1992) can also be used to assess the size of
liposome dispersions. The most common method employed to provide an absolute count
of particles in colloidal dispersions is the electric zone method (Chowan et al., 1972).
The commercially available apparatus is commonly known as the Coulter Counter.
The sizing procedures which have been employed for this study are described in the
methodology (section 3.3.8). For the theoretical background of these particle sizing
techniques, the reader is referred to the general books on particle size and the references listed therein (Allen, 1990; Washington, 1992).

3.1.6 Aims of the particle sizing study
The general aims of this study were two fold: the first purpose was to assess the particle size distribution of liposome dispersions with a high lipid concentration (60 mg/g) generated from pro-liposomes. The second aim was to investigate possible means of reducing the number of larger liposomes in these dispersions.

3.2 Materials

3.2.1 Materials for pro-liposomes
Absolute ethanol, AnalAr, B.N. various, BDH Chemicals Ltd., Poole, UK.
Egg phosphatidylglycerol (EPG), B.N. 10612-1/06, Lipoid, Ludwigshafen.
Epikuron 145 (referred to as soya PL in text), B.N. 1-5-9005, Lucas Mayer, Hamburg, Germany.
Epikuron 200 (referred to as soya PC in text), B.N. 1-4-9018, Lucas Mayer, Hamburg, Germany.
Glycerol, AnalAr, B.N. K213348 160 450, BDH Chemicals Ltd., Poole, UK.
Ovothin 180 (referred to as egg PL in text), B.N. 1-4-9240, Lucas Mayer, Hamburg, Germany.
Ovothin 200 (referred to as egg PC in text), B.N. 1-4-9248, Lucas Mayer, Hamburg, Germany.

3.2.2 Materials for sizing
Anhydrous glucose, GPR, B.N. 6275500 J, BDH Chemicals Ltd., Poole, UK.
Aseptically 0.2 μm filtered sodium chloride (0.45% w/v) and anhydrous glucose (2.5% w/v) solution. (Manufactured at School of Pharmacy, London)
Deionised water, pH approximately 5 from Elgastat, UHQ PS, Elguard.
Eppendorf combitips (5 ml) (Referred to as Eppendorf syringes in text)
PH meter, WPA CD 300 digital meter.
Sodium chloride, AnalAr, B.N. K22617733 604, BDH Chemicals Ltd., Poole, UK.
Standard solutions for pH meter: pH 4 and pH 7, BDH Chemicals Ltd., Poole, UK.
Tween 80, B.P. grade, B.N. 758, ICI, Macclesfield, UK.
3.3 Methods

3.3.1 Cleaning glassware and apparatus
All glassware, caps and syringes were carefully washed with filtered deionised water (using 0.22 µm Acrodiscs, Gelman sciences, Michigan, USA), prior to their contact with the pro-liposomes or liposome dispersions. Additionally, prior to the addition to pro-liposomes, the deionised water was filtered through 0.22 µm sterile filters (Acrodiscs). These two procedures helped reduce the level of extraneous particulate contamination introduced into the dispersion, and therefore lowered the likelihood of extraneous material interfering with the size measurements.

3.3.2 Pro-liposomes
3.3.2.1 Drug-free pro-liposomes
The objective of this study was to evaluate the size distribution of the carrier *per se*. Therefore, all size measurements were carried out with drug-free formulations, i.e. liposome dispersions converted from pro-liposomes without drug. Omitting a model drug circumvented potential drug-membrane interactions or drug precipitation, which could have adversely influenced the particle size distribution of the dispersions. The particle size of the carrier when associated with a model hydrophobic drug is investigated in chapter five.

3.3.2.2 Selecting pro-liposome components and manufacturing pro-liposomes
The pro-liposome method employed was developed from the method described by Perret (1991). All of the components used to formulate the pro-liposomes are currently employed in various intravenous formulations: the lipids are employed in parenteral emulsions, ethanol is accepted for intravenous administration as a cosolvent for hydrophobic drugs and glycerol is used as a tonicity adjuster for parenteral emulsions.

A clear ethanolic pro-liposome was produced by dissolving 3.0 g of the appropriate lipid(s) in 1.2 g of absolute ethanol in a cleaned 25 ml clear glass bottle. If the lipid was either egg PL or soya PL the solution was orange, in contrast, if the lipid was either egg PC or soya PC a yellow solution was produced. To these solutions, 1.3 g of glycerol was generally added. Rubber tops were used to seal the glass bottles and the mixes were shaken thoroughly for one minute and left to equilibrate at room temperature for
24 hours. The resultant fluid pro-liposomes were suitable for aseptic filtration through a 0.2 μm filter at room temperature, if required.

The lipids used in the studies varied, comprising one or more of the following: soya phosphatidylcholine (PC), soya phospholipids (PL), egg PC, egg PL, refined soya PC, refined egg PC or egg phosphatidylglycerol (EPG). The compositions of the main lipids were identified in the studies described in chapter two. The type of lipid used was dependent upon the particular experiment.

In this present study the abbreviation in the tables for a glycerol pro-liposome formulation is PRO.

3.3.2.3 Liposome isotonicity and lipid concentration

All pro-liposomes were converted into dispersions by the addition of deionised water either in one stage or two stages. The resultant dispersions had lipid concentrations of 60 mg/g and were isotonic to blood with respect to glycerol (Siegel, 1990). The contribution to tonicity of the dispersion from the phospholipids was ignored, because of their high MW (768 for egg PC: Mason and Huang, 1978; 786 for soya PC: Epikuron 200 data sheet, Lucas Mayer, Germany). The relatively high level of lipid (60 mg/g) was selected to enable the drug concentration in the final dispersion to be high. Thereby facilitating the handling and administration by reducing the volume of the final dispersion.

3.3.3 Number of experimental replicates

Each experiment was replicated a minimum of three times. In cases where the number of dispersions (n) was >3, the number of replicates are shown in the results tables.

3.3.4 Sample storage after conversion

All dispersions were refrigerated at 4 °C ± 2 °C until sizing measurements were taken. Measurements were made within 24 hours to avoid microbial spoilage, which may have biased the size distribution.

3.3.5 Statistical analysis

Two types of statistical analyses were employed on the data, which enabled liposome dispersions to be compared. If the dispersions were of the same lipid composition but converted in a different manner, a two tailed Student’s t-test was employed. However, if
the dispersions were of a dissimilar composition, a single factor analysis of variance (ANOVA) was used. The level of significance for both tests was 95%.

### 3.3.6 Protocol for particle sizing
In order to rationalise the sizing of liposome dispersions, the protocol as outlined in Figure 3.1 was used.

1. **Visual inspection**
2. **Light microscopy**
   - **Fine dispersion** (Few, if any particles visible under the light microscope)
   - **Coarse dispersion** (Many particles visible under the light microscope)
3. **Photon correlation spectroscopy (PCS)**
4. **Laser diffraction**
5. **Coulter Counter**
6. **Freeze fracture electron microscopy** (EM)
7. **Turbidity measurements**

Figure 3.1 Particle sizing protocol

*Indicates the sizing technique was optional.

See section 3.3.8 for explanation of all procedures
3.3.7 Diluting dispersions for particle size measurements

Most measurements made with photon correlation spectroscopy (PCS), laser diffraction or Coulter Counter were carried out by diluting the liposome dispersions in isotonic media. Isotonic media were selected for two reasons: firstly, it would reflect the dilution of the liposome dispersions in intravenous infusion fluids. Secondly, it did not create an osmotic gradient which may have damaged the osmotically sensitive liposomes. Generally, the most appropriate diluting medium was 50 mg/g glucose. If isotonic 0.9% w/v sodium chloride was used as the diluent, the reproducibility of the particle size measurements was poor. This was well illustrated by photon correlation spectroscopy (PCS). For a particular liposome dispersion measured using 50 mg/g glucose as the isotonic medium, the z average value was around 600 ± about 30 nm. However, if the same sample was measured in 0.9% w/v sodium chloride (NaCl) the z average results were over two fold higher: 1600 ± about 550 nm. The resultant diluted samples appeared visibly flocculated and cloudier to the naked eye compared to the samples diluted in 50 mg/g glucose. This aggregation could also be followed qualitatively using turbidity measurements of the liposome dispersions diluted in isotonic saline. The reason for this aggregation behaviour was probably attributable to the incompatibility of the high level of sodium cations with PL, which induce reversible liposome aggregation (Crommelin, 1984; Nagata et al., 1986).

This type of aggregation was particularly problematic when the Coulter Counter was employed, because a conducting ionic medium was required for the sample measurement. Typically, the Coulter Counter employs 0.9% w/v NaCl as the isotonic conducting medium. However, at this concentration dramatic liposome aggregation was caused. Although the level of NaCl was lowered to 0.45% w/v, this level of sodium ions still induced liposome aggregation. This aggregation prevented reproducible Coulter Counter readings from being obtained, particularly when a large volume of diluted dispersion was added to the media. To greatly reduce this aggregation and improve reproducibility, small quantities of the surfactant Tween 80 were added to all the dispersions. This was achieved by diluting the liposome dispersion ten fold in 50 mg/g glucose containing 0.06 mg/g Tween 80, prior to the addition to the conducting medium. Turbidity experiments indicated that the level of Tween 80 required to stabilise the samples was very low. Typically, only 1 part by weight of this surfactant to every
100 parts by weight of lipid was needed to keep the preparations from aggregating in the 0.45% w/v sodium chloride/2.5% w/v glucose conducting media. At this low level of surfactant, turbidity measurements remained constant. This stable level of turbidity indicated that neither liposome aggregation/fusion nor liposome solubilisation by Tween 80 was occurring.

### 3.3.8 Particle sizing techniques

#### 3.3.8.1 Visual inspection

The macroscopic properties of the dispersion provided a useful indication of the overall size characteristics of the dispersion. Very coarse dispersions containing a high proportion of large sized liposomes appeared opaque and started to sediment after only a few hours. In contrast, if the dispersion contained few large liposomes, the appearance was somewhat opalescent, like semi-skimmed milk, and no sedimentation was evident after 24 hours of storage.

#### 3.3.8.2 Light microscopy

Light microscopy provided a visual impression of the larger structures present in the dispersions. An Olympus BX50 optical microscope using x400 fold magnification with attached Sony CD TRI video camera was employed to rapidly assess the size characteristics of the dispersions. Approximately 2 μl of the test sample was examined under a glass slide with coverslip. Most of the sample was inspected and representative photomicrographs of the field of vision were taken with the Sony video graphic printer. Dispersions were usually examined in concentrated form, although in some cases the dispersion was diluted with 50 mg/g glucose. At a magnification of x400, the lower limit for determining the liposome diameter was about 5 μm. Hence, only larger liposomes above this diameter could be sized. Another disadvantage of this technique was the small sample size (few microlitres) which could be examined. It was therefore impractical to assess the presence of larger sized liposomes in fine dispersions with only a few large liposomes using this technique.

Dispersions were classified into five qualitative categories based on the frequency of visible liposomes counted in a representative photomicrograph (field size at x400 magnification approximately 150 μm x 120 μm). The emphasis centred around detecting the number of liposomes with diameters greater than 5 μm. The five categories are explained below:
Chapter three—Particle size of liposome dispersions

1) Very coarse: More than 100 liposomes had diameters ≥ 5 μm.
2) Coarse: Between 50 and 100 liposomes had diameters ≥ 5 μm.
3) Moderately coarse: Between 10 and 50 liposomes had diameters ≥ 5 μm.
4) Fine: Between 1-10 liposomes had diameters ≥ 5 μm.
5) Very fine: No liposomes were visible.

3.3.8.3 Photon correlation spectroscopy (PCS)

The Malvern Autosizer 2c (Malvern Instruments, UK) was employed to assess the average size and dispersity of the smaller sized liposomes. This technique is limited to detecting particles with a submicron diameter. All samples were diluted using 50 mg/g glucose as discussed in section 3.3.7. Each sample was prepared by adding a few microlitres to a clear sided 5 ml polystyrene cuvette (Kartell) and diluting with 5 ml of filtered 50 mg/g glucose. The cuvette was covered with parafilm and gently inverted to mix the liposome dispersion into the 50 mg/g glucose, without introducing bubbles. The measurements were carried out at 20 °C with the corresponding viscosity of 1.145 cP and refractive index of 1.340 for 50 mg/g glucose (Weast et al., 1988). The z average and polydispersity index (P.I.) were the only two parameters recorded. These measurements were made over a time period of 120 seconds, with an estimated size of 0. All PCS measurements were performed at least four times for each sample. The z average values and P.I. readings were averaged for each of the three repeat samples, and the mean and standard deviation of these averages were calculated and are stated in the appropriate table of results.

If the dispersion seemed moderately coarse, fine or very fine under the light microscope, reproducible results could usually be obtained from PCS. However, if the dispersion seemed coarse or very coarse under the light microscope, the PCS results were less reliable and the reproducibility was poorer, possibly due to the PCS laser being diffracted by the larger liposomes. It is also possible that during the 15 minutes required for repeated measurements on the same coarse sample, the larger liposomes may have started to sediment. Hence, in most instances when light microscopy indicated that the dispersion was coarse or very coarse, PCS measurements were not made.
3.3.8.4 Laser diffraction
Malvern 2600c laser diffraction (Malvern Instruments, UK) was employed to measure the volume median diameter of the larger sized liposomes in the dispersion. Since this particular technique is unsuitable for measuring the smaller liposomes, only the D-90 value was selected. The D-90 is an equivalent diameter which represents 90% of the undersize volume occupied by the particles. Since particles with a diameter of about 500 nm or less were not detected by this particular instrument, the true D-90 values reported here were probably considerably underestimated.

Before laser diffraction measurements were taken, the background contamination present in approximately 20 ml of 50 mg/g glucose in the laser diffraction cell had to be accounted for. Having measured the background, a sufficient amount of sample from a 100 μl Gilson was dispensed into the laser diffraction cell. The contents of this cell were stirred at a fixed moderate speed using the rotating magnetic stirrer. When a constant/stable obscuration of around 0.2 was obtained, readings were taken. The volumes of sample added to the laser diffraction cell to obtain the required obscuration were averaged and are individually recorded in the appropriate table of results in section 3.5.

The volume added to the laser diffraction cell to obtain the required obscuration value is a function of the number of particles and the size of these particles in a dispersion. Therefore, by comparing the volumes required to obtain a given obscuration, the relative number of large particles in different dispersions can be compared. For fine dispersions, a greater amount of dispersion had to be added to reach an obscuration of 0.2. In contrast, to obtain the same obscuration value, coarse dispersions required the addition of a smaller amount of dispersion.

Size measurements were performed at least five times for each sample and average D-90 values for each sample were calculated. The mean and the standard deviation of the average D-90 values for the three repeat dispersions were calculated and are stated in the appropriate table of results.

3.3.8.5 Coulter Counter
The Coulter Counter model TAI1 (Coulter, UK) with a 100 μm capillary was employed to count the number of large particles per ml in some dispersions. This particular capillary was suitable for detecting particles with diameters between 1 μm and 50 μm.
Chapter three-Particle size of liposome dispersions

However, only the number of oversized particles with diameters greater than 5 μm, 10 μm and 20 μm were counted. All glassware and apparatus involved in the dilution steps for Coulter Counter measurements were rinsed twice with freshly filtered (0.22 μm, Acrodiscs) deionised water. Although this washing procedure would not have eliminated all foreign particulate matter, it helped minimise counts emanating from extraneous contamination. The dispersions were diluted ten fold in filtered 50 mg/g glucose containing 0.06 mg/g Tween 80 prior to adding to the Coulter Counter conducting media.

After five background readings of the filtered NaCl (0.45% w/v)/glucose (2.5% w/v) solution were made, the appropriate volume of the 10 fold diluted sample was accurately added to 100 ml of this conducting media. The amount of sample added to the media depended upon the concentration of large particles in the dispersions: dispersions with a lower concentration of large particles required the addition of a greater volume of diluted sample. For optimal reproducibility the total number of counts were kept to below 10,000 per ml and the coincidence level was maintained below approximately 5%. The stirrer speed was fixed on a low to medium speed, which did not interfere with the measurements by generating turbulence. Ninety seconds after the addition of the diluted sample, ten measurements, each sampling two ml of the conducting medium containing the sample, were consecutively taken. After subtracting the background measurements and accounting for the dilution factors, the results of these ten measurements were averaged. Having calculated the average concentration of particles in the three repeat samples, these three averages were used to calculate the average and standard deviation of the concentration of particles in the dispersions. These averages and standard deviations are reported in the appropriate table of results.

3.3.8.6 Electron microscopy (EM)

Freeze fracture was employed to provide an impression of the size distribution of some dispersions. The technique described by Perret et al. (1993b) was employed. Briefly, 10-20 µl samples of dispersion were loaded onto small copper holdings and rapidly frozen in a liquid nitrogen jet freezer. The samples were freeze fractured and evaporation coated with carbon and platinum using Polaron E7500 freeze fracture devices (VG Microtech, Uckfield, East Sussex). The replicas were examined in a
Chapter three-Particle size of liposome dispersions

Jeol 100 CX II electron microscope. Representative photos were taken of the magnified field.

Due to the relatively small sample size being examined (about 10 µl) and the difficulty in producing replicas, this technique was unsuitable for routinely detecting large particles in fine dispersions. However, it was particularly useful when fine dispersions could not be observed using light microscopy.

3.3.8.7 Turbidity measurements

In this study, light absorbance was qualitatively employed to assess the turbidity changes which occurred immediately after dilution of various dispersions in various diluting media. Measurements were made by diluting a suitable amount of the dispersion to a volume of 10 ml using filtered media. A suitable volume of sample was an amount of sample which gave an absorption at 550 nm close to 0.5. The media employed was usually either 50 mg/g glucose or deionised water, unless otherwise stated. Typically, for fine dispersions about 100 µl of liposome concentrate was required. However, for coarse dispersions between 25-50 µl was required. The absorbance of the diluted sample was measured at 550 nm on the Perkin Elmer 554 UV vis. spectrophotometer against the corresponding diluting media as the blank. Absorbance measurements were taken at two to three minute intervals for the first five minutes, and at five minute intervals thereafter. Readings were continued for up to 30 minutes depending upon the behaviour of the sample upon dilution.

3.4 Studies performed

3.4.1 Measuring the size distribution of TPN

A commercially available TPN emulsion concentrate: Intralipid®, containing soya oil (10%) emulsified with egg phospholipids (1.2%), was used as a standard for sizing. The size of the oil droplets was determined by light microscopy, PCS, laser diffraction and Coulter Counter.

3.4.2 One stage conversion

The generation of a liposome dispersion by directly combining a pro-liposome with deionised water in one step is referred to in the text as one stage conversion. Unless otherwise stated, all conversions were carried out at room temperature (20 °C-25 °C) with filtered deionised water.
Various factors which may have potentially influenced the particle size of the dispersions converted in one stage were investigated individually. The conditions in these experiments were standardised by introducing 1.10 g of pro-liposome into 8.90 g of deionised water at room temperature (20 °C-25 °C) and handshaking vigorously for 60 seconds, unless otherwise stated. Other factors which could have altered the particle size such as the amount of the liposome dispersion (10 g) and the size of the glass container (25 ml) were fixed.

The factors potentially affecting the size of the dispersions generated in one stage have been classified into three separate categories in this method:

1) Agitation during one stage conversion.
2) Manipulation of the aqueous phase.
3) Manipulation of the pro-liposome.

3.4.2.1 Agitation during one stage conversion

The influence of energy input during one stage conversion was investigated by subjecting the pro-liposomes with bulk deionised water at room temperature to three different degrees of energy input. Two pro-liposomes: egg PL pro-liposome and the soya PL pro-liposome were converted into liposome dispersions by:

1) Vigorous handshaking, or
2) Moderate handshaking, or
3) No agitation.

In this context, a handshake is defined as the agitation of the container and its contents through a 90 ° angle and back again. Vigorous handshaking involved shaking the bottle violently for one minute at approximately 100 shakes per minute. In contrast, moderate handshaking involved gently inverting the bottle for one minute at a slower speed of 30 inversions per minute. No agitation involved leaving the pro-liposome to fully disperse in the bulk deionised water at room temperature without handshaking. The time taken for this pro-liposome to convert into a liposome dispersion without shaking was recorded.

Laser diffraction was used to measure D-90 values of both the egg PL and soya PL dispersions converted in one stage with varying energy inputs. However, the absolute number of particles was only counted by Coulter Counter for the egg PL dispersions.
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3.4.2.2 Manipulation of aqueous phase: Influence of aqueous phase temperature

The rationale for elevating the temperature of the aqueous phase was to introduce energy into the conversion process. Two temperatures were examined: 60 °C and 90 °C. Heating the aqueous phase was adopted, albeit for a different reason, for the first liposome product which was introduced onto the market in the early 1990s: Ambisome® (NeXstar, USA). Water for injections heated to 50 °C was added into the vial containing the freeze dried liposome/amphotericin B cake: this procedure was carried out to reduce the size and the lower the toxicity of the amphotericin B/liposome formulation (Profitt et al., 1989; Lance et al., 1995).

Rubber tops were loosely placed onto the vials containing 8.90 g of deionised water before placing the glass vials in a water bath at the temperature under examination. When the desired vial temperature had been reached, the vials containing the deionised water were immediately removed from the water bath, the rubber tops were removed and 1.10 g of egg PL pro-liposome at 25 °C was poured into the heated vials. The vials were recapped with the rubber tops and the vials were immediately vigorously handshaken for one minute. The sizes of the resultant dispersions were characterised using light microscopy, laser diffraction and Coulter Counter.

3.4.2.3 Manipulation of pro-liposome:

3.4.2.3.1 Effect of freezing the pro-liposome

The effect of freezing egg PL pro-liposome on the liposome particle size was investigated. This entailed freezing the egg PL pro-liposome in a 25 ml glass vial in a freezer for 12 hours. The conversion regime of the pro-liposome was altered somewhat for these experiments, because frozen pro-liposomes were too solid to be transferred. Instead of adding the pro-liposome to the aqueous phase, the converse was carried out: 8.900 g of deionised water was added to 1.100 g of the frozen egg PL pro-liposome in the glass vial at room temperature. To aid conversion, the vials containing the frozen pro-liposome and bulk deionised water were vigorously shaken for one minute. The resultant dispersions were sized by light microscopy, laser diffraction and Coulter Counter.

3.4.2.3.2 Effect of varying lipid composition

The effect of four different lipid compositions on size was examined by sizing liposomes converted in one stage from pro-liposomes with vigorous shaking for one
minute at room temperature. Each pro-liposome contained one of the four following phospholipids, which have been discussed and evaluated in chapter two: egg PL, soya PL, egg PC or soya PC. The resultant liposome dispersions were converted in the usual manner as described in section 3.4.2 by adding 1.10 g of the individual pro-liposome to 8.90 g of deionised water at room temperature. The vials containing these two components were immediately vigorously shaken for one minute to generate the liposome dispersions. Light microscopy, PCS and laser diffraction were employed to size most of the dispersions.

3.4.2.3.3 Effect of replacing soya PC with soya PL

The effect of blending soya PL with soya PC on the particle size of dispersions converted in one stage was examined. Liposome dispersions were converted in one stage from pro-liposomes. The soya PC: soya PL weight ratio of the six pro-liposomes was: 100:0, 93:7, 90:10, 75:25, 50:50 and 25:75. The pro-liposomes were made in the usual manner as described in section 3.3.2. The dispersions were generated by vigorously handshaking 1.10 g of pro-liposome in 8.90 g of deionised water for one minute at room temperature. All dispersions were sized with light microscopy and laser diffraction.

3.4.3 Effect of dilution on liposome dispersions converted in one stage

Soya phospholipid dispersions and egg phospholipid dispersions were converted at room temperature in one stage, described in section 3.4.2, by vigorously handshaking 1.10 g of the appropriate pro-liposome in 8.90 g of deionised water for one minute. The appearance of both sets of dispersions converted in one stage were qualitatively assessed by visual inspection and light microscopy. Additionally, a freeze fracture replica of the undiluted egg PL dispersion was produced and examined under electron microscope. The lipid concentration (60 mg/g) of both sets of dispersions was diluted at room temperature to 5 mg/g, 15 mg/g or 30 mg/g with 50 mg/g glucose and deionised water. These diluted dispersions were inspected visually and examined under the light microscope.

Turbidity experiments involving the dilution of egg PL dispersions in 50 mg/g glucose, deionised water and 50 mg/g glucose containing low levels of Tween 80 (1 part by weight of Tween 80 to 100 parts by weight of lipid) were also carried out using light absorbance, described in section 3.3.8.7.
3.4.4 Two stage conversion
Two stage conversion involved mixing a specified amount of deionised water with the pro-liposome prior to the addition to the bulk deionised water at room temperature. This first stage involving the addition of deionised water to the pro-liposome will be referred to as the hydration stage. Mixing the deionised water for hydration with the pro-liposome was achieved by repeatedly passing these two components between two Eppendorf syringes (5 ml) up to twenty times. This procedure is referred to in the text as Eppendorf mixing or mixing. This mixing usually resulted in the generation of a hydrated pro-liposome, which was transferred into a vial containing deionised water to give a final dispersion weight of 10 g. The hydrated pro-liposome was dispersed into the bulk aqueous phase by:
1) Syringing the pro-liposome in deionised water, or
2) Vigorously handshaking for 90 seconds, or
3) Leaving the pro-liposome to disperse in the deionised water without agitation.
This second stage involving the dispersion of the hydrated pro-liposome in bulk deionised water will be referred to in the text as the dispersion stage.

3.4.4.1 Effect of Eppendorf mixing during hydration stage
Experiments were carried out at room temperature (20 °C-25 °C) to elucidate the optimal amount of mixing required to form a homogeneous mix between the pro-liposome and deionised water for hydration. In order to standardise the amount of mixing, 1.265 g (1.100 g +15% overage) of egg PL pro-liposome and 0.633 g (0.550 g +15% overage) of deionised water for the first stage of hydration were passed between two Eppendorf syringes for a set number of times, ranging from 0 up to twenty times (section 3.4.4). Liposome dispersions were generated by adding and syringing 1.650 g of hydrated pro-liposome ten times in 8.350 g of deionised water. The resultant dispersions were examined by light microscopy and sized by laser diffraction.

3.4.4.2 Effect of varying the amount of deionised water for hydration
Varying amounts of deionised water were added at room temperature to a fixed amount of the fluid egg PL pro-liposome. The amount of deionised water added to 1.265 g (1.100 g +15% overage) of pro-liposome ranged from 0.115 g (0.100 g +15% overage) up to 10.235 g (8.900 g +15% overage) of deionised water. These two components were intimately combined by Eppendorf mixing (twenty times) and the appearance of the
resultant mix was recorded. Eppendorf syringes with a volume of 5 ml were used if the total weight of pro-liposome and deionised water was below 4 g. However, for larger amounts of pro-liposome and deionised water 20 ml Eppendorf syringes were employed to mix the components. If required, the mixed pro-liposome and deionised water were added at room temperature to the remaining deionised water to give a final dispersion weight of 10 g. The liposome dispersion was converted by syringing the hydrated pro-liposome in the bulk deionised water ten times (section 3.4.2). All samples were sized with light microscopy, laser diffraction and Coulter Counter (section 3.3.8). Freeze fracture replicas of liposome dispersions generated from pro-liposomes (1.265 g) hydrated with half the weight of weight (0.633 g) and an equal weight of deionised water (1.265 g) were made and examined under EM (section 3.3.8.7).

3.4.4.3 Turbidity of diluted dispersions generated in two stages
Turbidity measurements investigating the effect of diluting egg PL dispersions, produced using the methodology described in section 3.4.4.1, were carried out. These studies involved diluting 100 μl of the dispersions in 10 ml of 50 mg/g glucose and 10 ml of deionised water. The turbidities of these diluted dispersions were monitored for 20 minutes at 550 nm as described in section 3.3.8.7.

3.4.4.4 Effect of agitation during dispersion stage
The two extremes of agitation during the dispersion stage of a hydrated pro-liposome on liposome size were investigated: vigorous handshaking for 90 seconds and leaving to hydrate without any agitation. The hydrated egg PL pro-liposome was formed by passing 0.633 g (0.550 g +15% overage) of deionised water and 1.265 g (1.100 g +15% overage) of egg PL pro-liposome between two Eppendorf syringes twenty times, described in section 3.4.4. After Eppendorf mixing, 1.650 g of this gel was directly transferred into a glass vial containing 8.350 g of bulk deionised water. The deionised water and hydrated gel were immediately subjected to either:

**Vigorous handshaking**
The liposome dispersion was formed by vigorously handshaking the hydrated pro-liposome with the bulk deionised water at room temperature. The intensity of handshaking was identical to the handshaking adopted for the single stage conversion,
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described in section 3.4.2.1. The duration of shaking required to disperse the hydrated pro-liposome was 90 seconds. Or,

No agitation

The hydrated pro-liposome was left to hydrate in the bulk deionised water without agitation at room temperature. The time taken for the hydrated pro-liposome to disperse without agitation was noted.

The two sets of dispersions were compared by sizing with light microscopy, laser diffraction and Coulter Counter (section 3.3.8).

3.4.4.5 Effect of lipid composition

3.4.4.5.1 Comparing the particle size of liposomes generated in two stages using pure soya PC and pure egg PC

The influence of the difference in the degree of unsaturation between egg PC and soya PC on liposome particle size was investigated. This was achieved by comparing the size of liposomes generated from two pro-liposomes containing refined egg PC and refined soya PC. The pro-liposomes used to form these liposomes were produced at room temperature as described in section 3.3.2 by dissolving the requisite amount of either refined egg PC or refined soya PC in ethanol and subsequently adding glycerol. These two pro-liposomes were hydrated at room temperature by Eppendorf mixing 0.633 g (0.550 g +15% overage) of deionised water with 1.265 g (1.100 g +15% overage) of pro-liposome. After having Eppendorf mixed the two components twenty times, 1.650 g of the resultant gels were dispersed at room temperature in 8.350 g of bulk deionised water by vigorously handshaking the contents of the vial for 90 seconds. The resultant dispersions were sized by PCS, light microscopy and laser diffraction (section 3.3.8). To improve the reproducibility of the measurements, the isotonic media used for diluting these samples prior to sizing contained low amounts of Tween 80 (1 part by weight of Tween 80 to 100 parts by weight of lipid). The changes in turbidity were also qualitatively measured at 550 nm, described in section 3.3.8.7, after diluting 100 μl of the samples in 10 cm³ of 50 mg/g glucose, deionised water and 50 mg/g glucose with 0.006 mg/g Tween 80.

3.4.4.5.2 Effect of replacing soya PC with soya PL

The effect of blending soya PL with soya PC on the particle size of dispersions converted in two stages was examined. Six sets of liposome dispersions were generated
from pro-liposomes. The soya PC: soya PL weight ratios of the six pro-liposomes were 100:0, 93:7, 90:10, 75:25, 50:50 and 25:75. The pro-liposomes were made in the usual manner as described in section 3.3.2. The pro-liposomes were hydrated by Eppendorf mixing 1.265 g (1.100 g +15% overage) of each pro-liposome twenty times with 0.633 g (0.550 g +15% overage) of deionised water. The liposome dispersions were generated by vigorously handshaking 1.650 g of the hydrated pro-liposome in 8.350 g of deionised water for 90 seconds at room temperature. All dispersions were sized by light microscopy, PCS and laser diffraction, but only the number of particles in the dispersions with a soya PC: soya PL weight ratio of 90:10 were counted using the Coulter Counter (section 3.3.8).

3.4.4.5.3 Effect of including egg phosphatidylglycerol (EPG)

The two pro-liposomes containing EPG were manufactured by dissolving the sodium salt of EPG in the ethanolic solution of soya PC, before the addition of glycerol. The egg PC: EPG: ethanol: glycerol weight ratios of the two pro-liposomes were 59.7:0.3:36:26 and 58.5:1.5:36:26. Each pro-liposome was hydrated by passing 1.265 g (1.100 g +15% overage) of pro-liposome containing EPG and 0.633 g (0.550 g +15% overage) of deionised water between two Eppendorf syringes. After having formed a hydrated gel by mixing the components twenty times, 1.650 g of the resultant gels were dispersed in 8.350 g of deionised water by vigorously handshaking for 90 seconds at room temperature. The resultant dispersions were sized by light microscopy, laser diffraction and PCS (section 3.3.8).

3.5 Results and discussion

3.5.1 Particle size distribution of Intralipid®

The size results for the TPN sample are shown in Table 3.1.

<table>
<thead>
<tr>
<th>Table 3.1 Particle size results of Intralipid® concentrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPN sample</td>
</tr>
<tr>
<td>z average ± s.d. (nm)</td>
</tr>
<tr>
<td>P.I. ± s.d.</td>
</tr>
<tr>
<td>D-90 ± s.d. (μm)</td>
</tr>
<tr>
<td>Vol. added to LD cell (μl)</td>
</tr>
<tr>
<td>Microscope assessment</td>
</tr>
<tr>
<td>No. of particles ≥20μm ml⁻¹</td>
</tr>
<tr>
<td>No. of particles ≥10μm ml⁻¹</td>
</tr>
<tr>
<td>No. of particles ≥5μm ml⁻¹</td>
</tr>
</tbody>
</table>
Before discussing the particle size results of the various liposome dispersions, it is useful to put into perspective the size and number of particles present in commercially available emulsions for TPN. In TPN emulsions most of the oil droplets are approximately 250 nm in diameter (Table 3.1). This is approximately 20 fold smaller than the diameter of an average human erythrocyte, which is 7.5 μm (Ganong, 1991). Light microscopy revealed that there were rarely any droplets greater than 5 μm in the 10% triglyceride TPN concentrate. This observation was also reflected by the low D-90 value of 0.97 μm. One noticeable difference between the TPN and fine liposome dispersions, was the volume that had to be added to the laser diffraction cell to obtain an obscuration of 0.2: only 3 μl was required for the emulsion, compared to 60 μl for fine liposome dispersions (Table 3.10). This difference could be attributed to the high lipid concentration in the emulsion (23%) as well as the refractive index and opacity of the oil droplets. The Coulter Counter confirmed the presence of only a few particles by detecting only 240,000 particles greater than 5 μm per ml of 10% concentrate. This number was somewhat higher than figures quoted in the literature (Puntis et al., 1992), which may have been due to the non-laminar flow environment in which the measurements were taken. Puntis detected 37 000 particles between 2 μm and 100 μm in one day’s feed for an infant. These numbers should also be viewed in the context of the number of erythrocytes in blood, which is approximately 5.4 billion red blood cells per ml in an average healthy human male (Ganong, 1991).

3.5.2 One stage conversion
3.5.2.1 Effect of energy input during single stage conversion

The size results for the egg PL dispersions are shown in Table 3.2, and the size results for the soya PL dispersions in Table 3.3.

| Table 3.2 Effect of three different energy inputs during conversion of egg PL pro-liposomes in one stage |
|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| D-90 ± s.d. (μm)                     | Egg PL PRO converted in one stage with vigorous agitation (n=5) | Egg PL PRO converted in one stage with moderate agitation |
| Sample volume for LD (μl)            | 6                                               | 6                                               |
| Microscope assessment                | Coarse                                          | Coarse                                          |
| No. of particles ≥ 20μm ml⁻¹         | 2.2x10⁴±1.4x10⁴                                 | 3.6x10⁵±0.3x10⁵                                 |
| No. of particles ≥ 10μm ml⁻¹         | 4.9x10⁴±2.3x10⁴                                 | 7.2x10⁵±2.6x10⁵                                 |
| No. of particles ≥ 5μm ml⁻¹          | 8.0x10⁴±3.6x10⁴                                 | 8.0x10⁵±2.5x10⁵                                 |
| Time taken for pro-liposome to convert (min) | NA                                              | NA                                              | 30-45                                           |
The amount of shaking during conversion influenced the particle size distribution of both the egg PL liposomes (Table 3.2) and soya PL liposomes (Table 3.3). As the amount of shaking during the one stage conversion increased, the coarseness of both sets of dispersions decreased. This was reflected in the egg dispersions by the lower values for both the Coulter Counter and D-90 when shaking was vigorous. In the case of the Coulter Counter measurements for the egg PL liposomes, the number of particles in all three large size bands (≥5 μm, ≥10 μm and ≥20 μm) fell dramatically as the amount of shaking increased (Table 3.2). The number of particles greater than 5 μm significantly decreased over 10 fold from 1.3x10⁹ ± 0.10x10⁹ when the pro-liposome was converted without agitation, to approximately 8.0x10⁷ ± 3.6x10⁷ when converted by shaking vigorously for one minute (p = 0.002). Similar significant differences in numbers were also recorded for the other two oversize bands at 10 μm and 20 μm (p = 0.03; p = 0.045 respectively). These Coulter Counter results were paralleled by the results from the laser diffraction and light microscope: the average D-90 value (3.56 μm) for the egg PL dispersions converted whilst vigorously shaking was significantly lower than the average D-90 value (13.94 μm) of the egg PL dispersion left to convert in one stage without agitation (p = 0.003). For egg PL dispersions converted with moderate intensity, the values for the Coulter Counter and D-90 fell in between the values of these two extremes of energy input. Similarly, light microscopy visually confirmed the correlation between coarseness of egg PL liposome dispersions and energy input during conversion (Plate 3.1-3.3). From these plates it was evident that the number of larger liposomes decreased as the energy input during conversion increased. From these Coulter Counter results and light microscopy, it was evident that even the egg PL dispersions (Table 3.2) converted in one stage by vigorous shaking were considerably coarser than the TPN dispersion (Table 3.1). The Coulter Counter measurements for these egg PL liposomes (Table 3.2) were at least two orders of
magnitude greater than the equivalent measurements for TPN emulsions at the 5 μm size band (Table 3.1).

The size results for the soya PL dispersions were similar to the egg PL results in terms of the influence of shaking. However, the time for the soya PL pro-liposome to convert without agitation and the size of the resultant liposomes differed considerably. Without agitation soya PL pro-liposome took in excess of 12 hours to disperse in deionised water and the resultant dispersion yielded large D-90 values in excess of 60 μm. This D-90 value was significantly different from the D-90 value of 1.56 μm for the soya PL pro-liposome which was shaken vigorously during conversion (p = 5.7x10⁻⁵). This observation was verified with light microscopy, which qualitatively revealed the presence of giant MLVs (Plate 3.4) in addition to smaller vesicles. The difference in the conversion time was probably due to the thicker viscosity of soya pro-liposomes upon addition to deionised water, which hindered its own dispersion. This slow conversion probably enabled the glycerol to diffuse from the pro-liposome into the aqueous phase. This would have diminished the osmotic gradient between the pro-liposome structures and the aqueous phase, thereby removing one of the driving forces for smaller particle size.

For subsequent studies, most pro-liposomes added to deionised water in one stage were immediately subjected to vigorous handshaking for one minute, unless otherwise stated.

Plate 3.1 Typical light micrograph of egg PL dispersion converted in one stage by vigorously handshaking pro-liposome in excess deionised water (Bar = 25 μm), as described in section 3.5.2.1
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Plate 3.2 Typical light micrograph of egg PL dispersion converted in one stage by moderately handshaking pro-liposome in excess deionised water (Bar = 25 μm), as described in section 3.5.2.1

Plate 3.3 Typical light micrograph of egg PL dispersion converted in one stage from pro-liposome in excess deionised water without handshaking (Bar = 25 μm), as described in section 3.5.2.1
Chapter three-Particle size of liposome dispersions

Plate 3.4 Typical light micrograph of soya PL dispersion converted in one stage from pro-liposome in excess deionised water without handshaking (Bar = 25 µm), as described in section 3.5.2.1

3.5.2.2 Manipulation of aqueous phase: Effect of elevating the aqueous phase temperature on particle size

The particle size data for two sets of dispersions converted at 60 °C and 90 °C are shown in Table 3.4.

<table>
<thead>
<tr>
<th></th>
<th>Egg PL PRO converted in one stage at 60 °C with vigorous handshaking</th>
<th>Egg PL PRO converted in one stage at 90 °C with vigorous handshaking</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-90 ± s.d. (µm)</td>
<td>5.25±0.13</td>
<td>8.55±0.08</td>
</tr>
<tr>
<td>Volume added to LD cell (µl)</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Microscope assessment</td>
<td>Coarse</td>
<td>Very coarse</td>
</tr>
<tr>
<td>No. of particles ≥20µm ml⁻¹</td>
<td>1.0x10⁵±0.7x10⁴</td>
<td>15x10⁵± 0.4x10⁵</td>
</tr>
<tr>
<td>No. of particles ≥10µm ml⁻¹</td>
<td>2.0x10⁶±1.0x10⁵</td>
<td>1.8x10⁶ ± 0.7x10⁵</td>
</tr>
<tr>
<td>No. of particles ≥5µm ml⁻¹</td>
<td>1.1x10⁷±0.2x10⁷</td>
<td>5.5x10⁷±0.7x10⁷</td>
</tr>
</tbody>
</table>

The rationale for heating the aqueous phase was to encourage the formation of smaller liposomes by introducing more energy into the conversion process. However, as can be seen from Table 3.4, heating the aqueous phase appeared to have a detrimental effect upon the particle size of the liposomes. Indeed at an elevated temperature of 90 °C, the particle size distribution was significantly coarser than at 60 °C: the laser diffraction revealed that the dispersions converted at 90 °C had D-90 values of 8.55 ± 0.08 µm compared to D-90 values of about 5.25 ± 0.13 µm if converted at 60 °C. These laser diffraction results were verified by the Coulter Counter. At all three oversize bands, i.e. 5 µm, 10 µm and 20 µm, the egg pro-liposome converted at 90 °C had a significantly greater number of large particles compared to the same egg pro-liposome converted at 60 °C. Comparing the number of particles greater than 5 µm; heating the aqueous phase
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at 90 °C generated 550 ± 70 million particles, whereas heating the deionised water to 60 °C generated 110 ± 20 million particles (p = 0.02). A possible explanation for the larger number of liposomes at the 90 °C may be attributed to the speed of conversion. At 90 °C the pro-liposome converted rapidly, which perhaps resulted in liposome formation before handshaking dispersed the pro-liposome.

3.5.2.3 Manipulation of pro-liposome converted in one stage:

3.5.2.3.1 Freezing the pro-liposome

The rationale for freezing the pro-liposome was to precipitate out structured bilayers by lowering the solubility of egg PL in ethanol. Thereby enabling liposomes to be generated from a more ordered pro-liposome. The size results of this dispersion are shown in Table 3.5.

<table>
<thead>
<tr>
<th>Table 3.5 Effect of converting a frozen pro-liposome in one stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frozen egg PL PRO converted in one stage with vigorous handshaking (n=4)</td>
</tr>
<tr>
<td>D-90 ± s.d.(µm)</td>
</tr>
<tr>
<td>Volume added to LD cell (µl)</td>
</tr>
<tr>
<td>Microscope assessment</td>
</tr>
<tr>
<td>No. of particles ≥20µm ml⁻¹</td>
</tr>
<tr>
<td>No. of particles ≥10µm ml⁻¹</td>
</tr>
<tr>
<td>No. of particles ≥5µm ml⁻¹</td>
</tr>
</tbody>
</table>

The results in Table 3.5 reveal that the number of particles at 5 µm, 10 µm and 20 µm detected by the Coulter Counter were not significantly different from fluid egg PL pro-liposome converted in one stage (Table 3.2) (p = 0.186, p = 0.095, p = 0.818 respectively). However, the D-90 value of 6.15 ± 0.54 µm for the dispersion converted from the frozen pro-liposome with vigorous handshaking was significantly higher than the D-90 value of same unfrozen egg PL pro-liposome (3.56 ± 0.53 µm) converted at room temperature with vigorous handshaking (Table 3.2) (p = 0.001). Furthermore, light microscopy confirmed the coarse nature of the dispersion generated from frozen pro-liposome. These light microscopy and D-90 results suggested that freezing the pro-liposome may have increased the proportion of larger sized liposomes. After one minute of vigorous handshaking, the frozen pro-liposome had still not converted fully: ribbons of pro-liposome, visible to the naked eye, still remained. This unconverted proportion was left to disperse without further agitation and may have contributed to the generation of some of the larger liposomes in the dispersions.
3.5.2.3.2 Varying lipid composition

The four lipids described and evaluated in chapter two were used to form pro-liposomes. These four pro-liposomes were converted into liposome dispersions in one stage by vigorous handshaking for one minute. The resultant liposome dispersions were sized (Table 3.6) and the influence of the lipid compositions was assessed.

Table 3.6 Effect of lipid composition on particle size of liposomes converted in one stage

<table>
<thead>
<tr>
<th>Sample description</th>
<th>Soya PC PRO converted in one stage with vigorous agitation</th>
<th>Egg PC PRO converted in one stage with vigorous agitation</th>
<th>Soya PL PRO converted in one stage with vigorous agitation</th>
<th>Egg PL PRO converted in one stage with vigorous agitation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z average ± s.d. (nm)</td>
<td>546.6±53.3</td>
<td>710.9±59.9</td>
<td>307.0±12.0</td>
<td>418.3±13.6</td>
</tr>
<tr>
<td>P.I. ± s.d.</td>
<td>0.31±0.051</td>
<td>0.325±0.022</td>
<td>0.223±0.036</td>
<td>0.292±0.046</td>
</tr>
<tr>
<td>D-90 ± s.d. (μm)</td>
<td>3.50±0.09</td>
<td>3.77±0.04</td>
<td>1.56±0.18</td>
<td>3.35±0.08</td>
</tr>
<tr>
<td>Volume added to LD (μl)</td>
<td>7.5</td>
<td>10</td>
<td>60</td>
<td>12</td>
</tr>
<tr>
<td>Microscope assessment</td>
<td>Very coarse</td>
<td>Very coarse</td>
<td>Moderately coarse</td>
<td>Coarse</td>
</tr>
<tr>
<td>Dispersion characteristics</td>
<td>Fluid</td>
<td>Fluid</td>
<td>Flowable gel</td>
<td>Fluid</td>
</tr>
</tbody>
</table>

From the results in Table 3.6, both the z average and the D-90 value indicated that the liposomes generated from soya bean PL were significantly smaller than the liposomes generated from the egg yolk PL, egg PC and soya PC. The average z average of the soya PL liposomes was at least 100 nm smaller than the z averages of the egg yolk PL, egg PC and soya PC dispersions (p = 0.010, p = 0.004, p = 0.003 respectively). Furthermore, the average D-90 value for the soya PL (1.56 ± 0.18 μm) was approximately half the value of the egg yolk PL, egg PC and soya PC dispersions (p = 9.8x10⁻⁴, p = 3.1x10⁻⁵, p = 7.7x10⁻⁵, respectively). Light microscopy confirmed that there were far fewer larger liposomes in the soya PL dispersions (Plate 3.5) than in the corresponding egg PL dispersion (Plate 3.1). These results demonstrated that the lipid composition of the soya PL could significantly decrease the particle size of the liposomes converted from pro-liposomes in one stage. The visual appearance and the physical characteristics of the soya PL dispersion also differed. This soya PL produced visibly clearer and gel-like dispersions compared to the other lipids. Therefore, although the soya PL pro-liposome generated dispersions with smaller sized liposomes and a smaller proportion of large sized liposomes, this type of preparation would be unsuitable for intravenous administration because of its gel-like character. Formulations which possess a high viscosity are generally unsuitable for intravenous administration, because upon injection
circulatory vessels may become occluded and the preparation may not be rapidly diluted in the bloodstream.

The clarity, gel-like viscosity and the smaller liposome size of the soya PL dispersions could have perhaps been attributed to the presence of small but influential amounts of negative charge present in the soya PL. The charged species would have favoured the generation of smaller and more unilamellar structured vesicles (Perret et al., 1991). The smaller size and unilamellar nature of the liposomes would have made the dispersions seem clearer. Secondly, the generation of unilamellar structures created more liposomes. Both these factors, increased unilamellarity and an increase in the number of liposomes formed, may increase the aqueous capture volume (Talsma et al., 1992) and raise the viscosity of the dispersion.

Plate 3.5 Typical light micrograph of soya PL dispersion converted in one stage by vigorously handshaking pro-liposome in excess deionised water (Bar = 25 μm), as described in section 3.5.2.3.2

3.5.2.3.3 Replacing soya PC with soya PL

The size results for the dispersions converted in one stage from pro-liposomes containing various blends of soya PC and soya PL are shown in Table 3.7.

<table>
<thead>
<tr>
<th>Soya PC: soya PL weight ratio</th>
<th>D-90 ± s.d. (μm)</th>
<th>Sample added to LD cell (μl)</th>
<th>Microscope assessment</th>
</tr>
</thead>
<tbody>
<tr>
<td>100:0</td>
<td>3.75±0.26</td>
<td>7.5</td>
<td>Very coarse</td>
</tr>
<tr>
<td>93:7</td>
<td>5.58±0.15</td>
<td>40</td>
<td>Coarse</td>
</tr>
<tr>
<td>90:10</td>
<td>5.42±0.40</td>
<td>40</td>
<td>Coarse</td>
</tr>
<tr>
<td>75:25</td>
<td>2.54±1.21</td>
<td>60</td>
<td>Moderately coarse</td>
</tr>
<tr>
<td>50:50</td>
<td>2.17±1.21</td>
<td>60</td>
<td>Moderately coarse</td>
</tr>
<tr>
<td>25:75</td>
<td>2.09±0.62</td>
<td>60</td>
<td>Moderately coarse</td>
</tr>
</tbody>
</table>
Chapter three-Particle size of liposome dispersions

There appeared to be a relationship between the proportion of soya PL used to form the liposomes and the fineness of the dispersion (Table 3.7). Using light microscopy, a general trend was evident: as the level of soya PL increased, the dispersions became less coarse. Statistically, a significant drop in the D-90 value was noted in dispersions as the soya PL weight was raised to 25% of total lipid: the average D-90 value of dispersions which had a soya PC: soya PL weight ratio of 75:25 was significantly lower than the D-90 value of dispersions with soya PC: soya PL weight ratios of 90:10 and 93:7 (p = 0.033, p = 0.023 respectively). One anomaly in the average D-90 values was the low D-90 of the liposome dispersion formed solely from soya PC. In this instance, the average D-90 value for the soya PC liposomes was 3.75 ± 0.26 μm, which was significantly lower than the dispersions with soya PC: soya PL weight ratios of 90:10 and 93:7, which had average D-90 values of 5.43 ± 0.40 μm and 5.58 ± 0.15 μm (p = 0.007, p = 0.001 respectively). This contradicted the light microscopy results, which clearly revealed a greater number of larger sized liposomes in the soya PC dispersions. Perhaps this anomaly in the average D-90 value of the soya PC dispersion was linked to the shortcomings of laser diffraction, which only detected the relative size distribution and did not reflect the absolute number of particles in the dispersion.

3.5.3 Dilution of liposome dispersions converted in one stage

Egg PL pro-liposomes and soya PL pro-liposomes were converted into liposome dispersions at room temperature in one stage, by vigorously agitating the pro-liposomes with bulk deionised water for one minute. The appearances of the resultant dispersions are described and discussed in the following section.

3.5.3.1 Liposome dispersions before dilution

Macroscopically, the undiluted dispersion converted in one stage from egg PL pro-liposome appeared turbid. Examination of the freeze fracture replica of the undiluted egg PL dispersion (60 mg/g of phospholipid) under EM revealed that the size of the liposomes seemed highly polydispersed (Plate 3.6). The liposome diameters ranged from micrometer sized down to 100 nm or less. Two factors may have contributed to the turbid milky appearance: firstly, the larger sized liposomes in the egg dispersion diffracted more light than smaller particles. Secondly, compared to the soya PL, the more saturated nature of the egg PL fatty acid chains (section 2.4.3) may have conferred the dispersions with a more opaque appearance.
In contrast to egg PL pro-liposomes, soya PL pro-liposomes converted in one stage produced almost transparent orange semi-solid gels, which flowed poorly. In the undiluted state these soya PL liposomes were of a much smaller diameter than egg PL liposomes, and relatively few large spherical liposomes were observed per field in the soya PL dispersion (Plate 3.5). The main reason for the finer appearance of this soya PL dispersion may be related to the presence of charged species in the soya PL, which tends to favour smaller sized liposomes (Perret et al., 1991).

Plate 3.6 Typical freeze fracture replica of egg PL dispersion formed by vigorously handshaking pro-liposome in excess deionised water (Bar = 1 μm), as described in section 3.5.3.1

3.5.3.2 Liposome dispersions after diluting in 50 mg/g glucose

Diluting the concentrated liposome dispersions with 50 mg/g glucose to phospholipid concentrations of 5, 15 and 30 mg/g dramatically changed the appearance of the dispersions. Macroscopically, the soya PL dispersions became slightly turbid, and fine “lipid strands” could be visualised under the light microscope. These soya PL lipid strands ranged from 5 to 10 μm in length and were approximately 1 μm in breadth. A similar type of structure in liposome dispersions hydrated from films has been observed by Lasic (1993). The “lipid strand” is fully coated by a hydrated surface which prevents the hydration of its interior. The generation of these long lipid structures would have contributed to the increase in turbidity of the soya PL dispersions upon dilution and may have accounted for a proportion of the large liposomes in these dispersions upon
dilution. These “strands” floated freely underneath the microscope and were therefore unlikely to be artefacts of large liposomes, which had been “squashed” between the coverslip and the slide. Similar structures were also detected in the egg PL dispersions diluted with 50 mg/g glucose. However, the dimensions of these egg PL “strands” differed: their lengths were longer, exceeding 20 μm in some dispersions (Plate 3.7), and they appeared much broader than those found in the soya PL dispersions diluted in 50 mg/g glucose.

The generation of the lipid strands upon dilution in 50 mg/g glucose, suggested that some of the pro-liposome had not converted entirely into liposomes after the one stage liposome formation. Perhaps the addition of isotonic media was required to fully precipitate the remaining lipid into bilayered structures. The reason why the newly precipitated structures were in the form of lipid strands may have been attributed to the osmotic support provided by the diluent. The 50 mg/g glucose had an osmotic potential similar to the entrapped hydrophilic material, which meant there would have been no osmotic strain exerted on the bilayered structures upon dilution. Hence, there was no driving force to disrupt the newly precipitated structures and the formation of large lipid strands was encouraged.

Turbidity experiments revealed that when the liposome dispersions converted in one stage were diluted in 50 mg/g glucose, the cloudiness of these dispersions increased for two minutes. This is illustrated in Figure 3.2, which shows the turbidity of egg PL dispersions diluted in 50 mg/g glucose. The increase in turbidity was not abolished if the 50 mg/g glucose diluent contained a small level of surfactant such as Tween 80 (Figure 3.2). This indicated that the increase in turbidity was not due to aggregation/fusion of the liposomes. Therefore, it was likely that the increase in turbidity was attributable to the continued precipitation of large lipid structures and perhaps the gradual swelling of these structures upon equilibration in the media.
Figure 3.2 Turbidity of egg PL dispersion converted in one stage after diluting x200 fold in 50 mg/g glucose, deionised water and 50 mg/g glucose containing 0.003 mg/g Tween 80 (Each point is mean ± s.d., n=3)

Plate 3.7 Typical light micrograph showing the presence of lipid strands after two fold dilution of egg PL dispersion in 50 mg/g glucose (Bar = 25 μm), as described in section 3.5.3.2
3.5.3.3 Liposome dispersions after diluting in deionised water

The effects of changing the diluting media on the appearances of the soya PL dispersions were visible macroscopically by the naked eye. As described in section 3.5.3.2, dilution of the soya PL dispersion with 50 mg/g glucose to lipid concentrations of 5 mg/g, 15 mg/g or 30 mg/g resulted in the formation of hazy dispersions. However, if the same soya PL dispersion was diluted to the equivalent lipid concentrations with deionised water, the resultant diluted dispersions were translucent. These macroscopic observations were mirrored on a microscopic level when examined under the light microscope. If the soya liposome dispersion was diluted with deionised water, “lipid strands” were rarely detected. Similar observations were also recorded for the egg PL liposomes, although after dilution the liposomes were still markedly larger (Plate 3.8) than the soya PL liposomes.

The translucency of the soya PL dispersion after dilution in deionised water could have been partly attributed to the lowering of the PL concentration. However, the prevention of “strand” formation may have also partly contributed to the translucency of the dispersion. The reason for the absence of “stands” may be linked to osmosis. Like all semi-permeable membranes, phospholipid bilayers are sensitive to changes in their osmotic environment (Bangham et al., 1967). Therefore, diluting the dispersions with deionised water would have increased the osmotic potential of the external aqueous phase, thereby encouraging entry of water into the phospholipid structures. This addition of water may have swelled the membrane structures and caused the rupturing of any precipitated strands. Therefore, instead of coarse strands, liposomes would have been formed, and subsequently the dispersion would have appeared finer.

Evidence complementing the light microscope observations was provided by turbidity experiments (Figure 3.2). If the diluting media was deionised water, which had no osmotic support, the turbidity did not increase. Indeed for three minutes after the dilution, the turbidity started to decrease, perhaps reflecting the bursting of some closed bilayered structures.

Since it was apparent that a two stage dilution in deionised water dramatically affected the size of the liposome dispersions, it was decided to investigate the addition of deionised water in two stages as a means of generating finer liposome dispersions. Two main approaches could have been adopted to convert the pro-liposome in two stages.
The first approach would have involved diluting the one stage converted liposome dispersion in deionised water. However, this manipulation was considered to be unsuitable, because the concentration of lipid in the final dispersion would have been too dilute and the volume of the final dispersion would have been raised. It was therefore decided to investigate the second approach, which involved adding small amounts of deionised water to the pro-liposome in a first stage of hydration. This hydrated pro-liposome would be subsequently dispersed into the remaining bulk deionised water to form a liposome dispersion. This two step procedure for liposome formation is referred to in the text as two stage conversion. The resultant dispersions were isotonic with respect to glycerol (26 mg/g) and the PL concentrations were maintained at 60 mg/g.

Plate 3.8 Typical light micrograph of egg PL liposome dispersion generated in one stage after two fold dilution in deionised water (Bar = 1 μm), as described in section 3.5.3.3

3.5.4 Two stage conversion

3.5.4.1 Effect of mixing deionised water to hydrate pro-liposome

The degree of mixing required to generate a hydrated pro-liposome was studied. The egg PL pro-liposome and deionised water were passed between two 5 ml Eppendorf syringes 0, 3, 5, 10, 15 and 20 times. The subsequent gels were dispersed in bulk deionised water by syringing these two components 10 times. The particle size results of these various dispersions are shown in Table 3.8.
Table 3.8 Effect of passing egg PL pro-liposome and deionised water between two Eppendorf syringes prior to dispersing in bulk deionised water

<table>
<thead>
<tr>
<th>Number of passes between two Eppendorf syringes</th>
<th>0</th>
<th>3</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>z average (nm)</td>
<td>404.0±52.8</td>
<td>282.4±14.2</td>
<td>237.5±9.9</td>
<td>233.4±2.3</td>
<td>223.5±4.3</td>
<td>222.4±3.3</td>
</tr>
<tr>
<td>D-90 ± s.d. (μm)</td>
<td>5.47±0.03</td>
<td>3.57±0.05</td>
<td>2.58±1.21</td>
<td>2.51±0.21</td>
<td>2.06±0.13</td>
<td>2.00±0.08</td>
</tr>
<tr>
<td>Volume added to LD cell (μl)</td>
<td>12</td>
<td>30</td>
<td>30</td>
<td>35</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Microscope assessment</td>
<td>Very coarse</td>
<td>Coarse</td>
<td>Coarse</td>
<td>Moderately coarse</td>
<td>Moderately coarse</td>
<td>Moderately coarse</td>
</tr>
</tbody>
</table>

There was a relationship between the number of passes for the first stage of hydration and the particle size of the final liposome dispersion. Thoroughly mixing 0.633 g of deionised water for hydration with 1.265 g of egg PL pro-liposome using Eppendorf syringes reproducibly generated smaller sized liposomes. As shown in Table 3.8, it seemed that between 15 and 20 passes were required to generate gels, which when added to deionised water yielded liposome dispersions with the lowest z average and D-90 value. After 15 or 20 Eppendorf passes there appeared to be no significant difference between the z average of the dispersions (p = 0.743). This amount of intimate mixing was probably required because it redissolved large liposomes, which may have been generated during the initial addition of deionised water for hydration and pro-liposome. If thoroughly mixed, the resultant mixed pro-liposome would have consisted of organised stacks of precipitated bilayers containing evenly distributed amounts of deionised water, ethanol and glycerol.

Insufficient mixing, i.e. 10 passes or fewer, of egg PL pro-liposome and deionised water resulted in the hydrated pro-liposome appearing somewhat hazy. This haziness may have been due to localised regions of inhomogeneity within the hydrated pro-liposome. Imbalances within the gel, such as higher concentrations of deionised water, could have caused the precipitation of large liposomes, which upon dilution in the bulk deionised water would have increased the size distribution of the dispersion. The presence of larger liposomes in dispersions generated from hydrated pro-liposomes which had been mixed fewer than 10 times was confirmed by the coarser appearance under the light microscope.

3.5.4.2 Effect of varying the amount of deionised water added to the pro-liposome

The effect of varying the amount of deionised water added to the first stage of pro-liposome hydration was investigated. The physical appearance of the thoroughly mixed egg PL pro-liposome with deionised water was noted and is recorded in Table 3.9.
Chapter three-Particle size of liposome dispersions

Additionally, the particle size of the liposome dispersions generated from these pro-liposome/deionised water combinations was measured and is shown in Table 3.9 and Table 3.10. EM photographs of freeze fracture replicas of egg PL dispersions generated by hydrating 1.265 g pro-liposomes with 0.633 g and 1.265 g of deionised water, prior to dispersing in bulk deionised water, are shown in Plate 3.9 and Plate 3.10 respectively.

Table 3.9 Effect of adding varying amounts of deionised water to hydrate egg PL pro-liposome

<table>
<thead>
<tr>
<th>Pro-liposome: deionised water w/w</th>
<th>Appearance of pro-liposome/deionised water after mixing</th>
<th>No. of particles ≥20μm ml⁻¹</th>
<th>No. of particles ≥10μm ml⁻¹</th>
<th>No. of particles ≥5μm ml⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.100:0.100</td>
<td>Clear fluid</td>
<td>6.4x10⁶±5.2x10⁵</td>
<td>1.4x10⁶±0.5x10⁵</td>
<td>7.5x10⁵±1.8x10⁵</td>
</tr>
<tr>
<td>1.100:0.275</td>
<td>Clear firm gel</td>
<td>1.4x10⁶±1.6x10⁵</td>
<td>7.5x10⁵±2.3x10⁵</td>
<td>3.2x10⁵±0.2x10⁵</td>
</tr>
<tr>
<td>1.100:0.550</td>
<td>Clear firm gel</td>
<td>1.0x10⁶±0.3x10⁵</td>
<td>9.7x10⁵±4.4x10⁵</td>
<td>3.1x10⁵±0.5x10⁵</td>
</tr>
<tr>
<td>1.100:0.875</td>
<td>Milky firm gel</td>
<td>2.7x10⁶±2.2x10⁵</td>
<td>4.6x10⁵±0.5x10⁵</td>
<td>1.5x10⁵±0.9x10⁵</td>
</tr>
<tr>
<td>1.100:1.100</td>
<td>Milky firm gel</td>
<td>4.5x10⁶±2.0x10⁵</td>
<td>6.0x10⁵±4.3x10⁵</td>
<td>2.4x10⁵±0.9x10⁵</td>
</tr>
<tr>
<td>1.100:3.300</td>
<td>White flowable gel</td>
<td>6.1x10⁵±3.9x10⁵</td>
<td>2.2x10⁵±0.6x10⁵</td>
<td>4.2x10⁵±0.6x10⁵</td>
</tr>
<tr>
<td>1.100:8.900</td>
<td>White liquid</td>
<td>1.7x10⁵±5.9x10⁴</td>
<td>4.0x10⁴±0.8x10⁴</td>
<td>7.8x10⁴±2.0x10⁴</td>
</tr>
</tbody>
</table>

Table 3.10 Effect of adding varying amounts of deionised water to hydrate egg PL pro-liposome

<table>
<thead>
<tr>
<th>Pro-liposome: deionised water w/w</th>
<th>D-90 ± s.d.(μm)</th>
<th>Volume added to LD cell (μl)</th>
<th>Microscope assessment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.100:0.100</td>
<td>4.86±0.07</td>
<td>12.5</td>
<td>Coarse</td>
</tr>
<tr>
<td>1.100:0.275</td>
<td>1.58±0.15</td>
<td>30</td>
<td>Moderately coarse</td>
</tr>
<tr>
<td>1.100:0.550</td>
<td>1.87±0.05</td>
<td>35</td>
<td>Moderately coarse</td>
</tr>
<tr>
<td>1.100:0.875</td>
<td>3.77±0.17</td>
<td>25</td>
<td>Coarse</td>
</tr>
<tr>
<td>1.100:1.100</td>
<td>5.47±0.80</td>
<td>20</td>
<td>Coarse</td>
</tr>
<tr>
<td>1.100:3.300</td>
<td>7.32±1.57</td>
<td>15</td>
<td>Very coarse</td>
</tr>
<tr>
<td>1.100:8.900</td>
<td>7.90±0.52</td>
<td>10</td>
<td>Very coarse</td>
</tr>
</tbody>
</table>

There was a distinct relationship between the physical characteristics of the hydrated egg PL pro-liposome and the particle size of the liposomes in the final dispersion. Specifically, the clarity and thickness could be qualitatively used to estimate the fineness of the final dispersion. Clear and thick hydrated egg PL pro-liposomes yielded dispersions with fewer large liposomes. In contrast, opaque hydrated fluid egg PL pro-liposomes produced dispersions containing a greater number of larger sized liposomes. To generate a fine egg PL liposome dispersion, the ratio of egg PL pro-liposome to deionised water for the first stage of hydration had to be finely balanced (Table 3.9 and Table 3.10). This was achieved by Eppendorf mixing 1.265 g (1.100 g +15% overage) of egg PL pro-liposome with between 0.316 g (0.275 g +15% overage) and 0.633 g (0.550 g +15% overage) of deionised water twenty times. Thoroughly mixing this amount of deionised water with the pro-liposome enabled the egg PL to be precipitated into organised stacks of bilayers with an evenly distributed high concentration of glycerol. This entrapped glycerol created the large osmotic force which aided the
reduction in liposome size when the gel was added to the remaining bulk deionised water. The freeze fracture of the dispersion generated by hydrating the pro-liposome with half the weight of deionised water is shown in Plate 3.9. As can be seen from Plate 3.9, all liposomes were below 1 μm and most were between 200-250 nm in diameter.

If the pro-liposome: deionised water weight ratio was outside this range of 1.100:0.275 to 1.100:0.550, the benefit of the hydration stage was reduced. If insufficient deionised water was added, e.g. 0.100 g of deionised water to 1.100 g of egg pro-liposome, the resultant pro-liposome was clear but remained fluid and therefore possessed little bilayer structure. The absence/presence of bilayers in similar types of pro-liposomes has been elegantly demonstrated by Perret (1993) using $^{31}$P- NMR. Due to this inadequate bilayer precipitation, this two stage conversion, employing 0.100 g of water for hydration, resembled a one stage conversion. It was, therefore, unsurprising that the resultant dispersions had significantly higher numbers of particles greater than 5 μm compared to dispersions converted in two stages from egg PL pro-liposomes (0.550 g) hydrated with 1.100 g of deionised water (p = 0.03). The coarser nature of the dispersions converted from 1.100 g of pro-liposomes hydrated with 0.100 g of deionised water was also reflected by the significantly higher average D-90 value of 4.85 ± 0.02 μm (p = 6x10^{-6}).

If too much deionised water (≥ 0.875 g) was added to the pro-liposome (1.100 g), some large vesicular structures were generated. These structures were probably unable to be reorganised/redissolved even after mixing, because the ethanol level in the overall mix would have been too low. This probably explains why 1.100 g of pro-liposome mixed with more than 0.875 g of deionised water appeared opaque, even after mixing thoroughly. Upon dilution these large liposomes would have contributed to a proportion of the larger sized particles. The presence of larger sized liposomes was confirmed by examining the freeze fracture of the egg PL dispersion converted from a pro-liposome (1.100 g) hydrated with an equal weight of deionised water (Plate 3.10). Though many liposomes were smaller than 500 nm in diameter, a proportion of liposomes with a diameter larger than 1 μm were detected.

For subsequent sizing studies involving two stage conversion the pro-liposome: deionised water for hydration weight ratio was maintained at 1.100 g: 0.555 g, because this ratio generated liposome dispersions with fewer larger sized liposomes.
Plate 3.9 Typical freeze fracture replica of egg PL liposome dispersion converted in two stages by addition of half the weight of deionised water to pro-liposome and subsequent addition to bulk deionised water (Bar = 1 μm), as described in section 3.5.4.2

Plate 3.10 Typical freeze fracture replica of egg PL liposome dispersion converted in two stages by addition of an equal weight of deionised water to pro-liposome and subsequent addition to bulk deionised water (Bar = 1 μm), as described in section 3.5.4.2

3.5.4.3 Turbidity studies of diluted dispersions converted in two stages

The turbidity after dilution was examined by separately diluting the dispersions converted in two stages in deionised water and in 50 mg/g glucose. Figure 3.3 showed that the turbidities of diluted liposome dispersions converted in two stages did not fluctuate when added to either 50 mg/g glucose or deionised water. This probably
suggested that two stage conversion had encouraged the full precipitation of the PL and osmotic rupture was not occurring to a large extent upon further dilution in deionised water.

![Graph showing turbidity over time](image)

**Figure 3.3** Turbidity of egg PL dispersion converted in two stages after diluting x100 fold in 50 mg/g glucose and deionised water (Each point is the mean ± s.d., n=3)

### 3.5.4.4 Effect of agitation during dispersion stage

Egg PL pro-liposome was hydrated by the addition of deionised water and mixed by passing both components between two Eppendorf syringes twenty times. The resultant hydrated pro-liposome was dispersed in the bulk deionised water at room temperature either by vigorously handshaking for 90 seconds or by leaving the pro-liposome to disperse without any agitation. The particle size results for these two sets of dispersions are shown in Table 3.11.

<table>
<thead>
<tr>
<th></th>
<th>Hydrated egg PL PRO dispersed by vigorous agitation</th>
<th>Hydrated egg PL PRO left to disperse without agitation</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-90 s.d. (µm)</td>
<td>0.94±0.00</td>
<td>1.10±0.17</td>
</tr>
<tr>
<td>Volume of sample added to LD (µl)</td>
<td>60</td>
<td>45</td>
</tr>
<tr>
<td>Microscope assessment</td>
<td>Fine</td>
<td>Moderately coarse</td>
</tr>
<tr>
<td>No. of particles ≥ 20µm ml⁻¹</td>
<td>1.8x10⁵±1.2x10⁴</td>
<td>6.4x10⁵±1.0x10⁵</td>
</tr>
<tr>
<td>No. of particles ≥ 10µm ml⁻¹</td>
<td>2.8x10⁴±0.5x10⁴</td>
<td>7.1x10⁴±4.8x10⁴</td>
</tr>
<tr>
<td>No. of particles ≥ 5µm ml⁻¹</td>
<td>5.6x10³±2.1x10³</td>
<td>2.9x10³±1.1x10³</td>
</tr>
<tr>
<td>Time taken for hydrated egg PL PRO to disperse (min)</td>
<td>NA</td>
<td>15-20 minutes</td>
</tr>
</tbody>
</table>
The average D-90 value of liposomes formed by dispersing the gels in bulk deionised water by handshaking vigorously for 90 seconds (0.94 μm) was not significantly lower than the average D-90 value of dispersions generated without agitation (1.10 μm) \( (p = 0.076) \). Interestingly, the volume required to obtain an obscuration of 0.2 in the laser diffraction cell was consistently 25% less for the dispersion left to disperse without shaking than the dispersion formed by vigorously shaking for 90 seconds. This suggested that there were a greater number of large particles in this unshaken dispersion compared to the dispersions converted by vigorous shaking for 90 seconds. This fact was demonstrated quantitatively by the Coulter Counter, which significantly counted 6400 ± 1000 particles greater than 20 μm ml\(^{-1}\) in the vigorously shaken dispersions compared to only 1800 ± 1200 particles greater than 20 μm ml\(^{-1}\) in the dispersions left to disperse without agitation \( (p = 0.014) \).

Although shaking significantly reduced the number of large liposomes (greater than 20 μm ml\(^{-1}\)) generated by a two stage conversion, the influence was relatively small when compared to the number of particles generated from one stage conversion. Without agitation, the number of liposomes greater than 20 μm was 6400 ± 1000 per ml if two stage dilution was adopted (Table 3.11). In comparison, if one stage conversion without agitation was adopted, the number of particles greater than 20 μm would have been 6.0x10^6 ± 2.3x10^6 per ml (Table 3.2). This clearly demonstrated the benefit of the two stage conversion; it produced dispersions with fewer larger sized liposomes. Moreover, the number of larger sized liposomes in dispersions converted in two stages was considerably less influenced by the effects of shaking compared to the dispersions generated by one stage conversion.

Under the light microscope no liposomes greater than 10 μm in diameter were observed in 2-3 μl of any of the dispersions converted in two stages from pro-liposomes mixed and hydrated with deionised water in a 2:1 w/w ratio. The only particles in this size range and above were fibres and non-lipid debris. These contaminants were probably introduced into the liposome dispersion during the handling. In the context of the Coulter Counter, these contaminants were not important if the liposome dispersion was very coarse, because the number of non-liposome particles was small compared to the number of liposomes. However, if the concentration of large liposomes in the dispersion was small, the presence of non-lipid debris may have obscured the true concentration of
particles in the dispersion. Therefore, for maximum accuracy the liposome production should ideally be carried out with aseptically filtered components under aseptic and particle free conditions. Ideally, the Coulter Counter measurements should be also be carried out under laminar flow conditions.

3.5.4.5 Effect of lipid composition on liposomes converted in two stages

3.5.4.5.1 Comparing the particle size of liposome dispersions generated in two stages from purified egg PC and purified soya PC

The size of liposomes composed of soya bean PC and egg yolk PC was compared by sizing liposomes converted in two stages from purified soya PC pro-liposomes and purified egg PC pro-liposomes. The particle size data for these two sets of dispersions are compared in Table 3.12.

<table>
<thead>
<tr>
<th></th>
<th>Hydrated purified egg PC PRO dispersed in deionised water by vigorous agitation (n=5)</th>
<th>Hydrated purified soya PC PRO dispersed in deionised water by vigorous agitation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z average ± s.d. (nm)</td>
<td>265.6±12.5</td>
<td>251.3±3.1</td>
</tr>
<tr>
<td>P.I. ± s.d.</td>
<td>0.154±0.014</td>
<td>0.122±0.008</td>
</tr>
<tr>
<td>D-90 ± s.d. (μm)</td>
<td>1.26±0.14</td>
<td>1.07±0.22</td>
</tr>
<tr>
<td>Volume of sample added to LD (μL)</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Microscope assessment</td>
<td>Moderately coarse</td>
<td>Moderately coarse</td>
</tr>
</tbody>
</table>

The z average values and D-90 values of the two sets dispersions generated from high purity egg and soya PC appeared to be indistinguishable from one another. As can be seen from the laser diffraction results in Table 3.12, both purified lipids produced liposome dispersions with D-90 values close to the limit of laser diffraction detection. The D-90 values of 1.26 and 1.07 μm for the egg PC and soya PC dispersions respectively were not significantly different (p = 0.166). Light microscopy also indicated the presence of only a few larger sized liposomes. These sizing results suggested that the difference in the degree of unsaturation/fatty acid profile did not significantly influence the particle size of the liposome dispersion under the conditions tested.

One property of the purified PC dispersions which differed from the liposome dispersions used thus far was their turbidity upon dilution. Dilution of these high purity PC liposomes in either 50 mg/g glucose (Figure 3.4) or deionised water (Figure 3.4) resulted in detectable increases in turbidity. These increases in turbidity suggested that aggregation of the liposomes was occurring after dilution in both types of media. This aggregation event was confirmed by the fact that the addition of Tween 80 to 50 mg/g
glucose prevented this turbidity increase (Figure 3.4). Since this aggregation behaviour was not observed with the egg PL (Figure 3.2) and soya PL, it indicated that the non-PC lipids, such as other neutral lipids in egg PL or charged lipids in soya PL, prevented this aggregation.

Figure 3.4 Turbidity of purified egg PC dispersion converted in two stages after diluting x100 fold in 50 mg/g glucose, deionised water and 50 mg/g glucose containing 0.006 mg/g Tween 80 (Each point is the mean ± s.d., n = 3)

3.5.4.5.2 Effect of replacing soya PC with soya bean PL

The effect of replacing varying quantities of soya PC with equivalent weights of soya bean PL was investigated using dispersions converted in two stages. The two stage conversion was carried out in the usual manner by thoroughly mixing the pro-liposomes with deionised water to generate hydrated gels. These gels were dispersed in bulk deionised water by vigorously handshaking in bulk deionised water for 90 seconds. The particle size data for these sets of liposome dispersions are shown in Table 3.12 and Table 3.13.
Chapter three-Particle size of liposome dispersions

Table 3.13 Effect of soya PL content on the particle size of liposomes converted in two stages

<table>
<thead>
<tr>
<th>Soya PC: soya PL w/w</th>
<th>Z-average ± s.d. (nm)</th>
<th>P.I. ± s.d.</th>
<th>D-90 ± s.d. (μm)</th>
<th>Volume added to LD cell (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100:0</td>
<td>257.6±14.9</td>
<td>0.191±0.004</td>
<td>0.96±0.03</td>
<td>25</td>
</tr>
<tr>
<td>93:7</td>
<td>209.7±22.1</td>
<td>0.160±0.043</td>
<td>0.94±0.00</td>
<td>60</td>
</tr>
<tr>
<td>90:10</td>
<td>201.2±2.3</td>
<td>0.153±0.021</td>
<td>0.94±0.00</td>
<td>75</td>
</tr>
<tr>
<td>75:25</td>
<td>231.9±5.0</td>
<td>0.161±0.018</td>
<td>0.99±0.04</td>
<td>50</td>
</tr>
<tr>
<td>50:50</td>
<td>277.8±9.3</td>
<td>0.201±0.018</td>
<td>1.34±0.01</td>
<td>50</td>
</tr>
<tr>
<td>25:75</td>
<td>307.7±6.6</td>
<td>0.220±0.021</td>
<td>1.39±0.03</td>
<td>60</td>
</tr>
<tr>
<td>0:100</td>
<td>309.8±10.1</td>
<td>0.234±0.019</td>
<td>1.48±0.00</td>
<td>60</td>
</tr>
</tbody>
</table>

Table 3.14 Effect of soya PL content on the macroscopic and microscopic appearance of various soya dispersions

<table>
<thead>
<tr>
<th>Soya PC: soya PL w/w</th>
<th>Physical appearance of liposome dispersion after mixing</th>
<th>Microscope assessment after isotonic dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>100:0</td>
<td>Opalescent infusable dispersion</td>
<td>Coarse</td>
</tr>
<tr>
<td>93:7</td>
<td>Opalescent infusable dispersion</td>
<td>Fine</td>
</tr>
<tr>
<td>90:10</td>
<td>Opalescent infusable dispersion</td>
<td>Fine</td>
</tr>
<tr>
<td>75:25</td>
<td>Flowable gel</td>
<td>Moderately coarse</td>
</tr>
<tr>
<td>50:50</td>
<td>Clear viscous gel</td>
<td>Moderately coarse</td>
</tr>
<tr>
<td>25:75</td>
<td>Clear viscous gel</td>
<td>Moderately coarse</td>
</tr>
<tr>
<td>0:100</td>
<td>Clear viscous gel</td>
<td>Moderately coarse</td>
</tr>
</tbody>
</table>

From the studies involving one stage conversion, it was evident that raising the soya PL content reduced the liposome size if the energy input was standardised (Table 3.7). However, a somewhat different effect was observed if a two stage conversion was adopted. In contrast, the size reduction of the two stage conversion was compromised if the soya PL level of the liposomes was ≥ 25% (Table 3.13 and Table 3.14). This increased coarseness was confirmed by light microscopy, which revealed the presence of large liposomes upon diluting the dispersions in isotonic media. It seemed that raising the soya PL content of the dispersion encouraged the generation of coarser dispersions. The coarseness and appearance of the dispersions may be attributed to the presence of non-PC lipids present in soya PL, e.g. the charged acidic phospholipids. As described in section 3.5.2.3.2, these charged components encouraged the spacing out of bilayers to form vesicles with a fewer number of bilayers. However, if the level of charge was too high, at a total lipid concentration of 60 mg/g, it is possible that the vesicles with fewer number of bilayers can not be packed within the aqueous volume of the dispersion. Hence, unconverted pro-liposome structures probably co-existed with liposomes. Only upon further dilution were these pro-liposomes fully converted into liposomes. These liposomes formed upon further dilution in 50 mg/g glucose were larger than those already generated, because there was no osmotic stress exerted on the pro-liposomes when diluted in 50 mg/g glucose.
Chapter three-Particle size of liposome dispersions

The dispersions with the smallest z average value were obtained when the soya bean PC: soya bean PL weight ratio of the pro-liposome was 90:10. The z average of the dispersions with this particular soya PC: soya PL blend was significantly lower than the dispersions with a soya PC: soya PL weight ratio of 93:7 (p = 0.01) and the dispersions with a soya PC: soya PL ratio of 75:25 (p = 0.001).

It was also evident from the light microscopy, laser diffraction and PCS results that liposomes converted in two stages using the pro-liposome with a soya PC: soya PL weight ratio of 90:10 generated smaller sized particles than the oil droplets present in the TPN concentrate. The z average of these liposomes was approximately 200 nm (Table 3.13), which was approximately 50 nm smaller than the oil droplets of the TPN dispersion (Table 3.1). The D-90 value of the dispersions made from the 90:10 soya PL blend was 0.94 ± 0.00 μm, which was slightly lower than the D-90 value of 0.97 ± 0.01 μm for the TPN emulsion. These results were confirmed by light microscopy, which showed that there were very few large particles present in the both sets of dispersions.

b) Coulter Counter results of soya PL blend converted in two stages

The soya PL blend with a soya PC: soya PL weight ratio of 90:10 was selected for further particle size evaluation by Coulter Counter (Table 3.15).

<table>
<thead>
<tr>
<th>No. of particles ≥20μm ml⁻¹</th>
<th>Hydrated 90:10 soya PL blend PRO dispersed by vigorous handshaking</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.5x10⁶±0.7x10⁵</td>
</tr>
<tr>
<td>No. of particles ≥10μm ml⁻¹</td>
<td>1.1x10⁶±0.4x10⁵</td>
</tr>
<tr>
<td>No. of particles ≥5μm ml⁻¹</td>
<td>2.4x10⁶±5.6x10⁵</td>
</tr>
</tbody>
</table>

The number of particles present in the soya PL blend dispersions (Table 3.15) was similar to the number of droplets in the TPN sample (Table 3.1) in all three oversize bands. Further informative results would be obtained if the dispersions were manufactured and sized under aseptic conditions employing a smaller Coulter Counter capillary.

3.5.4.5.3 Effect of including egg phosphatidylglycerol (EPG)

Two levels of EPG were incorporated into soya PC pro-liposomes at soya PC: EPG weight ratios of 99.5:0.5 and 97.5:2.5. Both pro-liposomes were hydrated in the usual manner and dispersed by vigorous handshaking. The particle size data of these dispersions are compared in Table 3.16.
Chapter three-Particle size of liposome dispersions

As discussed in chapter two, the sodium salt of EPG was selected, because of its favourable toxicity profile and its appropriately low phase transition temperature. The inclusion of EPG at a soya PC: EPG weight ratio of 97.5:2.5 resulted in a thickening of the pro-liposome, even before the addition of deionised water for hydration. This increase in viscosity was likely to be due to the low solubility of EPG, which encourages the precipitation of a partially bilayered system (Perret, 1993). This partial structuring made handling difficult, particularly if the pro-liposome had to be filtered aseptically at room temperature.

The limited solubility of the charged lipid in ethanol also restricted the maximum quantity which could be incorporated into this pro-liposome. An EPG level exceeding the amount present in the soya PC: EPG weight ratio of 97.5:2.5 could not be satisfactorily dissolved in this particular pro-liposome formulation. This was probably due to the ionic structure of the EPG salt, limiting its solubility.

Coulter Counter could not be employed to count the number of large sized liposomes in dispersions generated in two stages from pro-liposomes containing EPG, because there were so few large liposomes. Similar dispersions sized previously (Table 3.15) and the fine appearance under the light microscope indicated that fine soya PC dispersions containing EPG would not have yielded meaningful results. Measured under non-laminar flow conditions, it was likely that non-lipid debris would have interfered with the Coulter Counter readings. The z averages of the two sets of dispersions with soya PC: EPG weight ratios of 99.5:0.5 and 97.5:2.5 were not significantly different (p = 0.386). It appeared that using a two stage conversion, the addition of only a small level about 0.5% EPG was sufficient to reduce the size of the liposomes to around 200 nm.

| Table 3.16 Effect of EPG inclusion on soya PC liposomes converted in two stages |
|--------------------------------|-----------|-----------|
| Soya PC:EPG weight ratio in pro-liposome | 99.5:0.5 | 97.5:2.5 |
| Z average ± s.d. (nm) | 197.5±4.4 | 190.8±6.3 |
| P.I. ± s.d. | 0.140±0.002 | 0.137±0.083 |
| D-90 ± s.d. (μm) | 0.94±0.00 | 0.94±0.00 |
| Volume added to LD cell (μl) | 90 | 90 |
| Microscope assessment | Fine | Very Fine |
3.6 Conclusions

Two factors significantly affected the particle size distribution of liposomes generated from pro-liposomes in one stage: the lipid composition and the energy input. A high proportion of soya bean PL and vigorous handshaking both encouraged the formation of liposomes with a smaller particle size. The inclusion of soya PL in the pro-liposome significantly reduced the particle size of the resultant liposomes. Dispersions generated from soya PL pro-liposomes had an average D-90 value of 1.56 \( \mu \text{m} \). However, the resultant soya PL dispersions were gel-like and thus probably unsuitable for intravenous administration. Furthermore, the particle size of the liposomes was significantly dependant upon the degree of agitation. Without agitation the average D-90 value of the dispersions generated in one stage from soya PL pro-liposomes and egg PL pro-liposomes was approximately 60 \( \mu \text{m} \) and 10 \( \mu \text{m} \) higher than the corresponding dispersions converted with vigorous intensity. As a consequence of this dependency on energy input, the particle size of dispersions was variable if the energy input was variable. Hence, dispersions generated in one stage would probably be unsuitable for production at the site of use, irrespective of the phospholipid composition, unless the energy input during conversion could be standardised.

In contrast, thoroughly mixing and hydrating the pro-liposome with deionised water prior to dispersing in bulk deionised water, dramatically reduced the particle size of liposomes and the concentration of large liposomes in the dispersion. However, careful optimisation of the amount of deionised water for hydration and thorough mixing of the first stage were crucial for optimal size reduction. Thoroughly mixing 1.100 g of pro-liposome with 0.550 g of deionised water for hydration by passing these components through two Eppendorf syringes twenty times generated a clear firm hydrated pro-liposome. This clear hydrated pro-liposome gel generated a fine liposome dispersion upon dispersion in bulk deionised water. The average liposome size was equal to or smaller than the average size of an emulsion droplet (approximately 260 nm in diameter) used in TPN feeds. However, the number of larger sized liposomes in two stage converted dispersions was still significantly affected by the degree of agitation. Without agitation, the concentration of large liposomes with diameters greater than 20 \( \mu \text{m} \) was significantly higher than in dispersions which were converted with vigorous
handshaking. However, in consideration of one stage conversion, this increase in the number of particles was relatively small. The \( z \) average diameter of these dispersions could be reduced further by optimising the lipid compositions: the individual inclusion of soya PL at a soya PC: soya PL weight ratio of 93:7 and 90:10 produced liposomes with a \( z \) average of approximately 200 nm. Similar sized liposomes could be generated if EPG was employed at a soya PC: EPG weight ratio of 99.5:0.5. However, the number of large sized liposomes in these dispersions could not be meaningfully assessed using the Coulter Counter with a 100 \( \mu \)m diameter capillary, due to the small number of large sized liposomes present.
Chapter four

Stability of unsaturated phospholipids
4.1 Introduction

4.1.1 Stability

Stability is a broad term relating to the resistance of a product to various chemical, physical and microbiological events, which may change the properties of the preparation over time (Pharmaceutical Codex, 1994c). It should be emphasised that stability not only relates to the active principle, but also to the product as a whole, including any excipients in the formulation.

The stability of a pharmaceutical may have profound ramifications on the three criteria used by regulatory authorities to assess the acceptability of a medicine for human use. Firstly, the efficacy of the active component may be adversely affected by chemical changes. Hence the regulations state that the amount of active component in the preparation has to remain within narrow limits of the original stated level throughout the shelf life of the product. Secondly, the safety may be compromised if any breakdown products in the preparation are toxic. In such cases the potentially toxic degradation products within the formulation will have to be tested and examined closely. Thirdly, poor stability affects the overall quality of the product.

The resistance of the product to all these events will dictate its shelf life. If a product is sensitive to chemical or physical change it may be necessary to adopt special storage conditions in order to extend its shelf life, e.g. refrigeration.

4.1.2 Chemistry of phospholipid breakdown

Phospholipid (PL) containing unsaturated fatty acids is susceptible to two types of chemical degradation: hydrolysis and oxidation.

4.1.2.1 Phospholipid hydrolysis

A simplified scheme of PL hydrolysis is provided in Figure 4.1. Any of the four ester bonds present in the PL molecule (Figure 2.1) may be hydrolysed by water. However, the ester bonds in the position 1 and 2 linking the fatty acids to the glycerol backbone are more susceptible to hydrolysis than the phosphate esters. The two single chained phospholipids formed by hydrolysis of either fatty acid chain are referred to as lyso-phospholipids (LPL). Hydrolysis of these ester bonds is catalysed in either an acidic or basic environment, and can occur if the esters are hydrated (Grit and Crommelin, 1993). An equilibrium state exists between these two isomeric breakdown products, which
favours the formation of the 1 acyl LPL. Migration of the acyl chain from 2 acyl LPL at alkaline pH encourages the formation of 1 acyl LPL in the molecular ratio of approximately 9 parts of 1-LPL to 1 part of 2-LPL (Plückthun and Dennis, 1982). LPLs are only intermediate products of PL hydrolysis, and further hydrolysis of these LPLs can continue by the removal of the remaining fatty acid to yield a glycerophosphosphero headgroup. The headgroup can be cleaved from this structure to yield glycerophosphoric acid. The hydrolysis of the final ester bond between the phosphoric acid and glycerol backbone tends to be exceedingly slow, and hence this glycerophosphoric acid structure is stable under pharmaceutically relevant conditions (Grit and Crommelin, 1993). Since phosphatidylcholine (PC) was the major constituent of the PLs under examination, only the hydrolytic breakdown products of PC were of concern in this investigation.

4.1.2.2 Phospholipid oxidation

The term autoxidation is frequently used to describe “oxidation occurring with atmospheric oxygen”. However, the term oxidation is preferred here, because it encompasses oxidative events not involving oxygen. Oxidation is a series of complex events which damage one or both fatty acids of the PL molecule. The events of oxidation predominate in unsaturated PL, because the double bonds of the fatty acid chains encourage the oxidation process. The key reactions of the oxidation process will be described under the following four sections, namely: conjugation, radical/peroxide formation, fatty acid fission and polymerisation.

i) Conjugation

This process results in the rearrangement of the double bond in fatty acids with two or more double bonds. In PL from natural sources these double or triple double bonds are arranged in an unconjugated fashion, i.e. a methylene group is spaced in between the double bonds. The rearrangement of the double bond occurs via the removal of a hydrogen atom from the unconjugated structure. This abstraction may occur without the participation of oxygen and results in the formation of a radical species. This radical is stabilised by the electron cloud of the double bonds, and can itself remove a hydrogen atom from another lipid molecule. However, the hydrogen will return to a different
carbon atom in order to adopt a more energetically favourable configuration. Hence the double bonds become conjugated, i.e. adjacent to one another.

Since this rearrangement commences in the initial stages of oxidation, detection of the two conjugated double bonds (dienes) and three conjugated double bonds (trienes) provides an indication that early oxidation is progressing.

**ii) Radical formation leading to peroxide formation**

A simplified scheme of the pathways involved in radical and peroxide formation is provided in Figure 4.2. For more detailed information regarding the mechanisms of peroxide formation and the structures of peroxides refer to Ando (1992). Radical formation occurs via the abstraction of a hydrogen ion from the fatty acid chain, which is encouraged by trace levels of transition metals and/or exposure to UV radiation. The free electron left behind, after this removal of a hydrogen ion, is stabilised by the double bonds of the fatty acid chain. These double bonds facilitate the delocalisation of the unpaired electron along the acyl chain. This is why radical formation is most likely to occur in polyunsaturated fatty acids, because the higher degree of unsaturation can support radical formation.

If molecular oxygen is present, further reactions can proceed via peroxide formation. Generally, two types of peroxide can be generated: hydroperoxides and heterocyclic peroxides (endoperoxides). These peroxide structures are created by the reaction between the free radical and molecular oxygen. The type of peroxide generated is largely dependant upon the structure of the unsaturated fatty acid. Compared to fatty acids with two double bonds, fatty acids with three or more double bonds are more likely to form endoperoxides. In these peroxide structures bound oxygen links two adjacent carbons to form a closed cyclic structure.

In contrast, fatty acid radicals with one or two double bonds are more likely to form hydroperoxides with oxygen. Structurally, the oxygen molecule of these hydroperoxides is attached to only one carbon atom, which is adjacent to a double bond. In fatty acids with two double bonds, the endoperoxide is not energetically favoured, because the formation could only occur if the conjugation is sacrificed.
Figure 4.1 Generalised scheme showing hydrolysis reactions of PL
(Taken from Grit et al., 1993)

Figure 4.2 Formation of peroxides in polyunsaturated fatty acid chains
(Taken from New, 1990b)
The detection of either the hydroperoxide or the endoperoxide is the basis of most stability assays. The hydroperoxide is usually detected by the oxidation of iodide into iodine, which can be measured colorimetrically. This principle is the basis of the Peroxide Value, which is the only Pharmacopoeial standard used to assess the oxidative status of the lipid. Officially, it is defined as “the milliequivalent of peroxidatively bonded oxygen found in 1.000 g of the sample”.

iii) Fatty acid fission
Fission of the fatty acid occurs as a result of the spontaneous break up of peroxide radicals. The decomposition products generated depend upon the type of fatty acid peroxide. Endoperoxides release aldehydes such as malondialdehyde (MDA), which shortens the fatty acid chain length by 2 carbons. The detection of this secondary product, MDA, is the basis of endoperoxide assays. The liberated MDA is reacted with thiobarbituric acid to yield a red pigment, which can be measured spectrophotometrically.

The decomposition of the hydroperoxide fatty acid can also result in the liberation of a variety of carbonyl cleavage structures (Arakane et al., 1995). The generation of these secondary products of oxidation contributes to the rancid olfactory changes which occur as lipid oxidation proceeds.

iv) Polymerisation
Polymerisation occurs when fatty acid radicals of separate PL molecules combine. This results in the termination of the radicals and the joining together of the individual PL molecules. However, if double bonds are still present in the fatty acids after polymerisation, the fatty acids may still be susceptible to further peroxidation, and hence continued PL polymerisation can occur.

4.1.3 Prevention of oxidation
The extent and rate of oxidation can be reduced by the inclusion of a variety of protectants (Yamamoto, 1985). These protectants include various antioxidants and chelating agents. However, for intravenous formulations, the employment of most of these antioxidants is restricted. The antioxidant which can be routinely used is \( \alpha \) tocopherol (commonly referred to as vitamin E). The protective effects conferred by the tocopherol antioxidants were not studied here, because only the behaviour of the PLs per se were under investigation.
Chapter Four-Stability of unsaturated phospholipids

4.1.4 Chemical stability of headgroups
There have been few reports published assessing the chemical stability of PL headgroups. This is probably because the most commonly used lipid is PC, which is physically stable. Lipids, such as phosphatidylethanolamine (PE) and phosphatidylserine (PS), which may be more susceptible to chemical changes are used relatively infrequently. The amine groups of these PLs can undergo Maillard type reactions with saccharides to form brown coloured complexes (Barenholz and Amselem, 1992). These reactions have been extensively studied in the food industry, and sometimes occur in other pharmaceutical contexts. For instance in TPN mixtures, browning can occur unless the amino acids, saccharides and emulsion are separated until just prior to administration.

4.1.5 Concerns associated with PL breakdown products
The presence of PL breakdown products in pro-liposome formulations is of concern for three reasons: toxicity, liposome integrity and drug stability.

4.1.5.1 Toxicity
i) Peroxides
Fresh PLs are well tolerated in vivo even when administered in large doses. However, this tolerability may be compromised if the PL contains degraded material. Indirect evidence from TPN studies has suggested that in certain clinical situations, parenteral administration of peroxides may induce deleterious effects (Helbock et al., 1993). Although deliberate administration of oxidised material is clearly undesirable, the toxic adverse effects may be reduced if the degraded fatty acid is still associated with the PL. If the fatty acid is free, it seems to be more toxic in vitro endothelial cell lines. Additionally, there appears to be differential toxicity between the unsaturated fatty acid peroxides: arachadonic acid peroxides are more cytotoxic when exposed to endothelial cells, compared to the shorter chain linoleic fatty acids (Kaneko et al., 1994).

ii) LPC
Experimentally, LPC has been associated with macrophage suppression and haemolysis (Brandl et al., 1994; Lutz et al., 1995). However, the level of LPC required to induce haemolysis is relatively high (Gjone, 1961; El-Harari et al., 1992). From in vitro studies it can be estimated that approximately 30% w/w of PL has to be hydrolysed to LPL before this single chain surfactant is haemolytically active (Zuidam et al., 1995).
Furthermore, a level of LPL below 10% w/w is unlikely to present any hazards systemically. This indirect evidence is provided from the clinical experience with PLs in TPN: 10% of these PLs are present as LPLs and further degradation products are detectable, indicating that considerable PL hydrolysis has occurred (Westesen and Wehler, 1992). Despite the presence of these hydrolytic products, these preparations which are often administered chronically in large quantities (more than 5 g of PL daily), appear to be well tolerated in vivo.

4.1.5.2 Liposome integrity

It is evident from turbidity studies that hydrolytic instability affects liposome integrity if the LPC and fatty acid contents exceed critical levels (Zuidam et al., 1995). The precise levels are dependant upon the PL composition and the acyl chain lengths of the PLs. However, if the bilayer is in a fluid state, and the amounts of fatty acids and LPLs exceed critical levels, mixed micelle structures will start to replace the disintegrating liposome structures.

Hydrated pro-liposomes and their resultant liposome dispersions are also likely to be affected in a similar manner, because both structures employ bilayered states. Therefore monitoring and controlling the components of hydrolysis is critical for the quality of the pro-liposomes and the resultant liposome dispersions.

4.1.5.3 Drug stability

No reports have been published on the possible interaction between oxidised products and the "active" component in liposome formulations. However, the highly reactive peroxide radicals may attack the drug entity, thereby adversely oxidising the organic structure of the water insoluble lipophile.

Therefore to minimise the problems associated with toxicity, liposome integrity and potential drug oxidation, it is clear that the level of oxidative and hydrolytic breakdown species should be kept to a minimum.

4.1.6 Reported investigations into phospholipid stability

In order to assess the suitability of PLs for parenteral applications, their chemical and physical stabilities have to be determined. Although there have been many reports in the literature investigating stability (Hernandez-Caselles and Gomez Fernandez, 1990; Grit and Crommelin, 1993; Lang and Vigo-Pelfrey, 1993), most studies have centred around
the stability of PC in aqueous liposome dispersions. It is evident from these reports that the presence of water in such systems may encourage PC degradation, particularly hydrolysis. Therefore, the widespread pharmaceutical use of these pre-formed liposome dispersions may be limited by the formation of PC breakdown products during storage.

The work reported here differs in two ways from the work which has been reported in the literature. Firstly, in this study two PLs incorporating PE and some non-phosphorus containing lipids were investigated in addition to soya PC and egg PC. Secondly, the three presentations of each of the four lipids under examination were anhydrous. In this context, the term anhydrous is used loosely to describe the deliberate omission of water from the formulations. It does not imply that the residual water present in the raw materials is absent. The rationale for this omission of water was to generate formulations with low water content so that the hydrolytic processes which typically occur in aqueous systems could be slowed.

4.1.7 Aims of the stability study

The purpose of this study was to assess the stability characteristics of the pro-liposomes. Therefore investigations were carried out without an active principle. Two separate sets of investigations were carried out. The first study investigated the behaviour of four commercial PL compositions (described in chapter two), in solid form, in an ethanol pro-liposome and in a glycerol containing pro-liposome under stress storage conditions at 55 °C. The rationale of these experiments was to determine the relative stabilities of the four lipids in the three different anhydrous forms. Stress storage conditions were chosen in order to deliberately hasten the decomposition processes, thereby enabling comparisons to be made rapidly.

The second objective of this stability study was to ascertain the oxidative stability of a pro-liposome formulation containing glycerol. This was achieved by investigating the effect of an accelerated stability test at 40 °C, storage at 20 °C and 4 °C on one specific soya PL pro-liposome containing glycerol during a 16 week period. The pro-liposome containing glycerol used in this particular study employed a blend of soya phospholipids, optimised for achieving small particle size (section 3.5.4.5.2). This particular pro-liposome is referred to in this text as the soya PL blend pro-liposome.
4.2 Materials

4.2.1 Lipids under investigation
Epikuron 145 (referred to as soya PL in text), B.N. 1-5-9005, Lucas Mayer, Hamburg, Germany.
Epikuron 200 (referred to as soya PC in text), B.N. 1-4-9018, Lucas Mayer, Hamburg, Germany.
Ovothin 180 (referred to as egg PL in text), B.N. 1-4-9240, Lucas Mayer, Hamburg, Germany.
Ovothin 200 (referred to as egg PC in text), B.N. 1-4-9248, Lucas Mayer, Hamburg, Germany.

4.2.2 Materials for conjugation assays
Absolute ethanol, AnalAr, B.N. various, BDH Chemicals Ltd., Poole, UK.

4.2.3 Materials for peroxide assay and standard curve
Acetic acid, AnalAr, B.N. K21773517 514, BDH Chemicals Ltd., Poole, UK.
Cadmium acetate, 98%, B.N. MF 06203EF, Aldrich Chemical Co., USA.
Chloroform, AnalAr, B.N. K22453741 550, BDH Chemicals Ltd., Poole, UK.
Cumene hydroperoxide, 80% technical grade, B.N. 0263675, Aldrich Chemical Co., USA.
Potassium iodide, (KI) 99% A.C.S, B.N. PF 06605 DF, Aldrich Chemical Co., USA.

4.2.4 Materials for TBARS and TBARS(F) assays and standard curves
Acetic acid, AnalAr, B.N. K21773517 514, BDH Chemicals Ltd., Poole, UK.
Anhydrous iron (III) chloride (FeCl\textsubscript{3}), GPR, B.N. 9562570K, BDH Chemicals Ltd., Poole, UK.
Butylhydroxytoluene (BHT), B.N. X, Sigma Chemical Co., USA.
Chloroform, AnalAr, B.N. K22453741 550, BDH Chemicals Ltd., Poole, UK.
1,1,3,3-Tetraethoxypropane (TEP), B.N. 123H2623, BDH Chemicals Ltd., Poole, UK.
Sodium lauryl sulphate, Laboratory reagent, B.N. 0977880, BDH Chemicals Ltd., Poole, UK.
Thiobarbituric acid, (TBA) B.N. 44H2621 & B.N. 105H3418, Sigma Chemical Co., USA.
Glycine, AnalAr, B.N. 1939420, BDH Chemicals Ltd., Poole, UK.
4.2.5 Materials for $^{31}$P-NMR spectroscopy
Chloroform-$d$, B.N. 00423MF, Aldrich Chemical Co., USA.
Triethylphosphate, B.N. 123H2623, Sigma Chemical Co., USA.

4.3 Methods

4.3.1 Manufacture of samples
The four commercial lipids evaluated in chapter two were studied without further purification in solid form and in two pro-liposome formulations. The solid lipid samples were transferred directly into glass ampoules and sealed. Pro-liposomes were produced by the method described in 3.3.2. The first pro-liposome formulation was an ethanol solution of PL (referred to as the ethanol pro-liposome), and the second formulation was an ethanol PL solution with glycerol (referred to as the glycerol pro-liposome).
Both pro-liposomes were produced in 50 ml glass Schott bottles: approximately 40 g of ethanol pro-liposome was produced by dissolving 30.0 g of the appropriate lipid in 12.0 g of absolute ethanol. Similarly, the glycerol pro-liposome was produced by dissolving 30.0 g of the appropriate lipid in 12.0 g absolute ethanol, prior to the addition of 13.0 g of glycerol. The weight ratios of the components used in this glycerol pro-liposome were identical to the ratios used for the pro-liposome examined in chapter three. All samples (approximately 4-5 g) were packaged under nitrogen into individual 5 ml clear glass ampoules, which were subsequently heat sealed.
The soya PL blend pro-liposome used for the accelerated and long term storage tests was produced in a 100 ml glass Schott bottle by dissolving 54.0 g of soya PC and 6.0 g of soya PL in 24.0 g of absolute ethanol, prior to the addition of 26.0 g of glycerol (section 3.4.5.4.2).

4.3.2 Analysis of oxidised phospholipid
Even after chromatographic purification, egg and soya PC are heterogeneous molecular mixes (Desai, 1996; section 2.4). Each PC comprises 20 molecular species of PC with different fatty acids. This diversity makes detailed analysis of the molecular breakdown species extremely difficult. The common assay techniques provide an indication of the general oxidative status of the PL, but do not assess the oxidative condition of the individual molecules.
As a result of the complex processes involved in oxidation, no single test provides an absolute indication of PL stability. A variety of tests were therefore employed in order
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to detect the different stages of PL oxidation. The tests and assays used in these investigations are described in detail in the following section:

4.3.2.1 Conjugated diene and triene assay
This test was one of the most informative and simple oxidative tests to perform. It involved the detection of conjugated double bonds using UV spectroscopy (Klein, 1970). The measurement of PL absorbance at wavelengths of approximately 230 nm and 275 nm provided an indication of the conjugated diene and triene level respectively. The exact wavelength for the maximum peak varied slightly from lipid to lipid, suggesting that different positioning of the double bonds could alter the precise wavelength at which the diene and triene peaks absorb. A similar observation was also noticed by Memoli et al. (1993).

In order to assess the relative changes in diene and triene levels with time, these absorbances had to be related to the total level of PL assayed. This was achieved by dividing the absorbance of the conjugated diene or triene by the absorbance of PL at 215 nm. These two ratios are referred to as the conjugated diene oxidation index and the conjugated triene oxidation index respectively.

Sufficient sample containing 5-10 mg of PL was accurately weighed into a 10 ml volumetric flask, dissolved and made up to 10 ml with absolute ethanol. The absorbance of this ethanolic PL solution was recorded over the wavelength 300-200 nm in matched cuvettes against absolute ethanol as the blank using a Perkin Elmer 554 UV spectrophotometer. The wavelengths for the maximum absorbance of the diene and trienes were verified before the absorbance at the wavelengths 215 nm, 230 ± 6 nm, 275 ± 6 nm was measured. To calculate the conjugated diene oxidation index the maximum absorbance of the ethanolic PL at 230 ± 6 nm was divided by the absorbance at 215 nm. Similarly, the conjugated triene oxidation index was calculated by dividing the maximum absorbance at 275 ± 6 nm by the absorbance at 215 nm.

4.3.2.2 Hydroperoxide assay
Traditionally, titrimetric assays are used to measure the hydroperoxide value of PLs. However, in order to detect low levels of peroxidation with the titrimetric assay, large quantities of PL (>6 g) is required for each sample. This quantity of PL was not practical in this study because the three forms were each measured in quadruplicate at five different time intervals. Therefore, an assay which required only milligram
quantities of PL was selected (Takagi et al., 1978). The peroxide assay employed in this study was based on the ability of hydroperoxide to oxidise the iodide ion into an I₃ complex. This yellow coloured complex could then be measured spectrophotometrically at 352 nm. The reliability and reproducibility of this assay depended upon: the purging of the assay solvents with nitrogen, sealing of the reaction container with nitrogen to exclude air and the protection of the reaction container from light.

All solutions for this peroxide assay were freshly produced just prior to the assay. The solvents for these solutions were deaerated by bubbling nitrogen through the liquids for 30 minutes. To reduce the evaporation of the volatile solvents during purging, deaeration of these solvents was carried out in containers with narrow necked vessels, whilst being cooled in a bucket of ice. Instead of assaying hydrated liposomes, the samples were tested for hydroperoxides in their anhydrous forms. This avoided any oxygen dissolved in water interfering with the iodometric assay.

Sufficient sample containing approximately 10 mg of PL was accurately weighed into the bottom of a 5 ml glass bottle. The precise amount depended upon the quality of the PL: highly oxidised material required only a few mg. To this lipid, 1.0 ml of deaerated acetic acid: chloroform (3:2 v/v) solution was added and the bottle was sealed under nitrogen. After the PL had fully dissolved, 50 µl of freshly made saturated potassium iodide solution (1.2 g in 1 ml purged deionised water) was added to the PL dissolved in acidified chloroform. The bottle was immediately stoppered, vortexed for 15 seconds and wrapped in foil to protect from light. The sample was placed in the dark for exactly five minutes. After the five minutes had elapsed, 4.0 ml of oxygen free 0.5% w/v cadmium acetate solution was added to the bottle and vortexed for ten seconds. The two phases were separated by centrifugation for 5-10 minutes at 2000 rpm.

Exactly 60 minutes after the addition of the potassium iodide solution, the absorbance of the upper phase was read at 352 nm, against a blank containing all the components minus PL.

A standard peroxide curve was created using cumene hydroperoxide as the peroxide standard (Appendix I). The cumene hydroperoxide (80%) solution was diluted ten fold by accurately diluting 1.00 ml of the peroxide solution to 10 ml in absolute ethanol (x10 fold dilution). This solution was further diluted by diluting 100 µl of this solution to 10 ml in absolute ethanol (x1000 fold dilution). The final dilution was made by
diluting 1.00 ml of this x1000 diluted cumene solution to 10 ml in absolute ethanol (final dilution x10,000 fold). The following amounts of the x10,000 fold diluted cumene hydroperoxide solution were individually assayed using the peroxide assay: 75 μl, 100 μl, 150 μl, 200 μl, 250 μl. The standard curve was constructed by plotting the amount of cumene hydroperoxide level (nmol) against the absorption of each cumene hydroperoxide level.

4.3.2.3 Thiobarbituric acid reactive substances (TBARS)

Two thiobarbituric acid (TBA) assays were employed to detect malondialdehyde (MDA). Since MDA is one of the major breakdown products of PL degradation, these assays provided an indication of the extent of oxidation. The reaction product of MDA and TBA generated an intense red colour which was detected at a wavelength of 532 nm. These reaction products are widely referred to as thiobarbituric acid reaction substances (TBARS).

The only difference between the two assays used in the stress study was the addition of iron (III) chloride in the TBARS(F) assay prior to heating. The addition of iron chloride to the reaction mixture encouraged the liberation of MDA from hydroperoxides. Hence this variant of the TBARS assay provided an indication of both the hydroperoxide and the secondary product content in the PLs. In contrast, by omitting iron chloride from the reaction mixture, only the secondary products present in the PL were detected (Asakawa and Matsushita, 1979 and 1980).

However, both assays required heating at 95 °C in order for the reactions to proceed. It was therefore necessary to protect the PL from oxidising during the reaction. This was achieved by the addition of the antioxidant butylated hydroxytoluene (BHT) (Asakawa and Matsushita, 1980) to both reaction mixtures prior to heating for 90 minutes at 95 °C.

4.3.2.3.1 Thiobarbituric acid reactive substances with iron (III) chloride (TBARS(F))

A sufficient amount of sample containing about 5 mg of PL was accurately weighed into a 20 ml glass bottle. To this sample, 100 μl of antioxidant butylated hydroxytoluene solution (110 mg BHT to 10 ml ethanol) was added. After dissolving the PL in this antioxidant solution, 500 μl of deionised water and 100 μl of FeCl₃ solution (27 mg of iron (III) chloride to 10 ml water) were added. This was followed by adding 1.75 ml of
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glycine-HCL buffer (0.2 M), pH 3.6. Finally, 1.75 ml of a solution containing 0.67\% w/v thiobarbituric acid and 0.3\% w/v sodium lauryl sulphate was added to the glass bottle. The bottle was sealed and vortexed for 15 seconds, before heating for 90 minutes at 95 °C. After heating, the bottles were cooled to room temperature by placing them in water. After cooling, 1.0 ml of acetic acid and 2.0 ml of chloroform were added to the contents of the bottle and handshaken vigorously. The samples were centrifuged at 5,000 rpm until a visibly clear upper phase was obtained. The absorbance of the top layer of the sample was measured at 532 nm, against a blank containing all components except for the PL. To ensure the top layer was clear and the absorbance was attributable to pink colouration, the optical density (O.D.) was measured at 560 nm. If the O.D. was low (0.02 and below), this verified that the solution was clear and had been satisfactorily centrifuged.

1,1,3,3-tetraethoxypropane (TEP) was employed as the standard substance for constructing the TBARS curve. This standard substance liberates malondialdehyde and ethanol under mild acid conditions. A standard stock TEP solution was produced by diluting 44 mg TEP to 250 ml with freshly deionised water. The TEP working solution was produced by further diluting 1.0 ml of stock TEP solution to 10 ml with deionised water. The following volumes of TEP working solution were assayed using the TBARS assay omitting the lipid and 500 µl of deionised water: 0 µl, 50 µl, 100 µl, 150 µl, 200 µl, 250 µl, 300 µl and 400 µl. Deionised water was added to the reaction mixture to ensure the volume of the TEP sample totalled 500 µl. A standard curve was constructed by plotting the amount of MDA released (in nmol) against the absorption of each TBARS reacted TEP sample.

4.3.2.3.2 Thiobarbituric acid reactive substances without iron (III) chloride (TBARS)
The method, described in section 4.3.2.4.1, was employed except that iron (III) chloride was omitted from the reaction mixture. The standard curve is not shown, because the curve was identical to TBARS(F) curve (Appendix II).

4.3.3 Visual appearance of formulations
The visual appearance of each sample was recorded at the specific test times.
4.3.4 Detection of lyso-phosphatidylcholine

There are a variety of techniques available to detect the hydrolytic breakdown products of PLs. Most techniques involve a two stage process. Firstly, chromatography, e.g. TLC and HPLC, is usually used to separate LPC from intact phospholipids. After this separation, LPC and PLs have to be analysed using spectroscopy or a phosphorus detecting technique.

The disadvantages of these two-stage techniques are three fold. Firstly, separation of the individual lipid components may not be complete. Secondly, detection of the PL is often inaccurate, because detection is often based upon UV detection of the double bonds. Slight variations in the profile of the double bonds may give misleading results. Thirdly, the detection of other breakdown products may be missed (Grit and Crommelin, 1993; Grit et al., 1993).

These difficulties have been largely overcome in this present study by adopting \(^{31}\text{P-NMR}\) (Henderson et al., 1974; Sotirhos et al., 1986). This technique enabled all the PL components of any mixture/blend to be analysed qualitatively and quantitatively without the need for separating the different PLs. The only preparative step required prior to the \(^{31}\text{P-NMR analysis} \) was the removal of most of the solvent/hydrophilic media associated with the PL. This was achieved by extraction and separation of the PL with chloroform, followed by evaporation of this volatile organic solvent (section 4.3.4.1). \(^{31}\text{P-NMR measurements} \) were measured only once on all the samples, because the reproducibility of the technique was good (Sotirhos et al., 1986).

All graphs showing PC and LPC contents were calculated as mol percentages of total PL content. Calculations based on overall lipid content could not be made using this technique, because lipids without phosphorous were not detected by \(^{31}\text{P-NMR}\).

Graphs plotting mol% PC based on total PL content against time in days were constructed for all stress stores samples. Similarly, LPC contents against time were shown for the soya PC, egg PC and egg PL samples. However, LPC graphs could not be constructed for soya PL samples, because the peaks representing the breakdown products overlapped and made the interpretation of this complex spectra difficult.

4.3.4.1 Sample preparation prior to \(^{31}\text{P-NMR analysis} \)

To prepare the lipid samples for \(^{31}\text{P-NMR analysis} \), the ethanol and ethanol/glycerol had to be removed from the ethanol pro-liposomes and glycerol containing pro-liposomes.
respectively. The ethanol was removed by evaporation under nitrogen at room temperature after depositing the PL in ethanol as a thin film on the inside of a glass watch glass. The glycerol was separated from the PL by the addition of 10 ml of chloroform in a 50 ml separating funnel. The lower immiscible layer containing the glycerol was removed, leaving an upper phase containing the PL in chloroform. The chloroform layer containing PL was reduced in volume down to about 5 ml by evaporating under nitrogen at room temperature. The remaining chloroform was removed by drying the PL under nitrogen on the inside of a large watch glass.

4.3.4.2 Experimental procedure for $^{31}$P-NMR spectroscopy
(See section 2.3.1 for details)

4.3.5 Repeat measurements
With the exception of $^{31}$P-NMR, all measurements were carried out a minimum of four times.

4.3.6 Data presentation
For clarity, units were expressed per mol of PL. The molar quantity of PL was calculated by dividing the mass of PL by an approximate average molecular weight (MW) of 750. This MW was not entirely accurate for the egg PC and the PLs, which contained PE and other lipids. However, the average MW of purified egg PC is 768 (Mason and Huang, 1979) and the average MW of purified soya PC is 786 (Lucas Meyer, Epikuron 200 data sheet). Therefore, 750 was a close approximation for comparative purposes.

The standard deviation for each sample point is recorded on the graphs as a bar above and below the mean value.

4.3.7 Stress test
4.3.7.1 Storage conditions for stress test
The four different phospholipids in the solid form, ethanol pro-liposomes and glycerol pro-liposomes were stored at 55 °C in a light proof incubator in order to investigate the relative stabilities of the lipids. Without deliberately stressing these phospholipid samples at this elevated temperature, it may have taken too long to obtain an impression of the relative stabilities.
4.3.7.2 Assay time points for stress test
The stability tests described above were carried out on all samples at five time points: time zero, 14 days, 28 days, 56 days and 84 days using the oxidation analysis tests described above.

4.3.8 Accelerated and long term storage stability test
4.3.8.1 Storage conditions for accelerated and long term storage stability test
The soya PL blend pro-liposome formulation which produced liposome dispersions with a desirable size distribution after a two stage conversion (section 3.5.4.5.2) was selected for accelerated and long term stability testing. The oxidation status of this pro-liposome was assessed at 0, 14, 28, 56, 84 and 112 days after manufacture. For the accelerated study, the soya PL blend pro-liposome (section 3.5.4.5.2) was stored at 40 °C ± 2 °C, and for the room temperature and reduced temperature studies the samples were stored at 20 °C ± 2 °C and 6 °C ± 2 °C respectively.

4.3.8.2 Assay time points for accelerated and long term storage stability tests
The soya PL blend pro-liposome (section 3.5.2.4.5.2) was analysed at 0, 14, 28, 56, 84 and 112 days at each of the three temperatures using the oxidation tests described above, except for the TBARS(F) test.

4.4 Results and discussion
4.4.1 Comparing formulations at time zero
4.4.1.1 Oxidation levels of the different formulations
No differences could be observed between the UV absorption profiles of the egg PC, egg PL and soya PL formulations at time zero. However, the formulations containing soya PC appeared to differ. Despite the fact that during processing, care was taken to protect the lipids from light and heat, at time zero, the UV measurements revealed a subtle difference between the three soya PC formulations. As can be seen from Figure 4.3, the ethanol pro-liposome (B) and glycerol pro-liposome (C) produced slightly more pronounced peaks at 227 nm, compared to the shouldered peak of the solid lipid (A). This observation indicated that a degree of rearrangement of the two double bonds had already occurred during the production of the soya PC ethanol pro-liposome and soya PC glycerol pro-liposome. Therefore, perhaps even more controlled handling procedures may be required during the processing of this sensitive soya PC.
During transferral/handling procedures it may be necessary to ensure this PC is kept away from all light and oxygen. This latter procedure could be achieved by dissolving the PC in nitrogen purged solvents during manufacture, and storing under nitrogen after production.

Figure 4.3 Comparison of the UV spectra for the three anhydrous soya PC presentations at time zero

4.4.1.2 Oxidation levels of lipids in solid form

At time zero, time = 0 on Figure 4.4 to Figure 4.23, the oxidative tests indicated that the four lipids had similar oxidation profiles, and there was little difference in the level of oxidation between the four lipids.
4.4.1.3 Visual appearance of the formulations

The purified PCs were bright yellow waxy solids, which produced clear yellow solutions when dissolved in ethanol. In contrast, the PLs were intensely coloured orange solids, which yielded clear golden orange solutions when dissolved in ethanol. The less intense colouration of the two PCs was probably due to the lower carotenoid content in the soya PC and egg PC. During commercial processing by acetone extraction and aluminium oxide purification, these coloured carotenoids were largely removed from PC, although trace amounts still remained.

4.4.2 Stress storage stability

4.4.2.1 Diene conjugation and triene conjugation ratios

The UV absorption characteristics of the different lipids could not be directly compared with one another, because different fatty acid profiles show different absorption characteristics (Holman and Burr, 1946). However, the changes in absorption for the different forms of a given PL, could be qualitatively compared because the same batch of lipid was used throughout the study.

All the diene and triene oxidation indices during the stress test were higher than the indices at the start of the stress test (time = zero in Figures 4.4- 4.11). This suggested that upon storage, diene and triene conjugation had increased in all samples. Virtually all of the ratios continued to increase throughout the stress test, which indicated that the degree of conjugation for a given phospholipid was increasing throughout the duration of the stress test.

After 56 days and 84 days of stress storage all the four lipids had higher levels of diene formation in the glycerol pro-liposome compared to their corresponding solid form. Similarly, at 56 days and 84 days the soya PL in the ethanol pro-liposome had higher levels of diene formation compared to the corresponding solid form (p = < 0.05; p = < 0.05). These results indicated that under the conditions tested, the glycerol pro-liposome and the ethanol pro-liposome may have encouraged diene conjugation.

A similar pattern was also observed for the conjugated trienes in egg PL and egg PC glycerol pro-liposomes after 84 days (p = < 0.05; p = < 0.05). The higher conjugation ratios in the glycerol pro-liposome may have been due to trace metal ions present in the glycerol. The presence of transition metals such as copper may have encouraged radical formation and hence double bond rearrangement (Ding and Chan, 1984).
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Figure 4.4 Development of conjugated dienes in three soya PL formulations with time at 55 °C (Each point is the mean ± s.d.)

Figure 4.5 Development of conjugated dienes in three soya PC formulations with time at 55 °C (Each point is the mean ± s.d.)
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Figure 4.6 Development of conjugated dienes in three egg PL formulations with time at 55 °C (Each point is the mean ± s.d.)

Figure 4.7 Development of conjugated dienes in three egg PC formulations with time at 55 °C (Each point is the mean ± s.d.)

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Figure 4.8 Development of conjugated trienes in three soya PL formulations with time at 55°C (Each point is the mean ± s.d.)

Figure 4.9 Development of conjugated trienes in three soya PC formulations with time at 55 °C (Each point is the mean ± s.d.)
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Figure 4.10 Development of conjugated trienes in three egg PL formulations with time at 55°C (Each point is the mean ± s.d.)

Figure 4.11 Development of conjugated trienes in three egg PC formulations with time at 55 °C (Each point is the mean ± s.d.)
4.4.2.2 Hydroperoxide levels
Both soya PC (Figure 4.13) and egg PC (Figure 4.15) in all three forms showed increases in peroxide levels over 84 days of stress storage. However, the extent of peroxidation in the egg PC samples during storage was much lower than the peroxide value of the soya PC samples at the corresponding time points. As can be observed from Figure 4.13, the highest peroxide increase occurred with the soya PC samples. The peroxide level of this particular PC in all three formulations increased throughout the study. After only 14 days of stress storage, the peroxide level of the soya PC formulations increased significantly compared to the corresponding formulations at time zero (p = < 0.05 for lipid formulation; p = < 0.05 for ethanol pro-liposome and p = < 0.05 for glycerol pro-liposome). In comparison, the peroxide value for the soya PL samples was markedly lower at all of the corresponding time points (Figure 4.12). Indeed, even after 84 days of storage, the peroxide level of the soya PL in solid form had not increased from its initial value at time zero (p = 0.397). This result suggested that the soya PL was less prone to oxidation under the conditions tested than both the egg PC and soya PC. The possible explanation for the soya PLs enhanced resistance to oxidation could perhaps be attributed to the formation of antioxidant by-products, which are discussed in section 4.4.2.5. This enhanced oxidative stability was unlikely to be due to the presence of residual antioxidants in the soya PL, since the extraction procedures would be expected to remove almost all of the naturally occurring antioxidants (Schneider, personal communication).

The egg PL behaved in a similar manner to the soya PL, being apparently more resistant to oxidation than the corresponding PC. As can be seen from Figure 4.14, the peroxide level for all formulations of the egg PL was very similar throughout the study, and remained virtually unchanged throughout the 84 day study period.

Although the overall degree of unsaturation may provide a general reflection of the lipids susceptibility to peroxide formation, it would perhaps be more informative to assess this susceptibility in view of the number of double bonds in the individual fatty acids. As explained in section 4.1.2.2, PLs with fatty acids with two or more double bonds would be more susceptible to oxidation than PLs with fatty acids with only a single double bond (Niki, 1992).
From the GC analysis of the fatty acids (Table 2.6), it was evident that the fatty acid profiles of the egg PC and soya PC differed considerably. Not only was the total percentage of unsaturated fatty acids lower in the egg PC (43% unsaturated fatty acids) than in the soya PC (83% unsaturated fatty acids), but the types of unsaturated fatty acids also greatly differed. As can be seen from Table 2.6, the percentage of fatty acids with three or more double bonds were similar (5% for the egg, and 9% for the soya). However, the percentage of fatty acids with two double bonds was over four fold higher in the soya PC than in the egg PC: in soya PC, the percentage of fatty acids with two double bonds was 69% compared to 15% in the egg PC. It was therefore unsurprising that under the conditions tested, the egg PC was more resistant to peroxide formation than the more unsaturated soya PC.

Figure 4.12 Change in peroxide level of three soya PL formulations with time at 55 °C
(Each point is the mean ± s.d.)
Figure 4.13 Change in peroxide level of three soya PC formulations with time at 55 °C (Each point is the mean ± s.d.)

Figure 4.14 Change in peroxide level of three egg PL formulations with time at 55 °C (Each point is the mean ± s.d.)
4.4.2.3 TBARS formation

The TBARS value did not dramatically change in any of the samples at 14 days of storage (Figure 4.16-4.19). However, after 28 days of storage, all the egg PC, soya PC and soya PL formulations had significantly increased TBARS values compared to the values at time zero (p = < 0.05 for all samples). In contrast, there were no changes in TBARS value with egg PL. This suggested that the formation of secondary oxidation products had started to occur in all PC samples and soya PL samples after 28 days, but was yet not detectable in the egg PL samples.

However, after 84 days of storage, the TBARS value for the egg PL glycerol pro-liposome (Figure 4.18) had increased significantly (p = 0.006) compared to the glycerol pro-liposome at time zero. This TBARS value had risen from about 0.6 mmol of MDA/mol PL at time zero to over 1.0 mmol of MDA/mol PL after 84 days. During the same time period, the TBARS level of the egg PL solid and ethanol egg PL pro-liposome had not increased significantly (p = 0.438; p = 0.271). This suggested that under the conditions tested, the egg PL in the glycerol pro-liposome formulation did not appear as stable as the egg PL in solid form or ethanol pro-liposome. A similar trend
was also observed with both the egg PC and soya PC samples; after 84 days of stress stability storage the TBARS value for the glycerol pro-liposomes was higher than the solid and ethanol pro-liposomes ($p = < 0.001$ for both egg PC and soya PC formulations).

### 4.4.2.4 TBARS(F) formation

As expected the addition of iron (III) chloride considerably increased the intensity of the pink colouration of the TBARS (Figures 4.20- 4.23). This was probably due to the release of MDA from the cyclic endoperoxides and hydroperoxides from the fatty acid chains. For example, without iron (III) chloride the TBARS value for soya bean PL at time zero was approximately 0.2 mmol MDA/mol PL (Figure 4.16). However, if the reaction was carried out in the presence of iron (III) chloride the TBARS(F) value was three fold higher (Figure 4.20).

At time zero, all samples had a TBARS(F) value ranging from about 0.7 to 1.2 mmol of MDA/mol PL. These values effectively remained constant even after 28 days of stress stability storage. After 84 days of storage, however, increases were observed in some samples. Notably the TBARS(F) value for the glycerol pro-liposomes composed of egg PC and soya PC increased significantly compared to the solid and ethanol pro-liposomes ($p = < 0.05$ for both soya PC and egg PC formulations). This indicated that a higher level of oxidation was occurring in these glycerol pro-liposome formulations, confirming that the pro-liposome formulation with glycerol was not stable as the solid PC or the ethanol pro-liposome.
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Figure 4.16 TBARS value of three soya PL formulations with time at 55 °C
(Each point is the mean ± s.d.)

Figure 4.17 TBARS value of three soya PC formulations with time at 55 °C
(Each point is the mean ± s.d.)
Figure 4.18 TBARS values of three egg PL formulations with time at 55 °C
(Each point is the mean ± s.d.)

Figure 4.19 TBARS value of three egg PC formulations with time at 55 °C
(Each point is the mean ± s.d.)
Figure 4.20 TBARS(F) values of three soya PL formulations with time at 55 °C
(Each point is the mean ± s.d.)

Figure 4.21 TBARS(F) value of three soya PC formulations with time at 55 °C
(Each point is the mean ± s.d.)
Figure 4.22 TBARS(F) values of three egg PL formulations with time at 55 °C
(Each point is the mean ± s.d.)

Figure 4.23 TBARS(F) value of three egg PC formulations with time at 55 °C
(Each point is the mean ± s.d.)
4.4.2.5 Visual appearance of the lipids

After 14 days of stress storage at 55 °C, the waxy powder of the solid egg PC and egg PL and the structured solid of the soya PC and PL had fused to form soft waxy masses. Whereas the ethanol and glycerol pro-liposome formulations remained fluid throughout the 84 day stress test. In terms of surface area, the solid lipids would clearly have a smaller area exposed than other solid forms, such as a PL film, a freeze dried preparation or a spray dried PL. Hence, it would be inappropriate to extrapolate the stress stability results of the solid lipid to these types of presentations.

After 28 days of stress storage, the colour of the ethanol egg PL and ethanol soya PL pro-liposomes had changed dramatically (Table 4.1): these PLs in all forms continued to darken throughout the study, until a dark brown colouration was reached after 56 days of storage. After 56 days of stress stability storage even the egg PC samples started to darken slightly. The only lipid which did not undergo any colour change in any of the three formulations during the stress study was soya PC.

The darkening of the PL samples was not directly related to the peroxide development or hydrolytic instability of the PL. It was probably related to the presence of saccharide (section 2.4.3) and the free reactive amine group of the PE. Under the temperature of the stress conditions employed in this study, the condensation reactions of these two components were promoted. These types of condensation reactions are known as Maillard reactions, and the formation of Maillard products probably caused the darkening of the PLs. Since the activation temperature of this process is 45 °C, the browning occurred rapidly at the stress stability storage temperature of 55 °C (Lange, personal communication). Therefore, in order to avoid this browning during extended exposure at elevated temperatures, it would have been necessary to either remove the saccharides or remove the PE from the PLs. The reason for the slight browning of the egg PC has not been established, since no PLs containing amine groups were detected along with this lipid. The darkening may have been attributable to the caramelisation of the saccharides present.

After 56 days of storage, all the egg PL and soya PL formulations had lower TBARS, TBARS(F) and peroxide values compared to the corresponding PCs. During the same time these PL formulations had also turned brown (Table 4.1), reflecting the formation of Maillard browning products. Perhaps the formation of Maillard browning products in
these formulations may have contributed to the apparent enhanced stability of both of
PLs. Park and Kim (1983) demonstrated that ethanolic fractions of similar "Maillard
browning agents" possessed considerable antioxidant properties and significantly
contributed to the enhanced stability of soya bean oil. Perhaps the Maillard browning
products formed during in this present study conferred both the soya PL and egg PL
formulations with antioxidant properties, which were absent in the soya PC and egg PC
formulations. This may explain why the soya PL and egg PL had lower TBARS,
TBARS(F) and peroxide values compared to the corresponding PCs.

<table>
<thead>
<tr>
<th>Time in days: Lipid formulation</th>
<th>Soya PL</th>
<th>Soya PC</th>
<th>Egg PL</th>
<th>Egg PC</th>
</tr>
</thead>
<tbody>
<tr>
<td>0: LIP</td>
<td>Orange</td>
<td>Bright yellow</td>
<td>Orange</td>
<td>Bright yellow</td>
</tr>
<tr>
<td>0: LA</td>
<td>Orange</td>
<td>Bright yellow</td>
<td>Orange</td>
<td>Bright yellow</td>
</tr>
<tr>
<td>0: GPRO</td>
<td>Orange</td>
<td>Bright yellow</td>
<td>Orange</td>
<td>Bright yellow</td>
</tr>
<tr>
<td>14: LIP</td>
<td>Orange</td>
<td>Bright yellow</td>
<td>Dark orange</td>
<td>Yellow</td>
</tr>
<tr>
<td>14: LA</td>
<td>Orange</td>
<td>Bright yellow</td>
<td>Dark orange</td>
<td>Yellow</td>
</tr>
<tr>
<td>14: GPRO</td>
<td>Orange</td>
<td>Bright yellow</td>
<td>Dark orange</td>
<td>Yellow</td>
</tr>
<tr>
<td>28: LIP</td>
<td>Orange</td>
<td>Bright yellow</td>
<td>Dark orange</td>
<td>Dark yellow</td>
</tr>
<tr>
<td>28: LA</td>
<td>Orange</td>
<td>Bright yellow</td>
<td>Dark orange</td>
<td>Dark yellow</td>
</tr>
<tr>
<td>28: GPRO</td>
<td>Orange</td>
<td>Bright yellow</td>
<td>Dark orange</td>
<td>Yellow</td>
</tr>
<tr>
<td>56: LIP</td>
<td>Brown</td>
<td>Bright yellow</td>
<td>Brown</td>
<td>Light orange</td>
</tr>
<tr>
<td>56: LA</td>
<td>Dark brown</td>
<td>Bright yellow</td>
<td>Brown</td>
<td>Light orange</td>
</tr>
<tr>
<td>56: GPRO</td>
<td>Brown</td>
<td>Bright yellow</td>
<td>Brown</td>
<td>Yellow</td>
</tr>
<tr>
<td>84: LIP</td>
<td>Brown</td>
<td>Bright yellow</td>
<td>Dark brown</td>
<td>Orange</td>
</tr>
<tr>
<td>84: LA</td>
<td>Dark brown</td>
<td>Bright yellow</td>
<td>Dark brown</td>
<td>Orange</td>
</tr>
<tr>
<td>84: GPRO</td>
<td>Brown</td>
<td>Bright yellow</td>
<td>Dark brown</td>
<td>Dark yellow</td>
</tr>
</tbody>
</table>

4.4.2.6 Lyso-phosphatidylcholine (LPC) level
At time zero (Figures 4.24-4.27), the soya PL and egg PL had a somewhat higher mol% of LPC contents than the corresponding soya PC and egg PC. The LPC content of soya PL and egg PL was 4 mol% and 2 mol% respectively, compared to 1 mol% for the soya PC and egg PC. The lower level of LPC in soya PC and egg PC was probably due to the aluminium oxide chromatography used to purify the lipids during manufacturing, which removed some of the LPC.

No clear relationship between the form of the PL and the level of LPC existed (Figures 4.24-4.27). However, there appeared to be a trend linking the PL type with the extent of hydrolysis. Even after 84 days of storage the only phospholipid hydrolytic breakdown product detected in the egg PC, soya PC and the egg PL was LPC. Breakdown products of glycerophosphorocholine and phosphoric acid were not present, which suggested that in these samples, severe hydrolytic degradation had not occurred. It was also clear that the PC samples were hydrolytically more stable than the PL samples. Even after 84 days
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of storage at 55 °C the LPC content of soya PC formulations and egg PC formulations had only increased by 1-5% and 3-8% respectively. In contrast, after 84 days of stress testing at 55 °C, the egg PC formulations showed greater increases in LPC content (between 10-23%).

The LPC content of three different forms of soya PL could not be calculated, because the overlapping multiple peaks in the \(^{31}\)P-NMR spectra were difficult to identify in some of the stored soya PL samples. Only the PC peak could be clearly established. Therefore, only the PC content of the soya PL was presented in Figure 4.24. As seen from Figure 4.24, after 84 days of stress storage the PC content declined from 75 mol% to 40-50 mol%. The spectra for the soya PL samples became increasingly difficult to interpretate as the test progressed with time. It was evident that severe hydrolytic breakdown had occurred in the soya PL samples, which was reflected by the number of peaks increasing from 4 at the start of the test (Figure 4.28) to 6 after only 28 days of storage of the glycerol soya PL pro-liposome (Figure 4.29). At the end of the 84 day stress study, many of the soya PL peaks were unidentifiable; there were 8 peaks in the solid lipid, glycerol pro-liposome and the ethanol pro-liposome (Figure 4.30). It was likely that these new peaks were attributable to hydrolysis of PE into LPE and the further hydrolysis of LPC and LPE. The reason for the difference in the extent of hydrolysis between the PCs and the PL was not clear. However, this hydrolytic instability may have been related to the PE content, since PE was the only component absent from the more hydrolytically stable PCs, but common to both PLs.
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Figure 4.24 Soya PC content of three soya PL formulations over 84 days at 55 °C

Figure 4.25 Soya PC and soya LPC content of three soya PC formulations over 84 days at 55 °C
Figure 4.26 Egg PC and egg LPC content of three egg PL formulations over 84 days at 55 °C

Figure 4.27 Egg PC and egg LPC content of three egg PC formulations over 84 days at 55 °C
Chapter Four-Stability of unsaturated phospholipids

Figure 4.28 $^{31}$P-NMR trace of soya PL at time zero

Figure 4.29 $^{31}$P-NMR trace of soya PL from the glycerol pro-liposome at 28 days
4.4.3 Stability of soya PL blend pro-liposome subjected to accelerated and long term storage

Upon storage the soya PL blend should have behaved in a similar manner to the soya PC glycerol pro-liposome, because the PC content of the soya PL blend was high. However, when subjected to accelerated storage conditions, the peroxide level (Figure 4.31) and TBARS value (Figure 4.32) did not rise greatly over 112 days. This suggested that, under the conditions tested, this soya PL blend in glycerol pro-liposome was relatively resistant to oxidation. Only the conjugated diene and conjugated triene assay detected signs of slight oxidation: after storing the samples for 112 days at 40 °C and 20 °C the conjugated diene ratio was significantly higher (p = 0.01; p = 0.001 respectively) than at the start of the study, time = 0 in Figure 4.31. Similarly, after storing the formulation for 112 days at 40 °C, 20 °C and 4 °C the conjugated triene ratio was significantly higher (p = < 0.05 at all three temperatures) than at the start of the study, time = 0 in Figure 4.31.
Physically, this soya PL blend remained golden brown throughout the study at all temperatures, demonstrating that the severe browning observed in the previous stress study was probably attributable to the elevated stress temperature of 55 °C. Since these browning agents did not seem to be extensively formed at 40 °C, the reason for the enhanced stability of the soya PL blend pro-liposome, particularly at accelerated stability conditions, was unclear. However, the apparent improvement in stability was likely to be linked with the addition of the soya PL. The addition of 10% soya PL by weight to the soya PL blend must have conferred some protective effect on the soya PC. As revealed during the stress testing, soya PC was found to be relatively susceptible to oxidation (Figure 4.13).

Figure 4.31 Development of conjugated dienes in soya PL blend pro-liposome with time at 40 °C, 20 °C and 4 °C (Each point is the mean ± s.d.)
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Figure 4.32 Development of conjugated trienes in soya PL blend pro-liposome with time at 40 °C, 20 °C and 4 °C (Each point is the mean ± s.d.)

Figure 4.33 Development of hydroperoxides in soya PL blend pro-liposome with time at 40 °C, 20 °C and 4 °C (Each point is the mean ± s.d.)
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Figure 4.34 Development of TBARS in soya PL blend pro-liposome with time at 40 °C, 20 °C and 4 °C (Each point is the mean ± s.d.)

4.5 Conclusions

Under the stress conditions, the egg PL and soya PL appeared more resistant to oxidation than their corresponding PCs. However, the physical appearance of both these PLs in all forms dramatically changed under the stress conditions. After only 28 days these PLs had darkened considerably. This was probably as a result of the formation of Maillard type complexes between phosphatidylethanolamine and the saccharides.

The form of the PL had an influence on the oxidative stability. After 56 days of stress stability storage, most of the assays detected differences between the different anhydrous formulations of PL. In terms of oxidative stability, the glycerol pro-liposome and ethanol pro-liposome formulations were generally less stable than the solid phospholipid.

From the $^{31}$P-NMR results no clear relationship between hydrolysis and the formulation was seen. However, the type of lipid used seemed to greatly influence the extent of hydrolysis. The egg PC and soya PC were more resistant to hydrolysis than the corresponding egg PL and soya PL after 84 days of stress storage. Of all the lipids, soya PL seemed the most susceptible to hydrolysis, after 84 days there was a large decrease in PC content and several unidentified peaks in the $^{31}$P-NMR spectra appeared. All the
other phospholipids appeared to only produce 1-LPC and 2-LPC as the hydrolytic breakdown products.

Finally, accelerated and long term stability tests revealed that the soya PL blend pro-liposome seemed to have a similar oxidation stability profile to the soya PL glycerol pro-liposome. This was despite the fact that only 10% w/w of this soya PL was added to the soya PC. Even after 112 days of storage at 40 °C this PL blend was remarkably stable, the only oxidative stability assays which rose slightly were the conjugated diene and conjugated triene oxidation index. The reason for this apparent enhancement of stability is unclear, but is likely to be related to the addition of the soya PL.
Chapter five

Solubilisation of cyclosporin A

by liposomes
Chapter five-Solubilisation of cyclosporin A by liposomes

5.1 Introduction

5.1.1 Selection of drug for investigation

Cyclosporin A (cyA) is a non-polar immunosuppressant of fungal origin. It is extensively used in organ transplantation to prevent allograft rejection (Cohen et al., 1984). This 1203 MW cyclic undecapolyopeptide was chosen as a model hydrophobic compound for a variety of reasons. Firstly, this drug possesses suitable solubility profiles for association studies. The aqueous solubility of cyA is quoted in the literature to be approximately 0.03 mg/ml at 20 °C (Ismailos et al., 1991), but is highly soluble in most organic solvents. Secondly, its interaction and association with artificial membranes has been thoroughly investigated (Fahr et al., 1994; Ouyang et al., 1995). Thirdly, cyA is just one of the many cyclosporin derivatives (Sadeg et al., 1993 and Fahr et al., 1995), which are difficult to formulate into a non-toxic intravenous preparation. Sandimmun® concentrate for IV infusion, (British National Formulary, 1996 (September)) is the only commercial IV formulation currently available for clinical use. This formulation employs cyA solubilised in ethanol/polyethoxylated castor oil, which has been implicated with unwanted adverse effects such as anaphylaxis (Howrie et al., 1985). Therefore, reformulating this peptide into a liposome carrier, free from this non-ionic surfactant may be of clinical benefit.

5.1.2 Importance of molecularly dispersing hydrophobes

The primary function of liposomes in this study was to molecularly solubilise cyA. Solid precipitates of unsolubilised hydrophobe were clearly undesirable, because of the uncertainty surrounding the fate of these particles after injection. These insoluble particles could potentially accumulate in the mononuclearphagocyte system (MPS) and/or potentially induce emboli by physically occluding capillaries.

5.1.3 Liposomes and cyclosporin A

The majority of studies in the literature concerning liposomal cyA have focused upon the parenteral administration of cyA liposomes, although the formulation of cyA into liposomes for pulmonary applications has been reported (Waldrep et al., 1993). Specifically, most of these parenteral studies relate to the IV administration of cyA liposomes in animals and the subsequent reduction in cyA toxicity (Luke et al., 1987; Smeesters et al., 1988; Akbarieh et al., 1993; Freise et al., 1994). Details relating to the
Chapter five—Solubilisation of cyclosporin A by liposomes

Complete solubilisation of cyA and other pharmaceutical aspects of cyA liposome formulations have largely been ignored in these published studies.

It is a common misconception that water insoluble lipophiles easily partition into the hydrophobic domains of the liposomal bilayer membrane. Indeed, as pointed out by Vadiei et al. (1989), the association of cyA is poor if traditional thin film techniques are adopted. In the literature no formulation studies have reported a cyA loading efficiency of 100%. Often further processing in the form of separation may have to be employed to remove the unassociated material from the liposome dispersion. For intravenous liposome products this separation introduces an undesirable extra step, which wastes drug and imposes an additional cost on an already expensive process. Ideally, cyA should therefore be fully associated with liposomes during their preparation, to avoid this separation step.

5.1.4 Location of cyA within membrane

The chemical structure of a drug dictates its location within a liposome (Juliano and Stamp, 1979a and 1979b). A drug which is completely non-polar and highly hydrophobic is likely to be “buried” within the hydrophobic domains of the liposome bilayer. In contrast, a drug which possesses entirely hydrophilic properties is likely to remain in the aqueous channels/core of the liposome. Drugs with both hydrophobic and hydrophilic properties may span these two regions, the hydrophilic moiety may be located at the lipid/water interface and the hydrophobic portion may be embedded within the hydrophobic region of the bilayer.

In the specific case of cyA, it seems that this non-polar hydrophobe is passively associated within the hydrocarbon region of the bilayer (Fahr, 1996). Unlike membrane proteins which naturally reside within cell membranes, cyA is not anchored to the membrane bilayer. The interactions between hydrophobic drugs and hydrocarbon regions of the membrane are often weak hydrophobic bonds and van der Waals forces (Wenk et al., 1996).

Only relatively modest amounts of this hydrophobe can be associated within the membrane, because of cyA’s bulky structure (Figure 5.1). This is perhaps unsurprising because the phospholipid bilayer is not designed to specifically carry this bulky molecule (Ouyang et al., 1995). Various membrane studies (Fahr et al., 1994) have estimated that a minimum of 19 molecules of PC are required to carry one molecule of...
cyA. This equates to approximately 12 parts by weight of egg PC to one part by weight of cyA. In the literature, the range of lipid: cyA weight ratios reported to carry cyA vary from 2:1 up to 25:1. None of these studies have reported absolute intercalation of cyA. Indeed often the amount of cyA molecularly associated with the lipid is not even reported, and it is assumed to be largely contained within the membrane of the liposome (Hsieh et al., 1985; Góreckie et al., 1991).

![Figure 5.1 Chemical structure of cyA](image)

**5.1.5 Methods for determining the degree of association**

The amount of drug carried by liposomes can be assessed by either directly measuring the amount of drug associated with liposome or indirectly measuring the amount of unassociated drug and subtracting this value from the total drug content. Typically, most studies adopt the former approach. This assessment is often most commonly achieved by centrifuging the liposome dispersion down to a pellet, followed by HPLC analysis or spectrophotometry of the drug in this pellet (Vadiei, 1989):

The “Entrapment efficiency” (EE) is calculated according to equation 5.1:

\[
\% \text{ EE} = \frac{[\text{Drug}] \text{ in pellet}}{[\text{Drug}] \text{ in liposome suspension}} \times 100
\]  

Equation 5.1

The centrifugation step of this technique may be inappropriate for assessing the association of hydrophobic drugs with liposomes. Centrifugation of the liposomes down to a pellet sediments any unassociated solid crystalline material along with liposomes.
solubilising hydrophobic drug. Therefore, when the pellet is analysed for hydrophobic
drug content, the amount of drug molecularly associated with the pellet may be
overestimated. It is for this reason that this technique was not employed in this current
study. This centrifugation technique is most applicable for assessing the entrapment of
water soluble compounds within the interior of liposomes. In this case, the amount of
hydrophilic drug in the pellet would probably be a truer reflection of entrapped drug.
Examination of the dispersion under the light microscope (denoted as LM in some
tables) may be used to assess the presence of unassociated drug precipitates. However,
initial light microscopy investigations of the cyA pro-liposomes and cyA liposome
dispersions generated in this study did not reveal the presence of any
crystals/precipitates. After these preliminary studies, it was realised that if this approach
was adopted, the probability of finding unassociated cyA may be remarkably low.
Particularly if the association of cyA with the liposomes was high. Hence, the hydrated
cyA pro-liposome and final liposome dispersion were not viewed under the light
microscope. Instead, the technique which was adopted for use in this association study
was a modification of the analytical filtration method described by Sharma and
Straubinger (1994). The advantage of this technique was that all insoluble material,
which had the potential to induce emboli, was retained on the filter. The details of this
method are described in section 5.3.5.

5.1.6 Aims of solubilising study
The primary aim of this study was to evaluate the solubilising capability of liposomes
produced from pro-liposomes using cyA as a model drug. Liposome dispersions
containing cyA were converted from various pro-liposome formulations in one stage
and two stages. The presence of cyA precipitates in these dispersions was determined
using analytical filtration (section 5.3.5). Factors potentially affecting the association
were investigated. Furthermore, factors potentially affecting both cyA association and
liposome particle size were studied.

5.2 Materials
Absolute ethanol, AnalA, B.N. various, BDH Chemicals Ltd., Poole, UK.
Cyclosporin A, USP grade, B.N. 201195, Ivax Corp., USA.
Deionised water, pH approximately 5, from Elgastat, UHQ PS, Elguard.
Egg phosphatidylglycerol (EPG), B.N. 10612-1/06, Lipoid, Ludwigshafen.
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Epikuron 145 (referred to as soya PL in text), B.N. 1-5-9005, Lucas Mayer, Hamburg, Germany.
Epikuron 200 (referred to as soya PC in text), B.N. 1-4-9018, Lucas Mayer, Hamburg, Germany.
Glycerol, AnalR, B.N. K213348 160 450, BDH Chemicals Ltd., Poole, UK.
Ovothin 180 (referred to as egg PL in text), B.N. 1-4-9240, Lucas Mayer, Hamburg, Germany.
Ovothin 200 (referred to as egg PC in text), B.N. 1-4-9248, Lucas Mayer, Hamburg, Germany.

5.3 Methods

5.3.1 CyA pro-liposome manufacture

Various pro-liposomes were made by the method described in section 3.3.1. For the sake of uniformity and convenience, the components of the CyA pro-liposomes are usually described as weight ratios in the following order: lipid: ethanol: optionally glycerol: cyA. The lipid composition and weight ratios of the individual CyA pro-liposomes are stated in the appropriate section described in section 5.4. CyA was incorporated into the pro-liposomes by dissolving the requisite amount in the lipid/ethanol solution. The CyA rapidly dissolved within five minutes and did not change the colour or clarity of the fluid pro-liposomes. If glycerol was incorporated into the CyA pro-liposome, this polyol was added after the CyA had fully dissolved in the lipid in ethanol solution.

For each experiment three sets of liposome dispersions were made from the same CyA pro-liposome batch and each dispersion was individually analysed for CyA precipitation. For each sample, sufficient liposome dispersion was made so that 10 mg of CyA could be passed through the filter.

5.3.2 Converting CyA pro-liposomes into liposomes in one stage

One stage liposome conversion (section 3.4.2) was carried out by directly adding the CyA pro-liposome to the bulk deionised water in a 25 ml glass bottle and immediately dispersing it by vigorous handshaking for one minute, unless otherwise stated. The weight of pro-liposome added to bulk deionised water was dependent upon the composition of the individual pro-liposome, which is detailed in the appropriate methodology in section 5.4. However, unless otherwise stated, the lipid concentration of
the liposome dispersions was 60 mg/g and the dispersions always contained 10 mg of cyA.

5.3.3 Converting cyA pro-liposomes into liposomes in two stages
Two stage conversion was carried out in a similar manner as described in section 3.4.4. Intimate mixing of the deionised water for hydration and cyA pro-liposome was achieved by passing these two components between two cleaned Eppendorf syringes twenty times. The amounts of deionised water for hydration and pro-liposome used in two stage conversion are detailed in the appropriate methodology in section 5.4. All hydrated cyA pro-liposomes were made with a 15% overage to account for the loss during the handling and processing of this gel. After thoroughly Eppendorf mixing the water and pro-liposome twenty times, a thick gel generally resulted, which was usually immediately added to the remaining bulk deionised water and dispersed by vigorous handshaking for 90 seconds. However, in some experiments investigating the robustness of a formulation, the cyA pro-liposome and deionised water for hydration were not mixed nor added to the bulk deionised water immediately. In these cases, the Eppendorf mixing and/or addition to bulk deionised water were delayed for 24 hours before converting the hydrated cyA pro-liposome into a liposome dispersion.

5.3.4 Cleaning syringes and containers
Care was taken to minimise the contamination of the dispersions with extraneous particulates by filtering the deionised water used to form liposomes. Additionally, all containers and non-sterile syringes were thoroughly washed with this filtered deionised water. Filtering was achieved by passing the deionised water through a sterile Acrodisc (Gelman Science, Michigan, USA), which is made up of a stacked 0.8 µm pre-filter and a 0.22 µm filter in a plastic filter and syringe holder.

5.3.5 Analytical filtration
Analytical filtration involved the separation of the solid material from the liposomes by filtering each dispersion through a polycarbonate filter with a 200 nm pore size (Cyclopore, Whatman Int. Ltd., Maidstone, England). The details of this filtration procedure are described in section 5.3.5.1. After drying the filters in an oven, the filters retaining the solids were weighed and inspected under light microscopy, and sometimes SEM to determine the nature of the residue. To ensure most of the solid material
originated from non-debris precipitate, care had to be taken to minimise the contamination from extraneous sources. Hence the deionised water employed for producing liposome dispersions, rinsing containers and flushing the filters was pre-filtered. If this deionised water was not filtered through a sterile 0.22 μm filter, the polycarbonate filter would have become excessively contaminated with debris after analytical filtration. Unless otherwise stated, analytical filtration was carried out on each of the three repeat samples at room temperature within six hours of generating the cyA liposome dispersions.

5.3.5.1 Filtration

Unless otherwise stated, each 25 mm diameter 0.2 μm polycarbonate filter was weighed on a Sartorius balance in milligrams to one d.p. before placing in the filter holder. For a greater degree of accuracy, the filters used in section 5.4.4 were weighed in milligrams to three d.p. on a Mettler M3 (Mettler Instrumente GMBH, Zürich, Switzerland) pan balance. After accurately weighing the filter, the filter was placed shiny side up on a filter support inside a stainless steel syringe and filter holder (Whatman Int., Maidstone, England). The filter holder was tightened and a 5 ml syringe containing sufficient liposome dispersion containing 10 mg of cyA was attached to the holder. The dispersion was passed through the holder containing the 0.2 μm polycarbonate filter by depressing the syringe plunger. The resistance or ease of filtration for each sample was noted and recorded. This subjective filtration resistance provided an indirect indication as to the extent of filter pore occlusion by solid residue within the liposome dispersion. This resistance was only an indirect indicator because the size of the liposome also affected the resistance somewhat: coarser drug-free dispersions were more difficult to filter than finer drug-free dispersions. The resistance encountered when extruding each dispersion through the filter was judged on a subjective scale from “I” to “III”, and is stated in the appropriate results tables below for each dispersion. “I” denoted a comparatively low resistance was encountered when the dispersion was passed through the filter, which implied the dispersion did not severely block the pores of the filter. At the other end of the scale, “III” denoted that a great resistance was encountered, indicating that the dispersion had occluded many of the filter pores.

After filtration of the dispersion, the syringe and filter support were both flushed with five x 5 ml aliquots of filtered deionised water. This aided the removal of any residual
liposomes containing cyA from the filter and left only the insoluble residues behind. After the liposomes had been passed through from the filter, the filter support was unscrewed and the moist filter with any solid residue was carefully removed with metal forceps. This filter was dried shiny side up on a cleaned watch glass in an oven heated to 50 °C for between five and ten minutes. The 10 μm thick polycarbonate filters dried rapidly and appeared completely opaque and white when dry. If the filter was improperly washed and residual cyA liposome dispersion remained, after drying, transparent greasy patches were visible on the filter with the naked eye. Under light microscopy at a magnification of x400 no large liposomes were visible on these greasy patches and it seemed that drying may have induced liposome collapse.

5.3.5.2 Filter analysis

i) Gravimetric analysis

All filters, after filtration, were carefully handled shiny side up with forceps to avoid loss of residue. The filters were re-weighed on a Sartorius balance in milligrams to one d.p. unless otherwise stated. The weight of retained residue from each filtrate was calculated using equation 5.2:

\[
\text{Weight of residue (mg)} = [\text{Weight of filter post filtration (mg)}] - [\text{Weight of filter pre-filtration(mg)}] \quad \text{Equation 5.2}
\]

The average and the standard deviation of the three repeats for each formulation were calculated. The weight of the filters used in section 5.5.4 were weighed with a greater degree of accuracy in milligrams to three d.p. using a Mettler M3 pan balance.

ii) Macroscopic and microscopic examination

After gravimetric analysis of the filter, the retained solid material on the filter was examined by:

i) Visual inspection: Large amounts of precipitate on the shiny upper surface of the filter appeared as a covering of fine white powder to the naked eye. Small amounts could not be visualised by the naked eye and examination under a light microscope or scanning electron microscope was required.

ii) Light microscopy: The light microscope, described in section 3.3.8.2, was employed to visualise the filters. The whole area of each filter was inspected under x100 magnification. At this magnification, 2-3 representative fields of each filter with precipitate formation were photographed. The smaller precipitate structures were
examined in greater detail, either by raising the light microscope magnification to x200 or x400 fold and/or employing SEM to view the structures.

iii) Scanning electron microscopy (SEM): A Phillips XL20 scanning electron microscope (Phillips Analytical, UK) was employed to provide a more detailed examination of the surface of some polycarbonate filters. The filters were mounted on double sided adhesive tape on aluminium stubs, and subsequently coated with a fine layer of gold using a sputter coater (Emitech K550) for approximately two minutes.

5.3.6 Sizing techniques
Sizing of the cyA liposomes was carried out prior to analytical filtration within six hours of manufacturing the dispersions. Chiefly, PCS and laser diffraction were employed to assess the particle size and particle size distribution of some cyA liposome dispersions. Details of PCS and laser diffraction are described in section 3.3.8.3 and section 3.3.8.4 respectively.

5.3.7 Statistical analysis
The statistical tests, described in section 3.3.5, were employed to compare the particle size of the dispersions in section 5.4.4.

5.4 Studies performed
5.4.1 Controls
5.4.1.1 Analytical filtration of drug-free egg PL dispersions
Egg PL pro-liposomes without cyA, as described in section 3.3.2, were converted into drug-free liposomes either in one stage or in two stages. The egg PL: ethanol: glycerol weight ratio was 60:24:26. One stage conversion was carried out, as described in section 5.3.2, by adding 0.550 g of egg PL pro-liposomes to 4.450 g of bulk deionised water in a 10 ml glass vial and handshaking vigorously. The lipid concentration of this dispersion was 60 mg/g.

Two stage conversion of egg PL pro-liposomes was carried out, as described in section 5.3.3, by passing 0.316 g (0.275 g + 15% overage) of deionised water for hydration and 0.633 g (0.550 g + 15% overage) of egg PL pro-liposomes between two Eppendorf syringes twenty times. Liposome dispersions yielding a lipid concentration of 60 mg/g were generated by vigorously handshaking 0.825 g of the resultant hydrated gel in 4.175 g of bulk deionised water for one minute.
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These drug-free dispersions were filtered through 0.2 μm polycarbonate filters to determine the amount of residue retained on the filter from liposomes without cyA. The increase in weight was measured in milligrams to one d.p. using the Sartorius balance.

5.4.1.2 Analytical filtration of drug-free soya PC/EPG dispersions

Drug-free dispersions were generated from pro-liposomes in two stages. Hydrated pro-liposomes were produced by passing 0.644 g (0.560 g ± 15% overage) of a pro-liposome with a soya PC: EPG: ethanol: glycerol weight ratio of 49.75:0.25:36:26 and 0.345 g (0.300 g ± 15% overage) of deionised water for hydration between two Eppendorf syringes twenty times. Liposome dispersions with a lipid concentration of 60 mg/g were generated by vigorously shaking 0.860 g of the hydrated pro-liposome in 4.140 g of bulk deionised water for 90 seconds. The resultant dispersions were analytically filtered and the filter was weighed in milligrams to three d.p. as described in section 5.3.5.

5.4.1.3 Diluting cyA dissolved in ethanol and glycerol

Two solvent systems were employed for cyA, having ethanol: glycerol: cyA weight ratios of 24:26:2 and 48:36:2. Both solvents systems were diluted with water to yield a cyA concentration of 2 mg/g. The resultant diluted systems were analytically filtered as described in section 5.3.5 and the filters were observed under the light microscope and SEM.

5.4.2 One stage conversion of cyA pro-liposomes into cyA liposome dispersions

5.4.2.1 Ethanol cyA pro-liposomes

CyA pro-liposomes containing egg PL: ethanol: cyA in a weight ratio of 60:24:2, were produced without adding glycerol to the formulation. Liposome dispersions were generated, as described in section 5.3.2, by vigorously handshaking 0.430 g of this glycerol-free cyA pro-liposome with 4.570 g of bulk deionised water for one minute. The lipid concentration and cyA concentration of these dispersions were 60 mg/g and 2 mg/g respectively. The resultant dispersions were analytically filtered, as described in section 5.3.5, to recover any precipitate from the dispersion.

5.4.2.2 Raising ethanol level in glycerol-free cyA pro-liposomes

The ethanol level of the pro-liposome was increased to give an egg PL: ethanol: cyA weight ratio of 60:36:2. Liposome dispersions were converted in one stage, described in
section 5.3.2, by vigorously handshaking 0.490 g of this particular cyA pro-liposome with 4.510 g of deionised water for one minute. The lipid concentration and cyA concentration were 60 mg/g and 2 mg/g respectively. The cyA liposome dispersions were subsequently analytically filtered as described in section 5.3.5.

5.4.2.3 CyA pro-liposomes containing glycerol
Glycerol was added to this egg PL cyA pro-liposome. The egg PL: ethanol: glycerol: cyA weight ratio of this particular cyA pro-liposome was 60:24:26:2. Liposome dispersions were generated by adding 0.560 g of this cyA pro-liposome containing glycerol to 4.440 g of bulk deionised water. These two components were converted into liposome dispersions by either:

i) Immediately vigorously handshaking for one minute, or

ii) Leaving to convert without external agitation.

Both sets of liposome dispersions containing 60 mg/g of lipid and 2 mg/g of cyA were filtered, as described in section 5.3.5, to recover any solid residues present.

5.4.2.4 Short term storage of cyA liposome dispersions
The pro-liposome with an egg PL: ethanol: glycerol: cyA weight ratio of 60:24:26:2, described in section 5.4.2.3, was converted into liposome dispersions in one stage, described in section 5.3.2, by vigorous handshaking for one minute. The resultant dispersions were stored for 24 hours at 4 °C and 20 °C to establish the effect of short term storage on residue formation. After 24 hours had elapsed, the dispersions were filtered and the filters were inspected as described in section 5.3.5.

5.4.2.5 Raising the ethanol level of cyA pro-liposomes containing glycerol
The ethanol level of the cyA pro-liposome containing glycerol was increased, to give an egg PL: ethanol: glycerol: cyA weight ratio of 60:48:26:2. Dispersions with a lipid concentration and cyA concentration of 60 mg/g and 2 mg/g were generated, as described in section 5.3.2, by adding 0.680 g of this pro-liposome to 4.320 g of bulk deionised water in one stage. These two components were vigorously handshaken for one minute to yield liposome dispersions, which were subsequently analytically filtered (section 5.3.5).
5.4.2.6 Raising the lipid: cyA weight ratio
The amount of cyA dissolved in the pro-liposome was halved to give an egg PL: ethanol: glycerol: cyA weight ratio of 60:24:26:1. To generate a liposome dispersion with a lipid concentration of 60 mg/g and a cyA concentration of 1 mg/g, 1.110 g of this cyA pro-liposome was added to 8.890 g of deionised water and vigorously handshaken for one minute, as described in section 5.3.2. Due to the halving of the cyA concentration in the final liposome dispersion, the amount of dispersion subjected to analytical filtration (section 5.3.5) was doubled to 10 g in order to maintain the amount of cyA filtered at 10 mg.

5.4.3 Two stage conversion
As discussed in section 3.5.4.2, adding a small amount of deionised water to form a hydrated pro-liposome improved the particle size distribution of the resultant liposomes. The impact of this two stage conversion on the association of cyA with liposomes was examined in the following study. The effect of the first stage of hydration was studied by adding varying levels of deionised water for hydration to various cyA pro-liposomes (section 5.3.3).

5.4.3.1 Conversion of cyA pro-liposomes in two stages
For the following study investigating two stage conversion, the egg PL: ethanol: glycerol: cyA weight ratio for the cyA pro-liposome was 60:24:26:2. To hydrate this pro-liposome, 0.316 g (0.275 g + 15% overage) of filtered deionised water and 0.644 g (0.560 g + 15% overage) of the pro-liposome were passed between two Eppendorf syringes twenty times. Liposome dispersions were generated by immediately vigorously handshaking 0.835 g of this hazy gel in 4.165 g of filtered deionised water for 90 seconds. The lipid concentration and cyA concentration of the resultant dispersions were 60 mg/g and 2 mg/g respectively. The liposome dispersions were analytically filtered in the usual manner (section 5.3.5), and after washing and drying the filters, the filters were weighed and examined under the light microscope.

5.4.3.2 Equilibrating hydrated cyA pro-liposomes
Two stage conversion was carried out with the amounts of cyA pro-liposome and deionised water for hydration described in section 5.4.3.1. The only difference was that the hydrated cyA pro-liposome was left to equilibrate at room temperature for 24 hours before it was added to the bulk deionised water. After dispersing the gel by vigorous
handshaking for 90 seconds, the cyA dispersions were immediately analytically filtered (section 5.3.5).

5.4.3.3 Hydration of cyA pro-liposomes with reduced levels of deionised water
The effect of reducing the level of deionised water for hydration added to the pro-liposome with an egg PL: ethanol: glycerol: cyA weight ratio of 60:24:26:2 was studied. The amount of deionised water added to hydrate 0.644 g (0.560 g + 15% overage) of this cyA pro-liposome was 0.265 g (0.230 g + 15% overage) and 0.173 g (0.150 g + 15% overage). The cyA pro-liposome and deionised water for hydration were mixed by passing the components between two Eppendorf syringes twenty times. To generate liposome dispersions, 0.790 g of the cyA pro-liposome hydrated with 0.265 g of deionised water and 0.710 g of the cyA pro-liposome hydrated with 0.173 g of deionised water were added to bulk deionised water, sufficient to give a final dispersion weight of 5 g. The gels were dispersed in the bulk deionised water by vigorous handshaking for 90 seconds. The resultant dispersions containing 60 mg/g of lipid and 2 mg/g of cyA were filtered and the surfaces of the polycarbonate filters were examined for the presence of solid material as described in section 5.3.5.

5.4.3.4 Delaying the mixing of pro-liposome with deionised water for hydration
For this study, the pro-liposome with an egg PL: ethanol: glycerol: cyA weight ratio of 60:24:26:2 was employed. Two factors potentially affecting cyA association were studied: firstly, the effects of delaying the mixing of deionised water for hydration with this cyA pro-liposome were investigated. Secondly, delaying the addition of the resultant hydrated cyA pro-liposome to bulk deionised water was evaluated. To hydrate the pro-liposome, 0.173 g (0.150 g + 15% overage) of deionised water for hydration and 0.644 g (0.560 g + 15% overage) of the pro-liposome were passed between two Eppendorf syringes twenty times. This mixing was carried out either immediately or after storing the pro-liposome and deionised water for hydration for 24 hours at room temperature. These two sets of gels (0.710 g) were subsequently added to 4.290 g of bulk deionised water either immediately or after delaying for another 24 hours to produce a total of four sets of dispersions. The egg PL concentration and cyA of the resultant dispersions were 60 mg/g and 2 mg/g respectively. The four sets of cyA dispersions were analytically filtered as described in section 5.3.5.

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5.4.3.5 Effect of ethanol level in cyA pro-liposomes on cyA association with liposomes

5.4.3.5.1 High ethanol level

The effect of a raised cyA pro-liposome ethanol level on the association of cyA with liposomes converted in two stages was investigated by doubling the ethanol level of the egg PL pro-liposome. Therefore, the egg PL: ethanol: glycerol: cyA weight ratio was 60:48:26:2. Three sets of hydrated cyA pro-liposomes were formed by Eppendorf mixing 0.161 g (0.140 g + 15% overage), 0.316 g (0.275 g + 15% overage) and 0.633 g (0.550 g + 15% overage) of deionised water for hydration with 0.782 g (0.680 g + 15% overage) of the high ethanol cyA pro-liposome twenty times. To generate liposome dispersions, 0.820 g, 0.955 g and 1.230 g respectively of the three hydrated pro-liposomes were immediately vigorously handshaken in deionised water, sufficient to produce final dispersion weights of 5 g. The resultant liposome dispersions had a lipid concentration of 60 mg/g and a cyA concentration of 2 mg/g. Analytical filtration was carried out on each of these dispersions, as described in section 5.3.5, to recover any solid residue present.

5.4.3.5.2 Medium ethanol level

The ethanol level of the egg PL pro-liposome was changed to 36 parts by weight, to give an egg PL: ethanol: glycerol: cyA weight ratio of 60:36:26:2. The pro-liposome was hydrated by passing 0.316 g (0.275 g + 15% overage) of filtered deionised water and 0.713 g (0.620 g + 15% overage) of this cyA pro-liposome between two Eppendorf syringes twenty times. Liposome dispersions with an egg PL concentration of 60 mg/g and cyA concentration of 1 mg/g were generated by vigorously handshaking 0.895 g of this hydrated pro-liposome in 4.105 g of filtered deionised water for 90 seconds. The resultant dispersions were analytically filtered, as described in section 5.3.5., to recover solid residue present.

5.4.3.6 Medium ethanol formulation with soya PL blend

This following pro-liposome employed a soya PC: soya PL: ethanol: glycerol: cyA weight ratio of 54:6:36:24:2. This soya PL blend cyA pro-liposome was hydrated by passing 0.316 g (0.275 g + 15% overage) of deionised water for hydration and 0.713 g (0.620 g + 15% overage) of soya PL blend cyA pro-liposome between two Eppendorf syringes twenty times. Liposome dispersions with a lipid concentration of 60 mg/g and a cyA concentration of 2 mg/g were generated by dispersing 0.895 g of this hydrated pro-
liposome with 4.105 g of deionised water. The resultant cyA liposome dispersions were analytically filtered to recover solid residues as described in section 5.3.5. The filters were examined under the light microscope and with SEM.

5.4.3.7 Ethanol cyA pro-liposomes

The effect of omitting glycerol from cyA pro-liposomes on the association of cyA with liposomes was investigated. A pro-liposome with a soya PC: soya PL: ethanol: cyA weight ratio of 54:6:36:2 was employed in the following study. This glycerol-free cyA pro-liposome was converted into a liposome dispersion in two stages. To form a hydrated pro-liposome, 0.316 g of deionised water (0.275 g + 15% overage) and 0.564 g (0.490 g + 15% overage) of the glycerol free pro-liposome were passed between two Eppendorf syringes twenty times. Liposome dispersions were formed by vigorously handshaking 0.765 g of this yellow cream-like cyA pro-liposome in 4.235 g of filtered deionised water for 90 seconds. The resultant dispersions with a lipid concentration and a cyA concentration of 60 mg/g and 2 mg/g respectively were subsequently analytically filtered as described in section 5.3.5.

5.4.3.8 Lowering the soya PL blend: cyA weight ratio

The effect of lower soya PL blend: cyA weight ratios on cyA association were investigated using two pro-liposomes with soya PC: soya PL: ethanol: glycerol: cyA weight ratios of 45:5:36:26:2 and 36:4:36:26:2. To generate hydrated pro-liposomes, 0.316 g (0.275 g + 15% overage) of deionised water and 0.656 g (0.570 g + 15% overage) of the pro-liposome which had a soya PL blend: cyA weight ratio of 50:2 were passed between two Eppendorf syringes twenty times. Liposome dispersions were generated by vigorously handshaking 0.845 g of this hydrated gel in 4.155 g of bulk deionised water for 90 seconds. The lipid concentration of these dispersions was 50 mg/g and the cyA concentration was 2 mg/g. The other pro-liposome, which had a soya PL blend: cyA weight ratio of 40:2, was converted in two stages in a similar manner: the pro-liposome was hydrated by passing 0.316 g (0.275 g + 15% overage) of deionised water and 0.598 g (0.520 g + 15% overage) of the appropriate pro-liposome between two Eppendorf syringes twenty times. Dispersions having a 40 mg/g lipid concentration were formed by vigorously handshaking 0.795 g of the resultant hydrated pro-liposome in 4.205 g of bulk deionised water for 90 seconds. The cyA dispersions
were filtered through polycarbonate filters, which were subsequently weighed and inspected by light microscopy as described in section 5.3.5.

5.4.3.9 Lowering the soya PL blend: cyA weight ratio without mixing
The experiments described in section 5.4.3.8 were repeated, except that instead of immediately mixing the cyA pro-liposomes and deionised water for hydration, these two components were left for 24 hours at room temperature without mixing. After the 24 hours had elapsed the two components were added without Eppendorf mixing to the bulk deionised water and dispersed by handshaking for 90 seconds. The resultant dispersions were analytically filtered to recover any solids present as described in section 5.3.5. The filters were examined with light microscopy and SEM.

5.4.3.10 Blending egg phosphatidylglycerol (EPG) with soya PC
The association of cyA in soya PC liposomes with three low levels of EPG was investigated. The soya PC: EPG: ethanol: glycerol: cyA weight ratios of the three pro-liposomes were 39.8:0.2:36:26:2, 39.6:0.4:36:26:2 and 39.4:0.6:36:34:2. In order to hydrate the three pro-liposomes, 0.316 g (0.275 g + 15% overage) of deionised water for hydration and 0.598 g (0.520 g + 15% overage) of each cyA pro-liposome were passed between two Eppendorf syringes twenty times. Liposome dispersions with a lipid concentration of 40 mg/g were generated by vigorously handshaking 0.795 g of each pro-liposome in 4.205 g of bulk deionised water. The dispersions were analytically filtered, as described in section 5.3.5, to recover any solid residue. The filters were examined with light microscopy and SEM.

5.4.4 Particle size and cyA association
The effects of individually varying the level of deionised water for hydration, ethanol and lipid on cyA liposome size and the cyA association were investigated. CyA pro-liposomes with a soya PC: EPG weight ratio of 99.5:0.5 were selected for the following sets of experiments.

5.4.4.1 Influence of varying the amount of deionised water for hydration
Three increasing levels of deionised water were added to 0.656 g (0.570 g + 15% overage) of a pro-liposome with a soya PC: EPG: ethanol: glycerol: cyA weight ratio of 49.75:0.25:36:26:2. The three levels of deionised water were 0.230 g (0.200 g + 15% overage), 0.345 g (0.300 g + 15% overage) and 0.460 g (0.400 g + 15% overage). Each
of the three cyA pro-liposomes with deionised water for hydration were passed between two Eppendorf syringes twenty times to form hydrated cyA pro-liposomes. Liposome dispersions were produced by vigorously handshaking 0.770 g, 0.870 g and 0.970 g respectively of the hydrated cyA pro-liposomes for 90 seconds in sufficient bulk deionised water to give a final dispersion weight of 5 g. The lipid concentration and the cyA concentration of the dispersions was 50 mg/g and 2 mg/g respectively. All cyA liposome dispersions were sized (section 5.3.6) before analytical filtration (section 5.3.5). Gravimetric analysis was carried out by measuring the weight of the filters in milligrams to three d.p. To account for debris introduced during the two stage hydration, the average weight increase of the dispersions without cyA (section 5.4.4.1) was subtracted from the average weight increase of the filters.

5.4.4.2 Influence of varying the ethanol level
The influence of raising the ethanol level of cyA pro-liposomes on liposome particle size and cyA association was examined using two pro-liposomes with soya PC: EPG: ethanol: glycerol: cyA weight ratios of 49.75:0.25:24:26:2, and 49.75:0.25:48:26:2. The two respective pro-liposomes were hydrated by Eppendorf mixing 0.587 g (0.510 g + 15% overage) and 0.725 g (0.630 g + 15% overage) of the pro-liposomes with 0.460 g (0.400 g + 15% overage) of deionised water twenty times. The liposome dispersions were generated by vigorously handshaking 0.910 g and 1.030 g of the respective hydrated pro-liposomes in filtered deionised water, sufficient to give a final dispersion weight of 5 g. The lipid concentration and cyA concentration of the dispersions were 50 mg/g and 2 mg/g respectively. The resultant cyA dispersions were sized, as described in section 5.3.6, prior to analytical filtration (section 5.3.5). The filters were weighed in milligrams to three d.p. To account for the introduction of non-cyA solids during the two stage hydration, the average weight increase of the dispersions without cyA (section 5.4.1.2) was subtracted from the average weight increase of the filters post cyA filtration.

5.4.4.3 Influence of varying the lipid: cyA ratio
The influence of reducing the lipid: cyA weight ratio was examined using two cyA pro-liposomes with soya PC: EPG: ethanol: glycerol: cyA weight ratios of 29.85:0.15:36:24:26:2 and 39.8:0.2:36:26:2. The two pro-liposomes were hydrated by Eppendorf mixing 0.541 g (0.470 g + 15% overage) and 0.598 g (0.520 g + 15%
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overage) of the respective pro-liposomes with 0.460 g (0.350 g) of deionised water for hydration twenty times. The two sets of liposome dispersions were generated by vigorously handshaking 0.870 g and 0.970 g of the respective hydrated pro-liposomes for 90 seconds in sufficient deionised water to give a final dispersion weight of 5 g. The lipid concentration of these two sets of dispersions was 30 mg/g and 40 mg/g respectively, the cyA concentration in both sets of dispersions was 2 mg/g. The resultant cyA dispersions were sized (section 5.3.6) prior to analytical filtration (section 5.3.5). The filters were weighed in milligrams to three d.p., and the average weight increase of the dispersions without cyA (section 5.4.2.2) was subtracted.

5.5 Results and Discussion

This section has been divided into four main parts. Part one (section 5.5.1) discusses the filtration of controls. Part two (section 5.5.2) focuses upon the degree of cyA association after converting cyA pro-liposomes into liposomes in one stage. Part three (section 5.5.3) discusses the association of cyA with liposomes generated from a two stage cyA pro-liposome conversion. The final part (section 5.5.4) concentrates upon factors which influence both the degree of cyA association and the particle size of cyA liposomes converted from cyA pro-liposomes in two stages.

5.5.1 Controls

5.5.1.1 Analytical filtration of drug-free liposome dispersions

The ease of filtration and amount of residue recovered from drug-free dispersions converted in one stage and two stages is shown in Table 5.1.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ease of filtration</th>
<th>Average increase in filter weight ± s.d. (mg)</th>
<th>Appearance of filter under the light microscope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg PL PRO (IS)</td>
<td>II</td>
<td>0.0 ± 0.0</td>
<td>No crystalline material. Small amount of debris</td>
</tr>
<tr>
<td>Egg PL PRO (2S)</td>
<td>I</td>
<td>0.0 ± 0.0</td>
<td>No crystalline material. A few long fibres and small amount of debris</td>
</tr>
</tbody>
</table>

The drug-free sample converted in two stages passed through the filter with somewhat more ease than the liposomes converted in one stage, perhaps this was attributable to the slightly larger average size of the liposomes converted in one stage. From previous particle size studies, discussed in section 3.5, it was evident that liposomes converted in one stage (section 3.5.2) were considerably larger than liposomes converted in two
stages (section 3.5.4). The liposomes converted in two stages have diameters close to 200 nm (the same size as the pore size of the filter employed in the analytical filtration), compared to over 400 nm for the one stage converted dispersions.

Although SEM and light microscopy detected a small amount of non-crystalline debris on the filters from both drug-free dispersions, the weight of the filters, to one d.p. of a milligram, did not increase after passage of blank liposome dispersions. This small amount of debris on the filter seemed unavoidable when non-aseptic laboratory conditions were used to manufacture and to process the pro-liposomes and the resultant liposomes. The debris mainly composed of long fibres. It was unclear whether or not this small quantity of debris was either present in starting materials and/or introduced during processing.

5.5.1.2 Analytical filtration of drug-free liposome dispersions converted in two stages
The M3 Mettler balance only became available during the study investigating the factors affecting particle size and association (section 5.5.4). This particular balance enabled the filters to be accurately measured in milligrams to three d.p.

The filter weight increase in section 5.5.1.2, measured using the more accurate Mettler balance, largely mirrored the weight increase obtained in section 5.5.3, which employed the less sensitive Sartorius balance. Drug-free liposome dispersions converted in two stages yielded a weight increase of less than 0.1 mg when measured using the M3 Mettler balance. In comparison, no weight increase was detected when similar filters (Table 5.1) were measured in milligrams to only one d.p. on the Sartorius balance.

<table>
<thead>
<tr>
<th>Table 5.2 Analytical filtration of soya PC: EPG dispersion without cyA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro-liposome weight ratio</td>
</tr>
<tr>
<td>---------------------------</td>
</tr>
<tr>
<td>39.85:0.15:36:26</td>
</tr>
</tbody>
</table>

5.5.1.3 Diluting cyA dissolved in ethanol and glycerol
The dilution of cyA dissolved in ethanol and glycerol resulted in major precipitation (Table 5.3). Diluting the solvent mixes with deionised water to a cyA concentration of 2 mg/g resulted in the immediate precipitation of cyA. The diluted mixes appeared opaque and flocculates of the precipitated powder were visible to the naked eye. Over 87% or more of the cyA was recovered from these diluted mixes. Under the light microscope this powder seemed fine and appeared as a fine dense mass. The unretained
cyA may have adhered to the surface of the filter holder during the processing of the diluted mix. Additionally, some of this cyA may have dissolved in the water used for diluting and washing the filter and some may have been in a fine precipitate capable of passing through the filter.

<table>
<thead>
<tr>
<th>Ethanol: glycerol: cyA weight ratio diluted with water to give 2 mg/g cyA</th>
<th>Ease of filtration</th>
<th>Average increase in filter weight ± s.d. (mg)</th>
<th>Appearance of filter under the light microscope</th>
</tr>
</thead>
<tbody>
<tr>
<td>24:26:2</td>
<td>III</td>
<td>8.7 ± 0.4</td>
<td>Dense mass of powder</td>
</tr>
<tr>
<td>48:26:2</td>
<td>III</td>
<td>8.9 ± 0.5</td>
<td>Dense mass of powder</td>
</tr>
</tbody>
</table>

5.5.2 One stage conversion

Egg PL pro-liposomes containing cyA were converted in one stage into liposome dispersions with a lipid concentration of 60 mg/g. The amounts of the components in the individual cyA pro-liposomes are outlined in section 5.4.

5.5.2.1 Ethanol cyA pro-liposomes

One stage conversion of a cyA liposome dispersion from the pro-liposome with an egg PL: ethanol: cyA weight ratio of 60:24:2 was rapid when the pro-liposome and bulk water were handshaken vigorously. The amount of precipitation recovered from this dispersion is shown in Table 5.4.

Table 5.4 Association of cyA with liposomes converted in one stage from an egg PL pro-liposome

<table>
<thead>
<tr>
<th>CyA pro-liposome weight ratio</th>
<th>Ease of filtration</th>
<th>Average increase in filter weight ± s.d. (mg)</th>
<th>Appearance of filter under the light microscope</th>
</tr>
</thead>
<tbody>
<tr>
<td>60:24:36:2 (IS)</td>
<td>III</td>
<td>1.4 ± 0.2</td>
<td>Vast amount of spherical precipitation. Diameter ranged from few to 50 μm. Though most were 10-25 μm in diameter</td>
</tr>
</tbody>
</table>

The liposome dispersions generated from this egg PL pro-liposome were difficult to filter. The large amount of force required to overcome this resistance suggested that many of the filter pores had become occluded during the filtration of the dispersions. Indeed upon visual inspection of shiny upper side of the filter, a covering of fine white powder, which weighed approximately 1.4 mg, could be observed macroscopically by the naked eye. The precipitate on the filter was distinct from the cyA powder (Plate 5.1), but similar in shape to the cyA precipitated from an ethanol: glycerol solution (Plate 5.2). Under x100 magnification of a light microscope, it was clear that this precipitate composed largely spherical individual particles with diameters between approximately 5 μm and 50 μm (Plate 5.3). The formation of this precipitate in this
dispersion demonstrated that even when there were approximately 50 molecules of lipid to every molecule of cyA, association of cyA into the liposome bilayer was disappointingly poor. This contrasts with the commercially available micelle formulation (Sandimmun® IV data sheet, 1996), which fully solubilises cyA when diluted in water to a cyA concentration of 2.5 - 0.5 mg/ml.

It is possible that the unsolubilised cyA precipitated along with phospholipid present in the dispersion. Subjective evidence for this type of precipitation is provided by SEM, which indicates that the surface of the spheres are “wax-like”. As a result of the possible precipitation of cyA with lipid, the gravimetric analysis of the filter could not be used to accurately determine the amount of cyA associated with the liposomes, since the lipid associated with precipitated cyA would have contributed to the increase in filter weight. Therefore, if the increase in filter weight after analytical filtration was subtracted from the total amount of cyA in the formulation, the level of cyA molecularly associated with the liposomes would have been underestimated. However, in this study the weights of the solids retained by the analytical filtration can be employed to indirectly assess the minimum level of association, if it is assumed that increase in the filter weight is entirely due to cyA. To quantitatively ascertain the degree of unassociated cyA with a greater degree of accuracy, it would be necessary to employ HPLC to analyse the cyA retained on the filter.
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Plate 5.2 Typical SEM of cyA precipitated from ethanol:glycerol (Bar = 2 μm), as described in section 5.5.2.1

Plate 5.3 Typical light micrograph of precipitate recovered from a liposome dispersion generated in one stage from an egg PL pro-liposome containing cyA (Bar = 100 μm), as described in section 5.5.2.1

5.5.2.2 Raising the ethanol level in ethanol cyA pro-liposomes

The results for the analytical filtration of the cyA dispersions converted in one stage from a pro-liposome with an egg PL: ethanol: cyA weight of 60:36:2 containing a raised ethanol level are shown in Table 5.5.

<table>
<thead>
<tr>
<th>CyA pro-liposome weight ratio</th>
<th>Ease of filtration</th>
<th>Average increase in filter weight ± s.d. (mg)</th>
<th>Appearance of filter under the light microscope</th>
</tr>
</thead>
<tbody>
<tr>
<td>60:36:2</td>
<td>III</td>
<td>2.5 ± 0.3</td>
<td>Copious amount of spherical precipitation</td>
</tr>
</tbody>
</table>

Table 5.5 Association of cyA with liposomes converted in one stage from an egg PL pro-liposome

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Raising the ethanol level in glycerol-free cyA pro-liposomes generated even larger amounts of precipitate. After analytical filtration, an average weight of 2.5 mg of residue was recovered from each liposome dispersion. This indicated that a higher level of ethanol in the glycerol free cyA pro-liposomes significantly \((p < 0.05)\) reduced the intercalation of cyA into the liposomes, if one stage conversion was adopted. It seemed that some of the cyA dissolved in the ethanol may have precipitated out upon the addition of the cyA pro-liposome to bulk deionised water.

5.5.2.3 CyA pro-liposomes containing glycerol

The pro-liposome with an egg PL: ethanol: glycerol: cyA weight ratio of 60:24:26:2 was added to deionised water and converted into liposome dispersions by either vigorously handshaking for one minute or leaving to convert without external agitation. The amounts of residue recovered by filtration from both sets of cyA liposome dispersions are shown in Table 5.6.

<table>
<thead>
<tr>
<th>CyA egg PL pro-liposome subjected to:</th>
<th>Ease of filtration</th>
<th>Average increase in filter weight ± s.d. (mg)</th>
<th>Appearance of filter under the light microscope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immediate vigorous handshaking</td>
<td>III</td>
<td>0.5 ± 0.1</td>
<td>Moderate amount of spherical precipitation</td>
</tr>
<tr>
<td>No agitation</td>
<td>III</td>
<td>0.8 ± 0.2</td>
<td>High level of spherical precipitation</td>
</tr>
</tbody>
</table>

Inclusion of glycerol into the cyA pro-liposome formulation had a beneficial influence on the incorporation of cyA into liposomes. As is evident from both the gravimetric analysis and inspection under the light microscope, the presence of glycerol in the cyA pro-liposome significantly \((p < 0.05)\) reduced the formation of the insoluble precipitate. The weight of precipitate was reduced from 1.4 mg if glycerol was omitted (Table 5.4), to about 0.5 mg if glycerol was incorporated into the cyA pro-liposome. The possible reason for this improved association is explained in section 5.5.3.8. Nevertheless, although the degree of cyA association was improved, even at this high lipid: cyA molecular ratio of 30:1, there was still a moderate amount of unsolubilised cyA in the dispersion as revealed by light microscopy (Plate 5.4). The dark circles seen in Plate 5.4 are the accumulation of fine precipitate around the unsupported areas of the filter.

It was apparent from the light microscopy results that the cyA dispersions converted in one stage without agitation had a greater amount of insoluble residue present compared
to the dispersions formed by vigorous handshaking. This indicated that the speed of the liposome formation may have influenced the intercalation of cyA into the bilayers of the liposomes. Perhaps without agitation, some cyA had time to diffuse and precipitate from the cyA pro-liposome before organised bilayers could be formed. However, this difference was not significantly different when the filter weight increase of the two sets of dispersions was compared (p = 0.08).

Plate 5.4 Typical light micrograph of precipitate recovered from liposome dispersion generated in one stage from an egg PL pro-liposome (Bar = 100 μm), as described in section 5.5.2.3

5.5.2.4 Short term storage of cyA dispersions
The cyA pro-liposome formulation, described in section 5.4.2.3, was converted into a liposome dispersion in one stage by vigorous handshaking and stored for 24 hours at 4°C and 20 °C. After 24 hours had elapsed, the dispersions were filtered and the filter was inspected in the usual manner. The details of the recovered residue are shown in Table 5.7.

Table 5.7 Association of cyA with liposomes formed in one stage from an egg PL pro-liposome after storage for 24 hours

<table>
<thead>
<tr>
<th>Storage conditions of cyA egg PL dispersion</th>
<th>Ease of filtration</th>
<th>Average increase in filter weight ± s.d. (mg)</th>
<th>Appearance of filter under the light microscope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stored for 24 hours at 4 °C</td>
<td>III</td>
<td>0.6 ± 0.1</td>
<td>Moderate amount of precipitation</td>
</tr>
<tr>
<td>Stored for 24 hours at 20 °C</td>
<td>III</td>
<td>0.5 ± 0.1</td>
<td>Moderate amount of precipitation</td>
</tr>
</tbody>
</table>

After analytical filtration, the light microscope and gravimetric examination of the filter revealed that the precipitate weight recovered from these dispersions was not different after 24 hours of storage at either 20 °C or 4 °C compared to those filtered immediately...
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after vigorous agitation (Table 5.6). This indicated that the precipitate formed during conversion did not equilibrate with the liposome dispersion within 24 hours of their formation. Once formed, this precipitate was not solubilised by the liposome dispersions under the conditions tested.

5.5.2.5 Raising the ethanol level in cyA pro-liposomes containing glycerol

The results of analytical filtration for the set of cyA dispersions converted in one stage from a pro-liposome with an egg PL: ethanol: glycerol: cyA weight ratio of 60:48:26:2, are shown in Table 5.8.

Table 5.8 Association of cyA with liposomes converted in one stage from an egg PL pro-liposome with raised ethanol level

<table>
<thead>
<tr>
<th>CyA pro-liposome weight ratio</th>
<th>Ease of filtration</th>
<th>Average increase in filter weight ± s.d. (mg)</th>
<th>Appearance of filter under the light microscope</th>
</tr>
</thead>
<tbody>
<tr>
<td>60:48:26:2</td>
<td>II</td>
<td>0.2 ± 0.1</td>
<td>Few spherical precipitates</td>
</tr>
</tbody>
</table>

After filtration of this dispersion, the filter weight increased slightly by 0.2 mg, which was markedly lower than the dispersion converted from cyA pro-liposomes with lower ethanol levels (Table 5.6). This reduction in the level of precipitate was confirmed by the presence of only a few 15-20 μm spherical particles under light microscopy. The higher amount of ethanol in the presence of glycerol clearly aided the intercalation of cyA into the liposome bilayers. This finding contrasted with the high ethanol cyA pro-liposome formulation omitting glycerol (section 5.5.2.2), which had reduced levels of cyA association. The improved cyA association was not due to cosolvency effects with ethanol, because when a solvent system containing ethanol: glycerol: cyA in a weight ratio of 48:26:2 was diluted in deionised water (Table 5.3), the solubility of cyA did not increase compared to the solvent system containing less ethanol.

5.5.2.6 Raising the lipid: cyA weight ratio of an egg PL pro-liposome

After analytically filtering 10 g of liposome dispersion containing 600 mg of egg PL and 10 mg of cyA, the amount of residue recovered from the filter was assessed (Table 5.9).

Table 5.9 Association of cyA with liposomes formed in one stage from an egg PL pro-liposome with lipid: cyA weight ratio of 60:1

<table>
<thead>
<tr>
<th>CyA pro-liposome weight ratio</th>
<th>Ease of filtration</th>
<th>Average increase in filter weight ± s.d. (mg)</th>
<th>Appearance of filter under the light microscope</th>
</tr>
</thead>
<tbody>
<tr>
<td>60:24:26:1</td>
<td>II</td>
<td>0.2 ± 0.1</td>
<td>Few spherical precipitates</td>
</tr>
</tbody>
</table>

Doubling the lipid weight ratio from 30 parts to 60 parts to carry 1 part of cyA clearly reduced the amount of precipitate formation. This was reflected by the smaller filter
weight increase after filtration of the dispersions. However, the filter weight increased by 0.2 mg and spherical deposits were still visible under the light microscope. Since a minimum of 19 molecules of phospholipid are reported to be required to incorporate 1 molecule of cyA (Fahr et al., 1994), the incomplete cyA association at a high molecular lipid: cyA ratio of approximately 100:1 showed that cyA loading into liposomes using one stage conversion was inefficient.

5.5.3 Two stage conversion

5.5.3.1 Conversion of cyA pro-liposomes in two stages

The egg PL cyA pro-liposome, which had an egg PL: ethanol: glycerol: cyA weight ratio of 60:24:26:2, was converted in two stages. The resultant cyA liposome dispersion was filtered in the usual manner, and after washing and drying the filter, the dried filter was weighed and examined under the light microscope. The results of which are shown in Table 5.10.

<table>
<thead>
<tr>
<th>Amount of cyA egg PL pro-liposome (g)</th>
<th>Amount of deionised water for hydration (g)</th>
<th>Ease of filtration</th>
<th>Average increase in filter weight ± s.d. (mg)</th>
<th>Appearance of filter under the light microscope</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.640</td>
<td>0.320</td>
<td>II-III</td>
<td>0.9 ± 0.2</td>
<td>Large number of spherical particles, some elongated structures.</td>
</tr>
</tbody>
</table>

It was evident from the increase in filter weight and the appearance of this filter under the light microscope, that a large amount of insoluble deposit was recovered from these cyA liposome dispersions after analytical filtration. The average weight of the fine white powder covering the surface of the filter was approximately 0.9 mg. The shape and dimensions of these deposits could be discerned and measured under the light microscope. Although most of the precipitate was spherical in shape and varied considerably in size from a few micrometers to 50 μm, some long strands of precipitate were also present (Plate 5.5). The dimensions of these slightly curved strands varied, the lengths ranged from about 5 μm up to 75 μm with breadths between about 5 μm to 10 μm. The surface properties of the precipitate could be visualised in greater detail under SEM: both the spherical (Plate 5.6) and elongated precipitate (Plate 5.7) possessed smooth rounded surfaces. Furthermore, this form of electron microscopy revealed the presence of fine micron sized crystalline precipitates covering the 0.2 μm pores of the filter (Plate 5.6), which collectively appeared as darkened circular regions.
under the light microscope and SEM (Plate 5.8). Perhaps this occlusion of the pores by the large precipitate and fine crystals explained why these dispersions were difficult to extrude through the filter.

Plate 5.5 Typical light micrograph showing precipitate strands recovered from a liposome dispersion converted in two stages from an egg PL pro-liposome (Bar = 25 μm), as described in section 5.5.3.1

Plate 5.6 Typical SEM of fine precipitate and a spherical precipitate recovered from a liposome dispersion converted in two stages from an egg PL pro-liposome (Bar = 5 μm), as described in section 5.5.3.1
Plate 5.7 Typical SEM of fine precipitate and a section of an elongated precipitate recovered from a liposome dispersion converted in two stages from an egg PL pro-liposome (Bar = 20 μm), as described in section 5.5.3.1

Plate 5.8 Typical SEM of precipitate recovered from a liposome dispersion converted in two stages from an egg PL pro-liposome (Bar = 100 μm), as described in section 5.5.3.1

5.5.3.2 Equilibrating hydrated cyA pro-liposomes

CyA pro-liposomes, with an egg PL: ethanol: glycerol: cyA weight ratio of 60:24:26:2, were hydrated and left to equilibrate for 24 hours before dispersing in bulk deionised water. The resultant liposome dispersions were analytically filtered. The results of this analytical filtration are shown in Table 5.11.
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Table 5.11 Effect of delaying the mixing of the deionised water for hydration and an egg PL pro-liposome for 24 hours on cyA association

<table>
<thead>
<tr>
<th>Amount of cyA egg PL pro-liposome (g)</th>
<th>Amount of deionised water for hydration (g)</th>
<th>Ease of filtration</th>
<th>Average increase in filter weight ± s.d. (mg)</th>
<th>Appearance of filter under the light microscope</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.640</td>
<td>0.320</td>
<td>III</td>
<td>1.0 ± 0.2</td>
<td>Vast number of spherical precipitate</td>
</tr>
</tbody>
</table>

The average weight of the insoluble residues of precipitate separated from the resultant liposome dispersions by filtration was about 1.0 mg. This increase in filter weight was no different from the filter weight increase of dispersions, which had been formed by immediately mixing the cyA pro-liposome and deionised water together and adding the resultant gel to bulk deionised water (Table 5.10). This demonstrated that even after leaving for 24 hours at room temperature, the hydrated cyA pro-liposome did not dissolve the precipitate under the conditions tested.

5.5.3.3 Hydrating cyA pro-liposomes with reduced levels of deionised water

Egg PL cyA dispersions converted from an egg pro-liposome, with an egg PL: ethanol: glycerol: cyA weight ratio of 60:24:36:2, were hydrated with reduced levels of deionised water. The resultant cyA liposome dispersions were analytically filtered. The results of this filtration procedure are shown in Table 5.12.

Table 5.12 Effect of reducing the amount of deionised water used for hydrating an egg PL pro-liposome converted in two stages on cyA association

<table>
<thead>
<tr>
<th>Amount of cyA egg PL pro-liposome (g)</th>
<th>Amount of deionised water for hydration (g)</th>
<th>Ease of filtration</th>
<th>Average increase in filter weight ± s.d. (mg)</th>
<th>Appearance of filter under the light microscope</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.640</td>
<td>0.260</td>
<td>II</td>
<td>0.4 ± 0.1</td>
<td>Moderate number of spherical and elongated precipitates.</td>
</tr>
<tr>
<td>0.640</td>
<td>0.170</td>
<td>II</td>
<td>0.2 ± 0.0</td>
<td>Few 5-10 μm spheres</td>
</tr>
</tbody>
</table>

Lowering the amount of deionised water for hydration added to the cyA pro-liposomes, resulted in the reduction of the amount of precipitate formed. If the amount of deionised water for hydration was lowered to 0.260 g, the weight of precipitate weight was lowered to 0.4 mg. Lowering the deionised water for hydration to 0.170 g further reduced precipitate formation. After analytical filtration of this cyA dispersion the filter weight increased by only 0.2 mg and a few precipitate spheres were seen under light microscopy. This improved cyA association with the liposomes could perhaps be attributed to the lipophilic balance of the milieu surrounding the cyA pro-liposome. In the cyA pro-liposome hydrated with 0.320 g of deionised water, this larger proportion of deionised water in the milieu probably discouraged the redissolving of the cyA...
precipitate - even after 24 hours of storage. In contrast, if this deionised water level was lowered to 0.170 g perhaps the media surrounding the cyA pro-liposome bilayers was sufficiently lipophilic either to virtually prevent this precipitation or to redissolve most of the precipitated cyA. These studies demonstrated the importance of selecting the correct level of deionised water for hydration to maximise the cyA association with liposomes.

5.5.3.4 Delaying mixing of cyA pro-liposome and deionised water for hydration

The four sets of egg PL dispersions used to investigate the effect of delayed mixing and equilibration were analytically filtered. The solid content present in the liposome dispersions was separated by analytical filtration. The results of these experiments are shown in Table 5.13.

Table 5.13 Effect of lowering the egg PL: cyA ratio in pro-liposomes converted in two stages on cyA association

<table>
<thead>
<tr>
<th>Eppendorf mixing of 0.640 g of cyA egg PL pro-liposome and 0.173 g of deionised water</th>
<th>Addition of hydrated pro-liposome to bulk deionised water after Eppendorf mixing</th>
<th>Ease of filtration</th>
<th>Average increase in filter weight ± s.d. (mg)</th>
<th>Appearance of filter under the light microscope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immediate mixing of cyA pro-liposome</td>
<td>Immediate</td>
<td>I-II</td>
<td>0.2 ± 0.0</td>
<td>Few spherical precipitates</td>
</tr>
<tr>
<td>Immediate</td>
<td>Immediate</td>
<td>I-II</td>
<td>0.2 ± 0.1</td>
<td>Few spherical precipitates</td>
</tr>
<tr>
<td>Delayed for 24 hours</td>
<td>Delayed for 24 hours</td>
<td>I-II</td>
<td>1.3 ± 0.3</td>
<td>Considerable number of spherical precipitates</td>
</tr>
<tr>
<td>Delayed for 24 hours</td>
<td>Delayed for 24 hours</td>
<td>I-II</td>
<td>0.3 ± 0.1</td>
<td>Few spherical precipitates</td>
</tr>
</tbody>
</table>

Immediate mixing of the egg PL pro-liposome with a small amount of deionised water for hydration generated a clear hydrated cyA pro-liposome. Moreover, if this hydrated cyA pro-liposome was left for 24 hours, the gel still remained optically clear. This clarity indicated that major precipitation had not developed during 24 hours of storage, which was confirmed by only slight increases in filter weight after analytical filtration of these cyA liposome dispersions. However, if the cyA pro-liposome and the deionised water for initial hydration were combined, but left for 24 hours before Eppendorf mixing, a hazy gel was generated. This haziness seemed to correlate with the formation of a considerable amount of precipitation in the final cyA liposome dispersions. If this hazy gel was immediately dispersed in the bulk deionised water, over 1.0 mg of solid precipitate was detected after analytical filtration of this cyA liposome dispersion. This indicated that delaying the mixing by 24 hours followed by immediate dispersion in bulk deionised water reduced the loading of cyA into the liposomes.
Equilibrating the hydrated hazy gel described above by delaying the addition of the hydrated pro-liposome gel for a further 24 hours at room temperature, resulted in the disappearance of the haziness. Additionally, conversion of this clarified gel to a liposome dispersion, resulted in an average filter weight increase of about 0.3 mg. This equilibration period demonstrated that during 24 hours of storage a large proportion of the deposits re-dissolved in the hydrated cyA pro-liposome. Thus, if only a small amount of deionised water was added to this cyA pro-liposome, the surrounding media was sufficiently lipophilic to redissolve most of the cyA precipitate.

5.5.3.5 Role of ethanol level for cyA association

5.5.3.5.1 High ethanol formulation

A high ethanol pro-liposome formulation, with an egg PL: ethanol: glycerol: cyA weight ratio of 60:48:26:2, was hydrated with three different levels of deionised water. The resultant dispersions were analytically filtered to assess the amount of solid precipitate present (Table 5.14).

<table>
<thead>
<tr>
<th>Amount of high ethanol cyA egg PL pro-liposome (g)</th>
<th>Amount of deionised water for hydration (g)</th>
<th>Ease of filtration</th>
<th>Average increase in filter weight ± s.d. (mg)</th>
<th>Appearance of filter under the light microscope</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.782</td>
<td>0.161</td>
<td>II</td>
<td>0.2 ± 0.1</td>
<td>Darkened grey circles of fine spherical precipitate</td>
</tr>
<tr>
<td>0.782</td>
<td>0.316</td>
<td>II</td>
<td>0.0 ± 0.0</td>
<td>Clear</td>
</tr>
<tr>
<td>0.782</td>
<td>0.633</td>
<td>II-III</td>
<td>1.3 ± 0.2</td>
<td>Vast number of spherical precipitates</td>
</tr>
</tbody>
</table>

The addition of insufficient deionised water to the pro-liposome reduced the amount of cyA associated with the liposomes. If only 0.161 g of deionised water was added to 0.782 g of cyA pro-liposome in the first stage of hydration, the association of cyA with liposomes was incomplete. Under the light microscope, dark circles of fine precipitate outlining the unsupported circular areas of the underlying filter support could be observed. The formation of precipitate in this case may have been attributed to the insufficient level of bilayer organisation prior to the dilution in bulk water. Therefore, the addition of insufficient amounts of water resembled a one stage dilution. However, if 0.316 g of deionised water for hydration was added and thoroughly mixed into the same amount of cyA pro-liposome, no precipitate was recovered from the final dispersions and the filter weight did not increase after filtering the dispersions. Hence, raising the ethanol level of the cyA pro-liposome enabled more deionised water to be
added without the formation of insoluble precipitate in the final cyA dispersions. The reason for this was probably due to the higher ethanol level increasing the lipophilic balance of the milieu surrounding the cyA pro-liposomes. However, even with this high ethanol formulation, over 1.0 mg of precipitate was formed if the amount of deionised water for hydration was raised to 0.633 g. These results again clearly illustrated the importance of adding the appropriate level of deionised water to the cyA pro-liposome in the first stage of hydration to maximise cyA association.

**5.5.3.5.2 Medium ethanol formulation**

The medium ethanol formulation, which had an egg PL: ethanol: glycerol: cyA weight ratio of 60:36:26:2, was converted into a liposome dispersion in two stages and analytically filtered (Table 5.15).

<table>
<thead>
<tr>
<th>Amount of medium ethanol cyA egg PL pro-liposome (g)</th>
<th>Amount of deionised water for hydration (g)</th>
<th>Ease of filtration</th>
<th>Average increase in filter weight ± s.d. (mg)</th>
<th>Appearance of filter under the light microscope</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.713</td>
<td>0.316</td>
<td>I-II</td>
<td>0.2 ± 0.1</td>
<td>Darkened grey circles of fine spherical precipitate</td>
</tr>
</tbody>
</table>

After filtration of the egg PL cyA liposome dispersion, traces of a fine white powder could be seen by the naked eye on the upper surface of the filters. In addition, the filter weight increased by 0.2 mg, which confirmed that association of cyA was compromised if there was either insufficient ethanol in the cyA pro-liposome and/or excess deionised water for hydration was added to the cyA pro-liposome. This demonstrated that the amount of deionised water added to the first stage of hydration should not be considered in isolation, but should be viewed in conjunction with the ethanol level of the cyA pro-liposome.

**5.5.3.6 Medium ethanol formulation with soya PL blend**

The same lipid: ethanol: glycerol: cyA weight ratio for the “medium” ethanol cyA formulation as used in section 5.5.3.5 was employed, but the egg PL was replaced with a soya PC: soya PL blend. The soya PC: soya PL: ethanol: glycerol: cyA weight ratio for the pro-liposome was 54:6:36:24:2. The analytical filtration results for this cyA dispersion are shown in Table 5.16.
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Table 5.16 Effect of adding deionised water for hydration to a soya PL blend pro-liposome converted in two stages on cyA association

<table>
<thead>
<tr>
<th>Amount of cyA soya PL blend pro-liposome (g)</th>
<th>Amount of deionised water for hydration (g)</th>
<th>Ease of filtration</th>
<th>Average increase in filter weight ± s.d. (mg)</th>
<th>Appearance of filter under LM and SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.713</td>
<td>0.316</td>
<td>1</td>
<td>0.0 ± 0.0</td>
<td>Clear</td>
</tr>
</tbody>
</table>

Replacing the egg PL with a soya PC/soya PL blend in a weight ratio of 90:10 (referred to in text as soya PL blend) improved the association of the cyA with the liposomes. This was shown by the fact that there were no increases in filter weight after analytical filtration of the soya PL blend dispersions containing cyA. Moreover, both light microscopy and SEM indicated that the polycarbonate filters were free from any form of precipitate after analytical filtration of the cyA dispersion (Plate 5.9). There are various possible explanations for the improved association of cyA with the soya PL blend liposomes. Firstly, the more unsaturated fatty acid chains of the soya PC may have been able to incorporate a higher cyA capacity than the more saturated fatty acids of egg PC. Secondly, the incorporation of cyA into the soya PC/soya PL blend bilayer may have been easier than the egg PL bilayer, as a result of the higher ethanol solubility of the soya lipids.

Due to the superior association of cyA with soya PL blend liposomes, this particular lipid blend was adopted for most of the subsequent association studies involving two stage conversion, unless otherwise stated.

Plate 5.9 Typical SEM of surface of polycarbonate filter used to filter a soya PL blend liposome dispersion containing cyA (Bar = 5 μm), described in section 5.5.3.6
5.5.3.7 Role of glycerol in two stage conversion

The glycerol-free soya PL blend pro-liposome formed an opaque yellow gel after being hydrated with deionised water. When shaken vigorously in bulk deionised water this hydrated cyA pro-liposome dispersed rapidly. The analytical filtration results of these cyA dispersions are shown in Table 5.17.

Table 5.17 Effect of adding deionised water for hydration to a glycerol-free soya PL blend pro-liposome on cyA association

<table>
<thead>
<tr>
<th>Amount of cyA glycerol-free soya PL blend pro-liposome (g)</th>
<th>Amount of deionised water for hydration (g)</th>
<th>Ease of filtration</th>
<th>Average increase in filter weight ± s.d. (mg)</th>
<th>Appearance of filter under the LM and SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.564</td>
<td>0.316</td>
<td>II</td>
<td>0.0 ± 0.0</td>
<td>Clear</td>
</tr>
</tbody>
</table>

As mentioned in section 5.5.2.1, omitting glycerol from cyA pro-liposomes reduced the association of cyA into liposomes when a one stage conversion was adopted. However, adopting two stage conversion for these glycerol-free cyA pro-liposomes resulted in the complete association of cyA with liposomes. By adding 0.316 g of deionised water to 0.564 g of the glycerol-free cyA pro-liposome, cyA could be completely incorporated into the liposome dispersion. After analytical filtration of these glycerol-free cyA liposomes, no increase in filter weight was detected and no precipitate was seen under the light microscope or SEM. Interpreted in light of the previous results of the glycerol-free cyA pro-liposomes converted in one stage (section 5.5.2.1), it was evident that the formation of the bilayered state by two stage conversion was crucial for efficient cyA loading. This enhanced loading of cyA into liposomes as a result of two stage conversion may also have explained why glycerol played a beneficial role in improving the association of cyA with liposomes converted in one stage. Glycerol was important for associating cyA with liposomes formed in one stage conversion, because it aids the formation of bilayer structures upon addition to bulk deionised water (In house data, Vandsons Research).

5.5.3.8 Lowering the soya PL blend: cyA weight ratio

The two sets of dispersions generated in two stages from pro-liposomes with soya PC: soya PL: ethanol: glycerol: cyA weight ratios of 45:5:36:26:2 and 36:4:36:26:2 were filtered through polycarbonate filters. Any precipitate retained on the filters from the two sets of dispersions was examined and inspected in the usual manner (Table 5.18).
Under the conditions employed, cyclosporin A (CyA) was efficiently solubilised at both soya phospholipid (PL) blend: CyA ratios, if the CyA pro-liposomes were immediately Eppendorf mixed twenty times with deionised water for hydration and added to bulk deionised water. This was reflected by the absence of precipitate on the filters and no increase in the filter weights after analytical filtration of the dispersions.

5.5.3.9 Lowering the soya PL blend: CyA weight ratio without mixing

The experiments as described in section 5.4.3.8 were repeated, except that the Eppendorf mixing of the two CyA pro-liposomes and deionised water for hydration was delayed for 24 hours, prior to the dispersion in bulk deionised water. The results for the analytical filtration of these two sets of CyA dispersions are shown in Table 5.19.

<table>
<thead>
<tr>
<th>CyA pro-liposome weight ratio</th>
<th>Amount of CyA pro-liposome (g)</th>
<th>Deionised water for hydration (g)</th>
<th>Ease of filtration</th>
<th>Average increase in filter weight ± s.d. (mg)</th>
<th>Appearance of filter under the light microscope</th>
</tr>
</thead>
<tbody>
<tr>
<td>45:5:36:26:2</td>
<td>0.656</td>
<td>0.316</td>
<td>I-II</td>
<td>0.1 ± 0.0</td>
<td>Few spheres</td>
</tr>
<tr>
<td>36:4:36:26:2</td>
<td>0.598</td>
<td>0.316</td>
<td>II-III</td>
<td>0.5 ± 0.1</td>
<td>Darkened grey circles of fine spherical precipitates</td>
</tr>
</tbody>
</table>

CyA association was reduced if the Eppendorf mixing of the CyA pro-liposomes was delayed for 24 hours. In the case of the pro-liposome with a lipid: ethanol: glycerol: CyA weight ratio of 50:63:26:2, delaying the Eppendorf mixing with deionised water for 24 hours resulted in the formation of a few individual spheres of precipitate on the filter. This precipitate was seen under the light microscope and the filter weight increased slightly by 0.1 mg. Lowering the soya PL blend: CyA weight ratio in the other pro-liposome to 40:2 increased the amount of precipitate formation by about 0.5 mg, if the mixing was delayed for 24 hours. This precipitate formation was confirmed by light microscopy, which revealed the presence of many fine spherical deposits. Therefore, the soya PL blend: CyA level could only be reduced to 40:2 without compromising the CyA association, if the CyA pro-liposome and deionised water for hydration were immediately mixed together.
5.5.3.10 Influence of EPG on cyA association

The influence of EPG on cyA association was examined using dispersions converted in two stages from three pro-liposomes with soya PC: EPG: ethanol: glycerol: cyA weight ratios of 39.8:0.2:36:26:2, 39.6:0.4:36:26:2 and 39.4:0.6:36:26:2. The results of the analytical filtration for the three sets of cyA dispersions with varying levels of EPG are shown in Table 5.20.

<table>
<thead>
<tr>
<th>CyA pro-liposome</th>
<th>Amount of</th>
<th>Amount of</th>
<th>Ease of</th>
<th>Average increase</th>
<th>Appearance of filter under LM and SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>weight ratio</td>
<td>cyA pro-liposome (g)</td>
<td>deionised water for hydration (g)</td>
<td>filtration</td>
<td>in filter weight ± s.d. (mg)</td>
<td></td>
</tr>
<tr>
<td>39.8:0.2:36:26:2</td>
<td>0.598</td>
<td>0.316</td>
<td>I</td>
<td>0.0 ± 0.0</td>
<td>Clear</td>
</tr>
<tr>
<td>39.6:0.4:36:26:2</td>
<td>0.598</td>
<td>0.316</td>
<td>II</td>
<td>0.0 ± 0.0</td>
<td>Clear</td>
</tr>
<tr>
<td>39.4:0.6:36:26:2</td>
<td>0.598</td>
<td>0.316</td>
<td>II</td>
<td>0.0 ± 0.0</td>
<td>Clear</td>
</tr>
</tbody>
</table>

At lipid: cyA weight ratios of 20:1, the cyA was fully solubilised in all three liposome dispersions containing EPG. There were no filter weight increases and no precipitates on the surface of the filters were seen under the light microscope or SEM, which suggested that the addition of EPG at these levels did not adversely affect cyA association.

5.5.4 Particle size and cyA association

The effects of varying the levels of deionised water for hydration, ethanol and lipid on cyA liposome size and cyA association were investigated.

5.5.4.1 Influence of varying the amount of deionised water for hydration

Three sets of liposome dispersions, converted in two stages from a soya PC/EPG pro-liposome with a soya PC: EPG: ethanol: glycerol: cyA weight ratio of 49.75:0.25:36:26:2, were used for this particular study. The size of the three sets of dispersions was measured prior to analytical filtration (Table 5.21).

<table>
<thead>
<tr>
<th>Amount of deionised water for hydration (g) added to 0.656 g of soya PC/EPG cyA pro-liposome</th>
<th>0.230</th>
<th>0.345</th>
<th>0.460</th>
</tr>
</thead>
<tbody>
<tr>
<td>z average ± s.d. (nm)</td>
<td>417.0 ± 43.2</td>
<td>229.6 ± 23.5</td>
<td>186.2 ± 2.3</td>
</tr>
<tr>
<td>P.I. ± s.d.</td>
<td>0.394 ± 0.056</td>
<td>0.187 ± 0.057</td>
<td>0.137 ± 0.019</td>
</tr>
<tr>
<td>D-90 ± s.d. (µm)</td>
<td>15.02 ± 4.85</td>
<td>1.54 ± 0.17</td>
<td>1.07 ± 0.20</td>
</tr>
<tr>
<td>Volume added to LD cell (µl)</td>
<td>40</td>
<td>60</td>
<td>120</td>
</tr>
<tr>
<td>Ease of filtration</td>
<td>II</td>
<td>II</td>
<td>II</td>
</tr>
<tr>
<td>Weight increase (mg)</td>
<td>0.057 ± 0.048</td>
<td>0.066 ± 0.021</td>
<td>0.178 ± 0.024</td>
</tr>
<tr>
<td>Appearance of filter under the light microscope</td>
<td>Few spheres</td>
<td>Clear</td>
<td>Spheres visible</td>
</tr>
</tbody>
</table>
It was evident that as the amount of deionised water for hydration was increased, the z
average value fell significantly. This finding paralleled the results discussed in
section 3.5.5.2. Adding 0.230 g of deionised water produced larger liposomes with z
averages greater than 400 nm. In contrast, if the amount of deionised water for hydration
was raised to 0.460 g, significantly smaller liposomes with z averages less than 200 nm
were generated (p = < 0.05). The probable reason for the reduction in particle size was
discussed in section 3.5.5.2. Addition of sufficient deionised water for hydration
encouraged bilayer precipitation, which favoured a smaller particle size and narrower
size distribution.

Raising the amount of deionised water for hydration to 0.460 g reduced the level of cyA
associated with the liposomes. After analytical filtration of these cyA dispersions, light
microscopy revealed that some spheres were recovered from this cyA dispersion with
small liposome size. However, these spheres were not seen after filtering the dispersions
generated in two stages by adding either 0.230 g or 0.345 g of deionised water for
hydration to the cyA pro-liposome. The reduction in cyA association of the dispersions
converted with 0.460 g of water for hydration is unlikely to be directly related to the
smaller size of the liposomes. From Table 5.21, although it appeared that the association
of cyA fell as the liposome size decreased, earlier studies with dispersions converted in
one stage demonstrated that larger sized liposomes did not fully solubilise cyA. The
reduced level of cyA association was probably related to the lipophilic balance of the
pro-liposome milieu. The addition of a higher level of water for hydration probably
lowered the degree of cyA association, because the relative lipophilicity of the milieu
surrounding the cyA pro-liposome was reduced. Addition of excess water for hydration
probably precipitated the hydrophobic drug and prevented this precipitate from
dissolving in the hydrated cyA pro-liposome. Hence after converting these hydrated pro-
liposomes in the remaining bulk water, precipitates were recovered from the resultant
dispersions.

In order to prevent the formation of this precipitate by manipulating the deionised water
for hydration, it was necessary to decrease the amount of deionised water for hydration.
However, lowering the amount of deionised water for hydration had a detrimental effect
on size characteristics by increasing the particle size of the liposome size and
broadening the size distribution.
The analytical filtration results of the soya PC/EPG dispersions in section 5.5.4.1, measured using the more accurate Mettler balance, largely mirrored the results obtained the filtration results of the soya PL blend dispersions in section 5.5.3.5.6, which employed the less accurate Sartorius balance. If the weight ratio of pro-liposome: water for hydration was 3:2, the solubilisation of cyA in the final dispersion was greater than 99%.

5.5.4.2 Influence of varying the level of ethanol

Two sets of liposome dispersions were generated in two stages from two pro-liposomes with soya PC: EPG: ethanol: glycerol: cyA weight ratios of 49.75:0.25:24:26:2, and 49.75:0.25:48:26:2. The particle size and cyA associated with both sets of liposome dispersions are stated in Table 5.22. For comparative purposes, the particle size data for the dispersions converted in two stages from pro-liposomes with a soya PC: EPG: ethanol: glycerol: cyA weight ratio of 49.75:0.25:36:26:2, described in section 5.5.4.3, is also shown in Table 5.22.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Amount of cyA pro-liposome (g)</td>
<td>0.587</td>
<td>0.656</td>
<td>0.725</td>
</tr>
<tr>
<td>Amount of deionised water for hydration (g)</td>
<td>0.460</td>
<td>0.460</td>
<td>0.460</td>
</tr>
<tr>
<td>z average ± s.d. (nm)</td>
<td>215.9 ± 7.68</td>
<td>186.2 ± 2.3</td>
<td>242.3 ± 15.3</td>
</tr>
<tr>
<td>P.I. ± s.d.</td>
<td>0.194 ± 0.015</td>
<td>0.137 ± 0.019</td>
<td>0.198 ± 0.038</td>
</tr>
<tr>
<td>D-90 ± s.d. (μm)</td>
<td>1.37 ± 0.04</td>
<td>1.07 ± 0.20</td>
<td>1.69 ± 0.23</td>
</tr>
<tr>
<td>Volume added to LD cell (μl)</td>
<td>100</td>
<td>120</td>
<td>60</td>
</tr>
<tr>
<td>Ease of filtration</td>
<td>II</td>
<td>II</td>
<td>II</td>
</tr>
<tr>
<td>Weight (mg)</td>
<td>0.218 ± 0.012</td>
<td>0.178 ± 0.024</td>
<td>0.064 ± 0.023</td>
</tr>
<tr>
<td>Appearance of filter under the light microscope</td>
<td>Spheres visible</td>
<td>Spheres visible</td>
<td>Clear</td>
</tr>
</tbody>
</table>

It was evident that raising the ethanol level in the cyA pro-liposome improved the association of cyA with liposomes converted in two stages. Raising the ethanol level in the pro-liposome with a soya PC: EPG: ethanol: glycerol: cyA weight ratio of 49.75:0.25:45:26:2 improved the association of cyA. The weight of residue was less than 0.06 mg, which equated to less than 1% of unassociated cyA. However, raising the ethanol level significantly increased the particle size of the cyA liposome dispersions converted in two stages. Two stage conversion of this pro-liposome generated cyA liposome dispersions with a z average of over 240 nm, which was significantly higher than the z average of the dispersions generated from pro-liposomes with soya PC: EPG: ethanol: glycerol: cyA weight ratio levels of 49.75:0.25:24:26:2 (p = < 0.05) and
Chapter five—Solubilisation of cyclosporin A by liposomes

49.75:0.25:36:26:2 (p = < 0.05). The reason for this increase in particle size and the broadening of the size distribution was probably due to the increased solubility of the lipid bilayers in this level of ethanol. The hydrated cyA pro-liposome bilayers, which were a prerequisite for fine particle size, were not fully precipitated in the presence of the raised ethanol level. This was directly indicated by the increased fluidity of the hydrated cyA pro-liposome with high ethanol level. Instead of possessing gel-like viscosity, the hydrated pro-liposome was a viscous fluid. Therefore, upon dilution this cyA pro-liposome with raised ethanol level generated somewhat larger liposomes.

These opposing effects of ethanol on the liposome dispersion illustrate the difficulties of generating a fine dispersion with complete cyA association. Low ethanol levels were required in order to generate a dispersion with fine particle size. However, high ethanol levels, which increased particle size, were needed for optimum cyA association. Therefore, the ethanol level of the cyA pro-liposome had to be carefully selected, which did not severely compromise either the fine particle size of the liposome dispersion or the association of cyA with the liposomes.

5.5.4.3 Influence of varying the lipid: cyA ratio

Liposomes were converted in two stages from pro-liposomes with soya PC: EPG: ethanol: glycerol: cyA weight ratios of 29.85:0.15:36:26:2 and 39.8:0.2:36:26:2. The details of the size and cyA associated with these two formulations are shown in Table 5.23. For comparative purposes, the particle size and association data for dispersions generated in two stages from a pro-liposome with a soya PC: EPG: ethanol: glycerol: cyA weight ratio of 49.75:0.25:36:26:2 (section 5.5.4.2) is also shown in Table 5.23.

Table 5.23 Influence of lowering lipid level in cyA soya PC/EPG pro-liposomes on cyA association and particle size of cyA soya PC/EPG liposomes

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Amount of cyA pro-liposome (g)</td>
<td>0.541</td>
<td>0.598</td>
<td>0.656</td>
</tr>
<tr>
<td>Amount of deionised water for hydration (g)</td>
<td>0.460</td>
<td>0.460</td>
<td>0.460</td>
</tr>
<tr>
<td>z average ± s.d. (nm)</td>
<td>172.1 ± 1.99</td>
<td>180.1 ± 15.5</td>
<td>186.2 ± 2.3</td>
</tr>
<tr>
<td>P.I. ± s.d.</td>
<td>0.187 ± 0.044</td>
<td>0.167 ± 0.033</td>
<td>0.137 ± 0.019</td>
</tr>
<tr>
<td>D-90 ± s.d. (µm)</td>
<td>1.32 ± 0.03</td>
<td>1.45 ± 0.26</td>
<td>1.07 ± 0.20</td>
</tr>
<tr>
<td>Volume added to LD cell (µl)</td>
<td>240</td>
<td>160</td>
<td>120</td>
</tr>
<tr>
<td>Ease of filtration</td>
<td>II</td>
<td>II</td>
<td>II</td>
</tr>
<tr>
<td>Weight (mg)</td>
<td>1.482 ± 0.127</td>
<td>0.368 ± 0.166</td>
<td>0.178 ± 0.024</td>
</tr>
<tr>
<td>Appearance of filter under the light microscope</td>
<td>Vast amount of precipitate</td>
<td>Precipitate present</td>
<td>Spheres visible</td>
</tr>
</tbody>
</table>
CyA association was severely compromised if the lipid to cyA weight ratio was lowered to 15:1. After the addition and thorough mixing of deionised water with the pro-liposome with a soya PC: EPG: ethanol: glycerol: cyA weight ratio of 29.85:0.15:36:26:2, the average weight of precipitate recovered from the liposome dispersion was close to 1.5 mg. Therefore, assuming the residue was entirely cyA, the level of liposome associated cyA was approximately 85%. Furthermore, prior to filtration, after one hour white flocculates sedimented from these cyA dispersions. In contrast, raising the lipid concentration to 50 mg/g, whilst maintaining the cyA concentration at 2 mg/g, resulted in the improved association of cyA with the liposomes, if the same proportions of deionised water for hydration and cyA pro-liposomes were employed. Only a few spheres were visible under the light microscope and the increase in weight was only 0.178 mg, which equated to a level of cyA association more than 98%. These findings were in line with previous experiments, which showed that cyA solubilisation was improved as the amount of lipid was raised (section 5.5.2.6).

5.6 Conclusions

Without phospholipid, cyA precipitated from ethanol: glycerol mixes upon dilution in deionised water. However, if the cyA was dissolved in pro-liposomes, this hydrophobic drug was largely solubilised when liposomes were generated, although the degree of solubilisation was dependent upon the composition of the pro-liposome and the method of conversion. The amount of phospholipid required to solubilise the cyA was relatively large: at least 20 parts by weight of phospholipid were required to solubilise 1 part by weight of cyA for two stage conversion.

Upon conversion of the pro-liposomes into liposome dispersions, if the cyA was not associated with the liposomes, precipitates were generated. These precipitates were generally smooth and spherical. The precipitate could be separated from the dispersions by filtration through polycarbonate filters and semi-quantified by weighing the increase in filter weight and microscopy.

This filtration method was useful for determining the presence or absence of hydrophobic drug in liposome dispersions. However, the possible co-precipitation of phospholipid with the cyA prevented this technique from being used to accurately quantify the amount of unassociated cyA.
If one stage liposome conversion was adopted, factors such as the inclusion of glycerol into the cyA pro-liposome, a high lipid: cyA weight ratio and vigorous handshaking all assisted cyA intercalation. However, one stage conversion did not yield dispersions which associated cyA completely. Even if the lipid: cyA weight ratio was raised to 60:1, the association was incomplete.

In contrast, if two stage conversion was selected- the correct balance of deionised water for hydration and the appropriate ethanol level of the cyA pro-liposome were critical factors for maximising cyA association. If these two levels were carefully controlled, the cyA association could be improved to a level where no precipitation was detectable in the final dispersion. However, inappropriate levels of water for hydration, inappropriately low ethanol levels in the pro-liposome or delayed mixing of pro-liposome and water for hydration reduced the level of cyA association.

Glycerol was not required for complete cyA intercalation, if two stage conversion was adopted. Dispersions converted in two stages by hydrating a glycerol-free pro-liposome with deionised water for hydration associated with cyA under the conditions tested. Although lower levels of deionised water and higher levels of ethanol favoured cyA association, from the particle size results it became apparent that these levels of the two variables were detrimental to the particle size distribution. An insufficient amount of deionised water for hydration added to the cyA pro-liposome and/or a high ethanol level of the cyA pro-liposome generated larger sized liposomes and more polydisperse liposome dispersions. Therefore, to fully associate cyA with small sized liposomes, it was evident that the levels of cyA, ethanol and lipid in the pro-liposome and the amount of deionised water for hydration had to be finely balanced. Using a pro-liposome formulation with a soya PC: EPG: ethanol: glycerol: cyA weight ratio of 49.75:0.25:36:26:2, cyA dispersions could be generated in two stages, which solubilised cyA and had an average liposome diameter of 230 nm.
Chapter six

Freeze drying liposome dispersions

generated from pro-liposomes
Chapter six-Freeze drying liposome dispersions

6.1 Introduction

6.1.1 Freeze drying

Freeze drying (FD) is essentially a two stage process, involving the freezing of an aqueous system and the sublimation of the ice from this frozen solid. Successful desiccation by freeze drying produces a porous cake, which has a large surface area and is hydrophilic in nature. Hence when a successfully freeze dried pharmaceutical is reconstituted by the addition of aqueous media, dissolution of the cake is rapid. As a result of this particular property, freeze drying is also referred to as lyophilisation, which is derived from the Greek meaning “liking to be dissolved”.

An increasing number of biologically active components are being developed as freeze dried preparations for IV administration (Skrabanja et al., 1994). This type of presentation is selected primarily to extend the shelf life of formulations by improving the stability of delicate molecules, e.g. water sensitive antibiotics, large MW proteins and some cytotoxics. The removal of water may slow both the chemical reactions and the physical reactions responsible for the deterioration of formulations (Murase and Franks, 1989). Therefore, freeze drying can potentially improve the stability of the active principle in a form which is elegant and easy to handle.

Although freeze drying is finding increasing employment in the formulation of molecules which are unstable in aqueous solution, not every formulation which requires desiccation is suited to this process. There are situations where freeze drying may not be appropriate: despite the fact that freeze drying removes water from a formulation, in some instances this removal may be deleterious to the stability of some molecules. The removal of water concentrates the components within the formulation and may generate an adverse environment which is detrimental to the active component. This is exemplified by the concentration of the species in glycine-glucose-glycerol mixes (Karel, 1975). In this particular case, low levels of water can encourage non-enzymatic browning, because there is no water to dilute the reactants and end product inhibition by water does not occur. Secondly, large volumes may also be unsuitable for freeze drying. The time and cost of energy required to remove the water from large volume parenterals may be economically prohibitive. Thirdly, as will be illustrated in greater depth by liposomes, freeze drying may damage sensitive formulations and compromise their properties upon reconstitution.
6.1.2 Sterilisation of freeze dried products

Parenteral pharmaceuticals have to be sterile, and freeze dried products are no exception. Terminal sterilisation of the freeze dried cake cannot usually be conducted, since the active principle is often thermo-labile and sensitive to ionising radiation. Therefore, sterilisation usually has to be carried out by aseptically filtering the liquid formulation through a 0.2 µm filter prior to freeze drying under sterile conditions (Evans and Grassam, 1986). Colloidal formulations with a significant proportion of droplets or particles greater than the filter diameter may therefore not be suited to this aseptic processing, because the colloidal particles will be retained by the filter. Hence prior to freeze drying, the individual particles of freeze dried IV colloidal dispersions usually have to be 0.2 µm or less in diameter, in order to facilitate their aseptic filtration.

6.1.3 Glass transition and glasses

An understanding of glass transition is of paramount importance in freeze drying. Formulating a stable freeze dried product is usually based upon the ability of the formulation to form a glass during processing, and the maintenance of this glassy state during storage. A glass develops as a liquid cools into a system, which has a viscosity usually in excess of $10^{14}$ Pa s (Franks, 1985). The temperature at which this transition occurs is referred to as the glass transition temperature, $T_g$. A special glass transition, denoted $T_g'$, has been defined as the $T_g$ of the maximally freeze concentrated system (Levine and Slade, 1988). The water content at this maximally freeze concentrated system is denoted as $W_g$.

6.1.4 Stages of freeze drying

There are four distinct stages of freeze drying, which are described in sequence below:

1) Freezing: During this first stage of lyophilisation, the solution/dispersion is cooled to below 0 °C in order to freeze the bulk water. Freezing conditions, such as the temperature and rate of freezing, should be carefully optimised to ensure the active principle in the formulation is not damaged (Fransen et al., 1986; Ausborn et al., 1992).

2) Primary drying (sublimation phase): This is the first stage of drying, which involves the sublimation of the unbound water from the frozen preparation, usually under reduced pressure and reduced temperature. To minimise the likelihood of cake collapse during the primary phase of drying, the product temperature should be kept below the $T_g'$ of the maximally freeze concentrate (Pikal and Shah, 1990). If the temperature rises
above the $T_g'$, the cake may collapse. Inadequate removal of free water can result in the resorption of water vapour back onto the drying cake. This process is known as meltback, which causes the collapse of the glass and ruins the cake.

3) Secondary drying (diffusion phase): The second phase of drying can commence after all the unbound water has been sublimated from the liposome dispersion. This stage of drying entails the removal, via diffusion, of water bound to the formulation components. Since removal of the plasticisizing bulk water raises the $T_g'$, this second drying stage can usually be carried out at a higher temperature, providing the temperature of the formulation is kept below the $T_g'$ of the glass. The level of water remaining in the formulation is important for long term product stability. As determined by DSC, moisture has been shown to plastisize the cake and dramatically lower the glass transition temperature, which adversely affects stability (Ahlneck and Zografi, 1990; Booy et al., 1992). Therefore, to obtain the highest possible $T_g$ for the product and to achieve optimal stability during storage, the residual water content in the cake should be as low as possible.

4) Storage: After the completion of freeze drying, storing the cake at the correct temperature relative to the $T_g$ is critical for successful long term stability (Sun et al., 1996). Since degradation kinetics are most likely to be accelerated above the $T_g$, ideally it is best if the product is stored below the $T_g$ of the product.

6.1.5 Freeze drying liposomes

Since the late 1970's, lyophilisation has been proposed as a means of improving the shelf life of aqueous liposome dispersions (I.C.I., 1978). Numerous studies have investigated freeze drying to enhance the chemical and physical stability of both liposomes and the active components entrapped/associated with liposomes (Harrigan et al., 1990). Most of these studies have focused upon the retention of water soluble compounds/markers encapsulated within the interior of the liposomes after lyophilisation (Van Bommel and Crommelin, 1984; Madden et al., 1985; Vemuri et al., 1991). However, the successful lyophilisation of these types of dispersions has proved to be difficult. The stresses exerted on the liposomes during the freezing and drying processes induce liposome fusion and bilayer disruptions, which result in the subsequent leakage of the entrapped water soluble drug.
In this study, only a hydrophobic drug associated with the membrane was of concern. The association of hydrophobic drugs with liposomes is usually less problematic than entrapment of water soluble compounds. In contrast to hydrophilic drugs, most water insoluble lipophiles appear to remain associated with the liposome bilayer after lyophilisation (Ausborn et al., 1992). Hence only two factors, namely particle size of the liposomes and unassociated drug after reconstitution have been examined.

6.1.6 Desirable properties for freeze dried liposomes

Ideally lyophilised liposome cakes should possess the following properties: firstly, the active principle should be completely associated with the liposome after reconstitution of the lyophilised cake. Secondly, after reconstitution the particle size of the liposome dispersion should be acceptable for infusion. Thirdly, after reconstitution the final dispersion should be isotonic. Finally, the cake should rehydrate rapidly upon the addition of water.

6.1.7 Liposome damage during freeze drying

Three main events may compromise the integrity of the liposome structure during freeze drying (Crowe et al., 1987): liposome fusion, development of non-bilayer phases and lateral phase separations within the bilayer. These three perturbations are outlined below:

i) Liposome fusion:

Fusion takes place when two or more liposomes coalesce and subsequently form a larger unified liposome. This event can occur when the water associated with the phospholipid headgroup is removed (Parsegian and Rand, 1979). In the absence of stabilisers, removing this hydration barrier generally induces the fusion of neighbouring bilayers. Evidence for the fusion of adjacent liposomes during dehydration has been provided by freeze fracture (Crowe et al., 1983), resonance energy transfer (Macdonald and Macdonald, 1981), and NMR (Strauss and Hauser, 1986). Liposome fusion results in the leakage of entrapped aqueous materials and increases both the size and the size distribution of the liposomes. Fusion differs from aggregation, which involves the coalescence of two or more liposomes and the maintenance of the individual liposome structures. However, in this study, fusion can not be readily distinguished from aggregation. Therefore, in these studies, the terms fusion and aggregation are used jointly to describe the measured increases in liposome size.
ii) Non-bilayer phases
Non-bilayer configurations may be generated upon dehydration of the bilayer. One of the most common non-bilayer phases is the hexagonal II phase ($H_{II}$). In this phase the phospholipid molecules are arranged in long cylinders with the polar head groups facing a water filled interior (Crowe and Crowe, 1982). Formation of these types of non-bilayer phases may damage membranes and cause leakage of entrapped molecules (Simon, 1974).

iii) Phase separation
Phase separation may arise if lipids with dissimilar headgroups or acyl chain lengths have poor miscibility in the gel phase (Matubaysi et al., 1986). Dehydration of these mixtures may result in independant transitions occurring within the bilayer, which again could lead to membrane defects and may displace integral membrane proteins (Crowe et al., 1983).

6.1.8 Minimising liposome damage during lyophilisation
There are a variety of factors which have to be considered to maintain the integrity of the liposome during lyophilisation. Firstly, components may be incorporated into the formulation to stabilise the liposome (Ausborn et al., 1992). Secondly, liposome damage during freeze drying can be minimised by carefully selecting the lipid composition. Addition of lipid containing carbohydrate to the bilayer has a tendency to improve membrane stability during freeze drying (Goodrich et al., 1988; 1991; Engel et al., 1994). Thirdly, the freezing (Fransen et al., 1986) and drying processes of lyophilisation can be optimised. Finally, liposomes with a small diameter can be selected which facilitate membrane stabilisation in the presence of protectants. Typically, liposomes with a diameter of 100 nm or less are most suited to stabilisation (Harrigan et al., 1990).

6.1.9 Stabilisers
In order to minimise the massive bilayer disruption and liposome fusion during lyophilisation, protectants have to be incorporated into the liposome dispersions prior to lyophilisation. Broadly speaking there are two types of protectant: a component which protects the liposomes during the freezing stage is known as a cryoprotectant, and a protectant which maintains the integrity of liposomes during the drying stage is referred to as a stabiliser. However, the term stabiliser is often used to generally describe an
excipient which protects the active ingredient throughout the whole freeze drying process. It is this latter definition which will be adopted in this context.

One of the most effective classes of stabilisers are the saccharides (Ausborn et al., 1992). The type of saccharide stabiliser and quantity of saccharide dictate the degree of liposome stabilisation. In the literature, the effect of different stabilisers and the level of stabilisers have been extensively studied in the context of retaining hydrophilic molecules/markers within liposomes. In these published studies, far higher quantities of stabiliser are required to prevent the leakage of entrapped hydrophilic material than are required to prevent liposome fusion. Compared to the quantity needed to prevent fusion, it was found that a 10 fold higher trehalose concentration was required to prevent the leakage of hydrophilic material from liposomes (Crowe et al., 1985).

The two main classes of saccharide employed for stabilising parenteral products are the monosaccharides and the disaccharides. The monosaccharides comprise only one saccharide subunit, e.g. glucose, whereas the disaccharides are made up of two saccharide subunits, e.g. lactose which is made up of a galactopyranosyl subunit and a glucopyranose subunit. It is widely accepted that to maximise the retention of hydrophilic molecules within liposomes, the disaccharides are generally superior to the monosaccharides (Crowe et al., 1987). However, the monosaccharides have been shown to be almost as effective as the disaccharides at preventing liposome fusion (Tanaka et al., 1992).

The exact mechanism by which saccharides stabilise liposomes is currently under debate. Two hypotheses have been proposed: vitrification and water replacement. Vitrification involves the formation of a stable glass which embeds the liposomes, and prevents fusion due to the high density of the glass (Koster et al., 1994). The other hypothesis, water replacement, suggests that saccharide molecules substitute for water by hydrogen bonding to the headgroup of the phospholipids as the water is removed (Crowe and Crowe, 1992). This interaction is believed to maintain the PL bilayer in a pseudo-hydrated state and reduce membrane perturbations during freeze drying. This interaction, via hydrogen bonding, may also explain why the disaccharides are superior at reducing the leakage of hydrophilic material from liposomes to the monosaccharides. The disaccharides have stronger interactive forces with the headgroups and additionally maintain the phospholipid membrane in a fluid state during the drying process.
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The evidence provided in the literature seems to demonstrate that vitrification alone does not seem to produce stable formulations (Crowe et al., 1994). However, a combination of water replacement and vitrification seem necessary for long term stability of the liposome formulation.

6.1.10 Aims of freeze drying study

From the results of chapter five, it was evident that to generate liposomes associating cyA from cyA pro-liposomes required the controlled hydration of a carefully balanced pro-liposome formulation. Deviations from the hydration protocol resulted in the compromising of particle size (chapter three) and/or cyA association (chapter five). Therefore, to ensure the formulation would consistently fall within definable parameters, any deviations from this protocol have to be avoided, which means that the controlled hydration step to generate the dispersions just prior to use has to be avoided. One possible way of avoiding this hydration step would be to form a liposome dispersion from a lyophilised liposome cake. This type of lyophilised presentation could be used to generate a liposome dispersion by the addition of water to the cake. Therefore, in this present study, pro-liposomes were investigated as a means of manufacturing finished liposome dispersions, which could subsequently be lyophilised to generate a cake for reconstitution just prior to administration.

The overall purpose of this investigation was to examine the feasibility of lyophilising liposomes generated from pro-liposomes. The experimental work presented can be conveniently subdivided into two sections. The first section investigates the lyophilisation of drug-free liposomes generated from various pro-liposomes. Omitting cyA avoided any potential interference caused by this hydrophobe during the freeze drying process. In these studies, the liposome size, before and after freeze drying, was assessed. Factors influencing liposome stabilisation, such as inclusion of various saccharides, lipid composition and freezing conditions were examined using drug-free liposome formulations. The feasibility of raising the lipid concentration of the final liposome dispersion by lyophilising a high lipid concentration and reconstituting cakes to a high lipid concentration was also studied.

The second part of the study assessed the effect of freeze drying on liposomes incorporating various levels of cyA. The quality of these reconstituted dispersions was assessed by determining the cyA association with liposomes after freeze drying and by
monitoring the size of the reconstituted cyA liposomes. Finally, the effect of freeze drying cyA liposome dispersions with different lipid composition was investigated.

6.2 Materials
Absolute ethanol, AnalaR, B.N. various, BDH Chemicals Ltd., Poole, UK.
Anhydrous glucose, GPR, B.N. 28450, BDH Chemicals Ltd., Poole, UK.
Deionised water, pH of approximately 5, from Elgastat, UHQ PS, Elguard.
Dextran MW 40,000, B.N. 116F-0067, BDH Chemicals Ltd., Poole, UK.
Epikuron 145 (referred to as soya PL in text), B.N. 1-5-9005, Lucas Mayer, Hamburg, Germany.
Epikuron 200 (referred to as soya PC in text), B.N. 1-5-9005, Lucas Mayer, Hamburg, Germany.
Glycerol, AnalaR, B.N. K213348 160 450, BDH Chemicals Ltd., Poole, UK.
Ovothin 180 (referred to as egg PL in text), B.N. 1-4-9240, Lucas Mayer, Hamburg, Germany.
Ovothin 200 (referred to as egg PC in text), B.N. 1-4-9248, Lucas Mayer, Hamburg, Germany.
PVP MW 44,000, B.N. 7099600K, BDH Chemicals Ltd., Poole, UK.
PVP MW 700,000, B.N. 1987 802, BDH Chemicals Ltd., Poole, UK.
Sucrose B.P., GPR, B.N. K21808007 539, BDH Chemicals Ltd., Poole, UK.

6.3 Methods
6.3.1 Manufacture of pro-liposomes
Pro-liposomes were made by dissolving the requisite amount of phospholipid in ethanol, as described in section 3.3.2 and section 4.3.1. The lipid to ethanol weight ratio was 60:36 for all of the pro-liposomes used in this study. This particular ratio was selected, because in the association studies carried out previously (section 5.5.3.7) a relatively high level of cyA loading into the liposome bilayers could be achieved with this lipid: ethanol ratio, if the amount of water for hydration was carefully controlled. Various lipid compositions were investigated generally employing combinations of two of the following lipids: soya phosphatidylcholine (PC), egg PC, soya phospholipid (PL) and egg phosphatidylglycerol (PG). The precise lipid composition used for each of the individual experiments is described in the appropriate methodology in section 6.4.
Unlike the pro-liposome formulations studied in chapter three and chapter five, glycerol was omitted from most of the formulations, unless otherwise stated. In these present studies, glycerol was generally replaced by one of two saccharides; either glucose or sucrose. The type of saccharide used for each of the individual experiments is detailed in the appropriate methodology in section 6.4.

If cyA was incorporated, it was dissolved in the pro-liposomes at varying lipid: ethanol: cyA weight ratios ranging from 60:36:1 to 60:36:3. The lipid: cyA weight ratio was kept above 20 parts of lipid to 1 part of cyA to ensure that the association of cyA with the liposomes was complete (section 5.5.4.3).

6.3.2 Producing liposome dispersions

Pro-liposomes were converted into liposome dispersions in either one or two stages. If cyA was omitted, the cyA-free pro-liposomes were added to bulk deionised water and converted in one stage as described in section 3.4.1. The resultant dispersions were extruded through membrane filters as described in section 6.3.3.2. However, if cyA was incorporated into the pro-liposome, the cyA pro-liposome was converted in two stages: the pro-liposome was hydrated with a small amount of a concentrated saccharide solution and the resulting gel was dispersed in bulk deionised water. This two stage conversion, described in section 5.5.3, was adopted in order to load cyA into the liposomes. The resultant cyA liposome dispersions were extruded through polycarbonate filters (section 6.3.3.2) to reduce the average liposome diameter and the size distribution prior to freeze drying.

At least 20 g of each dispersion was manufactured so that there was a sufficient weight of dispersion to freeze dry four 2.5 g samples. For dispersions which weighed 20 g in total, the liposome dispersions were made in 100 ml wide neck glass infusion bottles with appropriately fitting rubber tops. However, if the total weight of the liposome dispersion exceeded 50 g, the dispersion was made in 250 ml wide neck glass infusion bottles with appropriately fitting rubber tops.

6.3.3.1 Liposome size reduction

For all studies investigating freeze drying, the average liposome diameter in all dispersions was reduced to approximately 100 nm or less. The reasons for reducing the liposomes to this size were several fold. Firstly, it enabled the size of all liposome dispersions to be standardised, in order to facilitate comparisons between different
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dispersions. Secondly, liposomes of 100 nm or less can be more successfully freeze dried than larger vesicles. Thirdly, reducing the size of the liposomes to 100 nm or less lowered the aqueous capture volume of the liposomes, which enabled the lipid concentration of the dispersions to be raised, if required. Fourthly, the dispersions were translucent after size reduction, which meant that the development of turbidity in a liposome dispersion after reconstitution was probably attributable to an increase in liposome size or aggregation. Finally, the dispersions prior to freeze drying were suited to aseptic sterilisation through a 0.2 μm filter, if required.

6.3.3.2 Extrusion

A variety of post liposome production modification techniques could have been employed to reduce the size of the liposomes (section 1.1.4; Brandl et al., 1990; Bachmann et al., 1993). However, for the present studies extruding the dispersions through polycarbonate filters was chosen because the liposome size and distribution could be controlled within narrow definable limits by selecting the filter(s) with appropriate pore size (Olson et al., 1979). Furthermore, for laboratory scale production this simple technique was rapid, cost effective and reproducible for processing up to 100 g of dispersion.

Extrusions were carried out using a commercially available stainless steel filtration cell and filter holder with support (Liposofast, Avestin, Canada). All dispersions were poured into the filtration cell and forced through one or two stacked 47 mm diameter polycarbonate filters (Cyclopore, pore size 100 nm, Whatman Int. Ltd., Maidstone, England) under seven bars of pressure generated from a nitrogen cylinder (B.O.C., UK). The gas flow from the cylinder was maintained until all the dispersion had been extruded and collected in a cleaned glass bottle. Multiple extrusions were carried out by collecting the extruded dispersion, pouring it back into the filtration cell and repeating the whole procedure until the liposome filtrate had been extruded the desired number of times.

Two types of filter with 100 nm and 50 nm sized pores were investigated. Preliminary investigations revealed that both filters produced liposomes with diameters greater than the stated pore size after multiple passes. It was clear that the size of the liposomes, even after repeated passage through double-stacked 50 nm filters (Isopore polycarbonate filters, 47 mm diameter, Whatman Int. Ltd., UK) could not be lowered to below about

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80 nm. Similarly double stacked 100 nm filters (Cyclopore polycarbonate filters, 47 mm diameter, Millipore, USA) were unable to reduce the average liposome size to below approximately 120 nm after repeated extrusions. Similar results have been published by Harrigan et al. (1990). The reason for these incomplete size reductions could have been due to either imperfections in the filters or the ability of the "fluid" liposomes to squeeze through the small pores. Examination of the filters under SEM revealed the former possibility to be unlikely, because most pores appeared to be close to the stated diameter. Therefore, the larger liposome diameter after extrusion was probably due to the highly flexible nature of the "fluid" PL liposomes, which deformed and squeezed through the small diameter pores of the polycarbonate filter.

The extrusion procedure, which was adopted for producing uniformly sized liposome dispersions, involved two filtration stages. The first stage involved passing the dispersion through a single 100 nm polycarbonate filter three times. This first filtration step filtered any debris from the dispersion and facilitated subsequent extrusions by reducing the diameter of the liposomes. The second filtration step involved passing this dispersion through stacked 100 nm and 50 nm filters. The order of the two filters for the second stage of filtration was important. The 100 nm filter had to be placed on top of the smaller 50 nm filter, which enabled the liposomes to be reduced in size prior to their passage through the smaller 50 nm filter. The liposome dispersions were repeatedly passed through these double stacked polycarbonate filters 20 times, after this number of passes the average liposome diameter as measured by photon correlation spectroscopy (PCS) (section 3.3.8.3), could not be reduced any further.

6.3.4 Freeze drying protocol

Four aliquots of 2.5 g of each extruded drug-free dispersion were transferred into four 10 ml clear glass vials. The depth of the 2.5 g liposome dispersion in each vial was about 7 mm. The four vials of each liposome dispersion were frozen and subjected to the same freeze drying conditions. The protocol for freeze drying has been divided into two parts:

1) Freezing phase, and
2) Drying phase.

6.3.4.1 Freezing

The liposome samples were frozen either by:
i) Freezing in liquid nitrogen

Four 10 ml glass vials each containing 2.5 g of dispersion were sealed with rubber stoppers and placed into strengthened Edwards jars (200 ml). Liquid nitrogen was poured into the jars to a depth of about 3 cm. Initial evaporation of the liquid nitrogen was rapid but slowed as the containers cooled. Liquid nitrogen was replenished every 2-3 minutes. After freezing the samples in liquid nitrogen for 10 minutes, the remaining liquid nitrogen was poured from the Edwards jar. The frozen rubber tops were prised from the 10 ml glass vials before the jars were attached to the vacuum manifold of the freeze drier.

or ii) Freezing in freezer

The vials containing liposome dispersions were capped and placed in a freezer for 12 hours at about -20 °C. After the 12 hours had elapsed, the rubber tops were immediately removed from the vials and the open vials were placed upright in pre-cooled Edwards jars before attaching to the manifold of the freeze drier. Pre-cooling of the jars was achieved by adding liquid nitrogen to the inside of the jars for five minutes before attaching to the freeze drier. Without this pre-cooling, some frozen liposome dispersions appeared to melt during freeze drying.

6.3.4.2 Drying

The Edwards vacuum pump was switched on 30 minutes before lyophilisation to allow the oil to warm. Warming the oil reduced the likelihood of contamination by condensable gases. The output of the vacuum pump was set to high, with an intermediate gas throughput of I. The Edwards Micromodulyo condenser was also switched on 30 minutes prior to use, to enable the temperature within the condenser to be lowered to -55 °C.

After having frozen the liposome dispersions, each Edwards jar accommodating four upright vials was immediately attached to one of the arms of the freeze drier manifold. Hence, up to 24 samples could be freeze dried simultaneously if all of the six arms of the manifold were employed. Lyophilisation was initiated by engaging the vacuum to the jar. The vacuum immediately removed the air from the jar and reduced the internal pressure. After freeze drying the liposome dispersions by maintaining the jar under vacuum for 24 hours, the temperature of the jar and vials had warmed to room temperature. This whole freeze drying protocol was not ideal because the freezing
process and the drying process could not be adequately controlled. In particular, the temperature of the drying process could not be controlled and was entirely dependant upon the temperature of the room at the time of freeze drying (approximately 20 °C -25 °C).

6.3.5 Reconstitution of lyophilised cakes

After the completion of the freeze drying cycle, the Edwards jars were isolated from the vacuum by closing the output on the manifold arm. After releasing the vacuum, these glass jars could be removed from the manifold arms and the vials were immediately capped with rubber tops. Within two minutes of capping the vials, the air was evacuated from these glass vials by piercing the rubber top with a syringe needle attached to a vacuum pump. The tip of the needle was left inside the sealed jar for 15-20 seconds before carefully withdrawing it. The evacuation of the vials temporarily prevented the ingress of air into the vials by sealing the vials. Furthermore, if the vacuum was maintained, the rehydration was hastened when deionised water was added to the cake. All cakes were rehydrated within ten minutes of the evacuation. Prolonged exposure to air resulted in the hygroscopic cakes rapidly picking up moisture and becoming waxy. This behaviour was typical of hygroscopic freeze dried products, because lyophilisation creates a large dry surface area which attracts moisture from the air.

Unless otherwise stated, reconstitution at room temperature (20 °C -25 °C) was carried out by adding an appropriate amount of deionised water to the lyophilised cake. The appropriate amount of deionised water to reconstitute the cake was contained inside a five ml plastic syringe (Braun, Melsungen, Germany) with a 21G syringe needle (Beckton and Dickson, Ireland) attached to the Luer tip. The deionised water from the syringe was rapidly sucked into the vial after piercing the 21G needle through the rubber top of the evacuated vial. To facilitate the rehydration of the cake, unless otherwise stated, the vials containing the cakes were gently inverted thirty times for one minute. After the cake had appeared to fully rehydrate into a liposome dispersion, the vacuum was released.

6.3.6 Assessment of freeze dried cakes

The quality of the freeze dried cakes was characterised by assessing the physical properties of the liposome cake and the particle size of the reconstituted liposome dispersions. These two properties are described in section 6.3.6.1 and section 6.3.6.2.
respectively. Reconstituted dispersions containing cyA were additionally subjected to analytical filtration (section 5.3.5).

6.3.6.1 Properties of cake and reconstituted dispersion
Three cake properties were assessed. Firstly, the physical appearance of the cakes prior to reconstitution was noted. Cakes which had been successfully lyophilised appeared porous and brittle. In contrast, cakes which collapsed appeared compact and soft. Secondly, the relative ease and speed of cake rehydration upon the addition of deionised water was recorded. Cakes which rehydrated after gently inverting thirty times for one minute were classified as rapidly dispersing. Thirdly, the consistency of the resultant reconstituted dispersions was noted and is described in the appropriate tables of results either as a fluid or a gel.

6.3.6.2 Particle size of reconstituted liposome dispersions
In the literature, the particle size of most reconstituted lyophilised liposome dispersions has been characterised using only PCS (Vemuri et al., 1991; Tanaka et al., 1992). However, as discussed in section 3.3.8.3, due to the inability to detect large liposomes, PCS may not have been satisfactory for revealing the presence of a few large liposomes generated as a result of fusion/aggregation. Therefore, in this study other complementary sizing techniques were employed. The experimental details of the following techniques have been previously described in section 3.3.8. The size distribution of the dispersions before lyophilisation was assessed using light microscopy, PCS and laser diffraction. After lyophilisation all three techniques were only employed to characterise the size distribution of the liposomes, if the reconstituted dispersions were fluid and moderately translucent. Dispersions which appeared very turbid after freeze drying were generally not sized by PCS, because reproducible results could not be measured. Dispersions with gel-like properties after reconstitution were only assessed by light microscopy.

Light microscopy was employed to qualitatively assess the relative number of the larger liposomes in the individual dispersions using the scale described in section 3.3.8.2. The assessment of each dispersion based on this scale is given in the appropriate results tables in section 6.5. The appearance of the extruded dispersions under light microscopy, prior to lyophilisation, was not stated in the table of results, because all
dispersions were very fine and no large liposomes were observed under light microscopy at x400 magnification. When dispersions were sized by PCS and laser diffraction, the measurements were carried out at least four times on each of the four samples of each dispersion. The mean of the z average, polydispersity index (P.I.) and 90% undersize (D-90) values for each sample were determined. The means of these four replicates were in turn averaged to calculate the mean z average with standard deviation, mean P.I. with standard deviation and mean D-90 value with standard deviation. The average sample volume of the four replicates added to the laser diffraction cell is quoted as a mean volume in microlitres (μl) in the results tables in section 6.5.

**6.3.6.3 Analytical filtration of reconstituted dispersions**

All liposome dispersions containing cyA were subjected to analytical filtration as described in section 5.3.5. However, the amount of dispersion filtered was greater than in section 5.3.5. The four replicate dispersions were combined and analytically filtered, therefore the filtrate contained approximately 20 mg of cyA. The increase in filter weight for the formulations is stated in the appropriate tables in section 6.5.8. The relative ease of filtration and the appearance of the filter under light microscopy are also presented in the results tables.

**6.3.6.4 Statistical analysis**

The methods of statistical analysis, described in section 3.3.5, were used to compare the particle size of the various reconstituted liposome dispersions. The confidence limits of the statistical tests were set at 5%.

**6.4 Studies performed**

The particle size of the following dispersions was measured prior to and post lyophilisation. The quality of the freeze dried and reconstituted cakes was also assessed (section 6.4.8). Liposome dispersions incorporating cyA were analytically filtered (section 5.3.5).

**6.4.1 Stabilising phospholipid vesicles with saccharides**

The reasons for incorporating saccharide into the following liposome dispersions were two fold. Firstly, the saccharide acted as a stabiliser during lyophilisation to aid
liposome integrity. Secondly, it was added to render the formulation isotonic with serum.

Two saccharides; glucose (a monosaccharide) and sucrose (a disaccharide) were individually incorporated into soya PL blend liposomes as potential stabilisers. Throughout these experiments, investigating the stabilising properties of these two sugars, the soya PC: soya PL weight ratio of 90:10 (referred to in the text as the soya PL blend) was used to form the liposomes (section 3.5.5.5.2). The details of the three soya PL blend levels tested with glucose and sucrose are described in section 6.4.1.1 and section 6.4.1.2 respectively.

6.4.1.1 Incorporating glucose into liposome dispersions

Three different soya PL blend: glucose weight ratios were tested. Prior to freeze drying, the lipid concentrations of the extruded liposome dispersions were 30, 60 and 90 mg/g, and the glucose concentration for all three dispersions was 50 mg/g. These dispersions were made by adding 0.960 g, 1.920 g and 2.880 g of the soya PL blend pro-liposome to 1.000 g of anhydrous glucose dissolved in an amount of deionised water sufficient to give a final dispersion weight of 20 g. The dispersions were vigorously handshaken for one minute and the resultant coarse dispersions were subsequently extruded through polycarbonate filters as described in section 6.3.3.2. Freeze drying was carried out by freezing the samples in liquid nitrogen for ten minutes and immediately freeze drying for 24 hours. After freeze drying four 2.5 g of samples of each dispersion, all four cakes were immediately rehydrated at room temperature with sufficient deionised water to yield a final dispersion weight of 2.5 g. To hasten cake rehydration, the vials containing the rehydrating cakes were gently inverted for one minute.

6.4.1.2 Incorporating sucrose into liposome dispersions

Three formulations containing 30, 60 and 90 mg/g by weight of soya PL blend were made each containing 90 mg/g of sucrose. The dispersions were produced in a similar manner to the glucose dispersions described in section 6.4.1.1. However, the weight of sugar in these dispersions was higher. On a weight for weight basis almost twice the amount of sucrose (90 mg/g) was required to render the dispersions isotonic compared to glucose (50 mg/g). This was due to the fact that sucrose is a disaccharide with a MW (342) nearly double that of the monosaccharide anhydrous glucose (180). The three dispersions were produced by adding 0.960 g, 1.920 g and 2.880 g of the soya PL blend
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pro-liposome to 1.800 g of sucrose dissolved in deionised water, sufficient to give a total dispersion weight of 20 g. Liposome conversion was aided by vigorously handshaking the pro-liposome with this aqueous phase. The resultant dispersion was repeatedly extruded as described in section 6.3.2.2. Four 2.5 g samples of each extruded dispersion were frozen in liquid nitrogen for ten minutes and immediately freeze dried for 24 hours. The resultant cakes were immediately reconstituted at room temperature with deionised water to produce a final dispersion weight of 2.5 g. These reconstituted dispersions had lipid concentrations of 30, 60 and 90 mg/g and contained 90 mg/g of sucrose. To facilitate cake rehydration, the vials were gently inverted for one minute.

6.4.2 Varying lipid: sucrose concentration of liposome dispersions

From previous studies (section 6.4.1.2) it was found that soya PL blend liposome dispersions produced good lyophilised cakes if the soya PL blend: sucrose weight ratio was 2:3. Hence this ratio was adopted for studies investigating the effect of altering the lipid: sucrose concentration. In the following set of experiments, four different concentrations of soya PL blend were lyophilised, whilst the lipid: sucrose weight ratio was maintained at 2:3. The soya PL blend: sucrose weight concentrations in the four dispersions were 15:22.5, 30:45, 60:90 and 100:150 mg/g. These four dispersions were produced by respectively adding 0.480, 0.960, 1.920 and 3.200 g of soya PL blend pro-liposome to an aqueous phase sufficient to give a final dispersion weight of 20 g. Each of the four aqueous phases contained 0.450, 0.900, 1.800 and 2.700 g of sucrose respectively. The four dispersions were vigorously handshaken and the resultant liposome dispersions were processed by extrusion as described in section 6.3.3.2. The extruded dispersions were frozen in liquid nitrogen for ten minutes and freeze dried as described in section 6.3.4. After freeze drying these dispersions, the resultant cakes were immediately reconstituted at room temperature with deionised water to a final dispersion weight of 2.5 g. Hence the soya PL blend concentration of these reconstituted dispersions was 60 mg/g and the sucrose concentration was 90 mg/g. In order to facilitate the rehydration of the cakes, the vials were gently inverted for one minute.

6.4.3 Factors affecting size of reconstituted liposomes

6.4.3.1 Half isotonic dispersions

The term “half isotonic” in this context refers to a dispersion which possesses a tonicity, prior to freeze drying, half that of serum. These dispersions were produced to enable the
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l lipid concentration of the reconstituted dispersions to be raised to 120 mg/g, whilst maintaining isotonicity (90 mg/g).

A total weight of 75 g of this particular “half isotonic” dispersion was produced by adding 7.2 g of soya PL blend pro-liposome to 3.38 g of sucrose dissolved in 64.43 g of deionised water. The dispersion was vigorously handshaken for one minute and the resultant “half isotonic” liposome dispersion comprising 60 mg/g of soya PL blend and 45 mg/g of sucrose was extruded to reduce the average liposome size and distribution (section 6.3.3.2). This dispersion was used to study the effects of a variety of factors which could have potentially affected the particle size and size distribution of the liposomes. The details of these experiments are described in sections 6.4.3.2 and 6.4.3.3.

6.4.3.2 Influence of freezing history and agitation during reconstitution

The “half isotonic” soya PL blend liposome dispersion containing sucrose (section 6.4.3.1) was either frozen in the freezer for 12 hours (denoted F in Table 6.5) or frozen in liquid nitrogen for ten minutes (denoted N in Table 6.5) prior to freeze drying (section 6.3.4). After freeze drying these samples for 24 hours, the cakes were immediately reconstituted with deionised water to a final dispersion weight of 2.5 g. These cakes were reconstituted at room temperature by either gently shaking the vial or leaving the cake to disperse without any shaking. Gentle shaking involved immediately inverting the vials and their contents for one minute (denoted M in Table 6.5). In contrast, no shaking involved leaving the cakes to rehydrate in the deionised water for reconstitution until the cakes had completely dispersed (denoted S in Table 6.5). Photomicrographs of rehydrated dispersions which were either freezer frozen or liquid nitrogen frozen prior to lyophilisation are shown in Plate 6.2 and Plate 6.3 respectively.

6.4.3.3 Concentrating dispersions by adding a reduced amount of deionised water to the lyophilised cake

The rationale behind the following experiment was to determine the feasibility of raising the lipid concentration of the dispersion to 120 mg/g, whilst maintaining isotonicity. Raising the lipid level to 120 mg/g may have enabled the cyA concentration to be increased, which would have facilitated handling by reducing the volume of the dispersion.
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Samples from the same batch of the “half isotonic” soya PL blend dispersion containing sucrose (section 6.4.3.1) were either frozen in the freezer or liquid nitrogen before freeze drying. However, instead of reconstituting the cakes to their original weight with deionised water, the cakes were rehydrated by adding deionised water to half the weight of the original dispersion and gently inverting the vials for one minute. This rehydration regime concentrated the soya PL blend level in the final dispersion to 120 mg/g, whilst rendering the reconstituted dispersion isotonic (90 mg/g sucrose).

6.4.4 Effect of freezing regime on liposome size
The effects of the freezing regime on the particle size of the soya PL blend liposome dispersion without lyophilisation were studied. A fresh 30 g batch of liposome dispersion containing 60 mg/g of soya PL blend and 45 mg/g of sucrose was produced as described in section 6.4.3.1. Four 2.5 g aliquots of dispersion were either frozen in liquid nitrogen for 10 minutes or freezer frozen at approximately -20 °C for 12 hours (section 6.3.4). After freezing, the samples were left to defrost simultaneously at room temperature (20 °C -25 °C) prior to measuring the particle size.

6.4.5 Freeze drying liposome dispersions incorporating sucrose and glycerol
Six combinations of glycerol and sucrose were incorporated into soya PL blend liposome dispersions to investigate their roles as stabilisers during freeze drying. Each combination of glycerol and sucrose was added to the aqueous phase in quantities which yielded dispersions with a tonicity half that of serum before freeze drying. The six levels of glycerol added to the aqueous phase, ranged from 100% (13 mg/g) to 10% (1.3 mg/g) of the tonicity, the remaining tonicity was provided by the sucrose. These levels of glycerol and sucrose were selected so that after freeze drying, the soya PL blend level in the final dispersion could be doubled to 120 mg/g whilst maintaining isotonicity. The doubling of the soya PL blend concentration in the final dispersion was achieved by reconstituting the lyophilised cakes at room temperature with deionised water to 1.25 g. The six dispersions were produced by adding 1.920 g of soya PL blend pro-liposome to the six combinations of glycerol and sucrose dissolved in sufficient deionised water to give a final dispersion weight of 20 g. The pro-liposome and aqueous phase were vigorously handshaken to generate the liposome dispersions. The resultant dispersions were extruded as described in section 6.3.3.2, frozen in liquid nitrogen and freeze dried as described in section 6.3.4. The resultant cakes were immediately rehydrated at room
temperature with deionised water to half the weight of the dispersion prior to freeze drying. To aid reconstitution the vials containing the rehydrating cakes were gently inverted for one minute. The final dispersions had a soya PL blend concentration of 120 mg/g and a sucrose concentration of 90 mg/g.

6.4.6 Effect of lipid composition on the size of reconstituted liposome dispersions

6.4.6.1 Effect of blending soya PL with soya PC

Pro-liposomes containing varying ratios of soya PC and soya PL were produced by dissolving the requisite amount of lipid (60 parts by weight) in absolute ethanol (36 parts by weight). The soya PC: soya PL weight ratios of the seven pro-liposomes were: 100:0, 90:10, 75:25, 50:50, 75:25, 10:90 and 0:100. Each of the seven dispersions was produced by adding 1.920 g of the pro-liposome containing the appropriate soya PC: soya PL ratio to 0.900 g of sucrose dissolved in 17.18 g of deionised water. To facilitate liposome formation, the pro-liposome and aqueous phase were vigorously shaken for one minute. Prior to lyophilisation, the lipid concentration of these dispersions was 60 mg/g and the sucrose concentration was 45 mg/g. These dispersions were processed as described in section 6.3.3.2 by extruding the liposomes through a 100 nm filter three times, followed by two stacked 100 nm and 50 nm filters twenty times. The extruded dispersions were frozen in liquid nitrogen and freeze dried as described in 6.3.4. After lyophilisation of these “half isotonic” dispersions, the lipid and sucrose concentrations were doubled to 120 mg/g and 90 mg/g respectively by rehydrating the cake at room temperature to half the weight of the original dispersion. Cake rehydration was aided by gently inverting the vials for one minute. Light photomicrographs of rehydrated dispersions with soya PC: soya PL weight ratios of 100:0 and 75:25 are shown in Plate 6.5 and Plate 6.6 respectively.

6.4.6.2 Effect of blending soya PL with egg PC

The experiments as described in section 6.4.6.1 were repeated, except that egg PC was substituted for soya PC.

6.4.6.3 Effect of blending EPG with soya PC

EPG was blended along with soya PC to investigate the effect of a charged anionic PL on the size and size distribution of liposome dispersions reconstituted to a high lipid concentration after freeze drying. Specifically, four pro-liposomes with soya PC: EPG weight ratios were tested: 99.5:0.5, 99.0:1.0, 97.5:2.5 and 95:5. These pro-liposomes
Chapter six-Freeze drying liposome dispersions

were converted into liposome dispersions by adding 1.920 g of the appropriate pro-liposome to 0.900 g of sucrose dissolved in deionised water sufficient to give a dispersion weight of 20 g. The pro-liposomes and aqueous phases were handshaken vigorously at room temperature for one minute to yield dispersions which had a lipid concentration of 60 mg/g and a sucrose concentration of 45 mg/g. These dispersions were extruded as described in section 6.3.3.2, frozen in liquid nitrogen and freeze dried as described in section 6.3.4. The resultant cakes were rehydrated at room temperature to half the weight of the original dispersion to yield isotonic dispersions with a lipid concentration of 120 mg/g. To facilitate the rehydration of the cakes, the vials containing the cakes and deionised water for rehydration were gently inverted for one minute.

6.4.7 Effect of adding water soluble polymers to liposome dispersions

Polymers have been used to stabilise freeze dried pharmaceuticals. These large MW molecules are used to raise the $T_g$ of a formulation, thereby enhancing the stability of the cake during lyophilisation and storage (Skrabanja et al., 1994).

In the following studies, the effect of three different polymers, dextran and two different MW PVPs, on liposome particle size after freeze drying was investigated. The polymers were included in the liposome formulations by individually dissolving varying levels of these polymers in an extruded liposome dispersion prior to freeze drying. A “half isotonic” soya PL blend liposome dispersion was produced with the same ratios as described in section 6.4.3.1, except the total weight of soya PL blend dispersion was 100 g. Prior to freeze drying, the soya PL blend concentration of this liposome dispersion was 60 mg/g and the sucrose concentration was 45 mg/g. Three types of polymers were separately added to this liposome dispersion: dextran MW 40,000, PVP MW 44,000 and PVP MW 700,000. The dextran MW 40,000 was added at three concentrations: 1% w/w, 2.5% w/w and 5% w/w. The PVP MW 44,000 was added at 1% w/w and 2.5% w/w and PVP MW 700,000 was added at 1% w/w. The amount of polymer added to the dispersions was above the aqueous solubility of the polymer. Addition of the polymers to the liposome dispersions after polycarbonate extrusion (section 6.3.3.2) kept the polymer external to the liposome interior. The resultant dispersions containing polymers were frozen in liquid nitrogen for 10 minutes and freeze dried as described in section 6.3.4. After freeze drying, the cakes were rehydrated.
at room temperature with deionised water to give a final dispersion weight of 1.25 g. The soya PL blend concentration of these reconstituted dispersions was 120 mg/g, the sucrose concentration was 90 mg/g and the polymer concentration was doubled. To facilitate reconstitution the vials containing the rehydrating cakes were gently inverted for one minute.

6.4.8 Freeze drying liposomes containing cyA

6.4.8.1 Effect of varying cyA levels

The effect of lyophilising liposome dispersions containing varying levels of cyA was assessed in the following experiments. CyA was incorporated into soya PL blend pro-liposomes at the following three lipid: ethanol: cyA weight ratios: 60:36:1, 60:36:1.3 and 60:36:2.4. These three pro-liposomes were converted using a two stage conversion to form liposome dispersions with a cyA concentration of 1 mg/g, 1.3 mg/g and 2.4 mg/g respectively. In these studies, the liposome dispersions containing 60 mg/g of the soya PL blend were stabilised with either sucrose or glucose. Details describing the manufacture of these dispersions are provided in section 6.4.8.1.1 and section 6.4.8.1.2. Liquid nitrogen freezing and freeze drying of both sets of dispersions was carried out in the usual manner as previously described in section 6.3.4.

6.4.8.1.1 CyA liposomes incorporating sucrose

All three sets of soya PL blend liposome dispersions containing varying amounts of cyA were generated from pro-liposomes (section 6.4.8.1) using a two stage conversion (section 3.4.2). Firstly, some of the concentrated sucrose solution was Eppendorf mixed with each of the soya PL blend: ethanol: cyA pro-liposomes twenty times. Care had to be taken to ensure no more than 1.150 g (1.000 g + 15% overage) of sucrose solution (500 mg/g) was mixed with the appropriate amount of pro-liposome. The appropriate amounts of pro-liposome were: 2.231 g (1.940 g + 15% overage) of the pro-liposome with the lipid: cyA weight ratio of 60:1; 2.238 g (1.946 g + 15% overage) of the pro-liposome with the lipid: cyA weight ratio of 60:2 and 2.263 g (1.968 g + 15% overage) of the pro-liposome with the lipid: cyA weight ratio of 60:2.4. Greatly exceeding this level of aqueous phase resulted in the formation of precipitate in the dispersions. The hydrated pro-liposomes containing cyA were converted into liposome dispersions by adding 2.940 g, 2.946 g and 2.968 g respectively of the hydrated pro-liposomes to 0.800 g of sucrose dissolved in bulk deionised water, sufficient to give a total dispersion
weight of 20 g. To aid the dispersion of the hydrated cyA pro-liposome, the vials were vigorously handshaken for 90 seconds. The liposome size was reduced by extruding the dispersions as described in section 6.3.3.2. After extrusion, the first 100 nm filter and second 100 nm filter used for extruding each of the dispersions were examined under the light microscope to verify that the cyA had not precipitated during manufacture of the soya PL blend dispersions. CyA loss via precipitation was clearly undesirable, because the actual cyA content in liposomes would have been misleadingly lower than the stated cyA content. The extruded dispersions were subsequently lyophilised by freezing the samples in liquid nitrogen for ten minutes and freeze drying as described in section 6.3.4. The lyophilised cakes were rehydrated at room temperature by adding deionised water to a final dispersion weight of 2.5 g. These reconstituted dispersions had a soya PL blend concentration of 60 mg/g and a sucrose concentration of 90 mg/g. Rehydration of the cake was facilitated by gently inverting the vials for one minute.

6.4.8.1.2 CyA liposomes incorporating glucose
The sucrose in the above final dispersion was replaced with an equivalent molar weight of glucose for the following experiments. Therefore, before freeze drying and after rehydration of the liposome dispersion, the soya PL blend concentration was 60 mg/g and the sucrose concentration was 50 mg/g. These soya PL blend liposomes were made by passing 1.150 g (1.000 g + 15% overage) of a glucose solution (500 mg/g) and the appropriate weight of pro-liposome between two Eppendorf syringes twenty times. The appropriate weights of pro-liposome were: 2.231 g (1.94 g + 15% overage) of the pro-liposome with a lipid: cyA weight ratio of 60:1; 2.238 g (1.946 g + 15% overage) of the pro-liposome with a lipid: cyA weight ratio of 60:2 and 2.263 g (1.968 g + 15% overage) of the pro-liposome with a lipid: cyA weight ratio of 60:1. The hydrated soya PL blend pro-liposomes containing cyA were converted into liposome dispersions by adding 1.970 g, 1.973 g and 1.984 g respectively of the hydrated pro-liposomes to bulk deionised water, sufficient to give a total dispersion weight of 20 g. To facilitate the dispersion of the hydrated gels in bulk deionised water, the vials were handshaken for 90 seconds to generate liposome dispersions with soya PL blend concentrations of 60 mg/g. The subsequent liposome dispersions were extruded, as described in section 6.3.3.2, to narrow the size and size distribution. After examining the polycarbonate filters as described in section 6.4.8.1.1, the extruded dispersions were subsequently
frozen in liquid nitrogen for ten minutes and freeze dried as described in section 6.3.4. The cakes were rehydrated at room temperature by adding deionised water to the cakes to yield glucose concentrations of 50 mg/g. Rehydration of the lyophilised cakes was aided by gently inverting the vials for one minute.

6.4.5.2 Effect of varying lipid composition

The association of cyA with two liposome compositions before and after freeze drying was investigated. The two lipid compositions had a soya PC: EPG weight ratio of 99.5:0.5 and a soya PC soya PL weight ratio of 90:10. The lipid: ethanol: cyA weight ratio of both cyA pro-liposomes was 60:36:2.4. The dispersions were converted from pro-liposomes in two stages as described in section 6.4.8.1.1. Hydrated pro-liposomes were produced by passing 2.263 g of (1.968 g + 15% overage) of the appropriate pro-liposome and 1.150 g (1.000 g + 15% overage) of sucrose solution (500 mg/g) between two Eppendorf syringes twenty times. After Eppendorf mixing, 2.968 g of the resultant gel was added to 0.800 g of sucrose dissolved in deionised water, sufficient to give a total dispersion weight of 20 g. The lipid: cyA: sucrose weight ratio of the resultant dispersions was 60:2.4:90 mg/g. The resultant dispersions were extruded (section 6.3.3.2) and the filters were examined for the presence of precipitation (section 6.4.8.1.1). The extruded dispersions were frozen in liquid nitrogen for 10 minutes and freeze dried as described in section 6.3.4. The cakes were immediately reconstituted at room temperature with deionised water to yield liposome dispersions with a lipid concentration of 60 mg/g. Rehydration of the freeze dried cakes was aided by gently inverting the vials for one minute.

6.4.5.3 Effect of raising lipid concentration and cyA concentration

The effect of raising the cyA concentration in two soya PL blend dispersions was assessed. Two pro-liposome formulations with lipid: ethanol: cyA weight ratios of 60:36:3.0 and 60:36:2.4 were used to produce liposome dispersions containing lipid: ethanol: sucrose: cyA concentrations of 60:36:90:3.0 and 60:36:45:2.4 mg/g respectively. Both dispersions were generated from pro-liposomes using a two stage conversion. The pro-liposome with a lipid: ethanol: cyA weight ratio of 60:36:3.0 was used to produce the dispersion containing 3.0 mg/g of cyA dispersion. This dispersion was generated by passing 2.277 g (1.98 g + 15% overage) of the pro-liposome and 1.150 g (1.000 g + 15%) of sucrose solution between two Eppendorf syringes twenty
Chapter six-Freeze drying liposome dispersions

times, and adding 2.980 g of the hydrated pro-liposome to 0.8 g of sucrose in 16.22 g of deionised water. The cyA dispersion containing 2.4 mg/g prior to freeze drying was produced from the pro-liposome with a lipid: ethanol: cyA weight ratio of 60:36:2.4. The dispersion was generated by passing 2.263 g (1.968 + 15%) of the pro-liposome and 1.035 g (0.900 g + 15% overage) sucrose solution (500 mg/g) between two Eppendorf syringes twenty times, and adding 2.868 g of the hydrated pro-liposome to 17.132 g of deionised water.

The resultant dispersions were extruded (section 6.3.3.2) and the filters were examined for precipitation. The dispersions were subsequently frozen in liquid nitrogen and freeze dried as described in section 6.3.4. Both sets of liposome cakes were rehydrated at room temperature with deionised water to yield sucrose concentrations of 90 mg/g. Therefore, the soya PL blend: sucrose: cyA concentrations of the two reconstituted dispersions were 60:90:3 mg/g and 120:90:4.8 mg/g respectively. To facilitate reconstitution, the vials were gently inverted for one minute.

6.5 Results and Discussion

6.5.1 Incorporating saccharides into liposome dispersions

6.5.1.1 Incorporating glucose into soya PL blend liposomes

The particle size of the soya PL blend liposome dispersions incorporating glucose prior to and post freeze drying is shown in Table 6.1.

<table>
<thead>
<tr>
<th>Soya PL blend concentration (mg/g) before FD</th>
<th>30</th>
<th>60</th>
<th>90</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose concentration (mg/g) before FD</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>D-90 ± s.d. before FD (µm)</td>
<td>0.94±0.00</td>
<td>0.94±0.00</td>
<td>0.94±0.00</td>
</tr>
<tr>
<td>z average ± s.d. before FD</td>
<td>86.5±0.7</td>
<td>81.7±3.4</td>
<td>82.5±3.7</td>
</tr>
<tr>
<td>P.I. ± s.d. before FD</td>
<td>0.087±0.030</td>
<td>0.091±0.044</td>
<td>0.082±0.050</td>
</tr>
<tr>
<td>Soya PL blend concentration (mg/g) after reconstitution</td>
<td>30</td>
<td>60</td>
<td>90</td>
</tr>
<tr>
<td>Glucose concentration (mg/g) after recon.</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>D-90 ± s.d. after reconstitution (µm)</td>
<td>9.94±1.30</td>
<td>16.20±6.08</td>
<td>-</td>
</tr>
<tr>
<td>Vol. added to LD cell after reconstitution (µl)</td>
<td>1400</td>
<td>120</td>
<td>-</td>
</tr>
<tr>
<td>z average ± s.d. (nm) after reconstitution</td>
<td>91.2±3.5</td>
<td>126.4±2.5</td>
<td>-</td>
</tr>
<tr>
<td>P.I. ± s.d. after reconstitution</td>
<td>0.176±0.020</td>
<td>0.243±0.008</td>
<td>-</td>
</tr>
<tr>
<td>Appearance of reconstituted dispersions under LM</td>
<td>Fine</td>
<td>Coarse</td>
<td>Very coarse</td>
</tr>
<tr>
<td>Characteristics of reconstituted dispersions</td>
<td>Fluid</td>
<td>Fluid</td>
<td>Gel</td>
</tr>
</tbody>
</table>

Reconstituting the cakes to a final lipid concentration of 90 mg/g generated a thick gel with unhydrated regions of lipid/glucose. As a consequence of this inadequate liposome rehydration, it can be concluded that there was insufficient glucose to stabilise this high
level of lipid. However, liposome stabilisation at a lipid concentration of 90 mg/g could not have been improved by raising the level of glucose above 50 mg/g, without generating a hypertonic dispersion.

Lowering the lipid: glucose weight ratio by reducing the concentration of lipid in the dispersion improved the quality of the reconstituted liposomes. At the lower lipid concentrations of 30 mg/g and 60 mg/g, fluid dispersions were generated after rehydration. Nevertheless, even at a lower lipid level of 60 mg/g with 50 mg/g glucose, a large degree of fusion/aggregation had taken place. This was verified by light microscopy, which revealed the presence of many large liposomes with diameters between 5-10 μm (Plate 6.1). The lowest level of liposome fusion/aggregation seemed to have occurred in the sample with the lowest concentration of lipid (Plate 6.2). The z average of the 30 mg/g lipid dispersions was significantly smaller than the z average of the dispersions with 60 mg/g lipid (p = 0.0003). Although the D-90 of these 30 mg/g lipid dispersions had risen from 0.94 μm to 9.94 μm after lyophilisation, the volume of reconstituted dispersions added to the laser diffraction cell to obtain the necessary obscuration was 1400 μl. This large volume clearly indicated that the number of large liposomes in the reconstituted sample was relatively small. This fact was confirmed by light microscopy which revealed the presence of only a few large liposomes around 5 μm (Plate 6.1).

Plate 6.1 Typical light micrograph of a reconstituted soya PL blend dispersion containing 60 mg/g of lipid and 50 mg/g of glucose (Bar = 25 μm), as described in section 6.5.1.1
6.5.1.2 Incorporating sucrose into soya PL blend liposomes

The particle size of the soya PL blend liposome dispersions incorporating sucrose prior to and post freeze drying is shown in Table 6.2.

Table 6.2 Effect of sucrose on the particle size of three different soya PL blend liposome dispersions

<table>
<thead>
<tr>
<th>Soya PL blend concentration (mg/g) before FD</th>
<th>30</th>
<th>60</th>
<th>90</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose concentration (mg/g) before FD</td>
<td>90</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>D-90 ± s.d. before FD (µm)</td>
<td>0.94±0.00</td>
<td>0.94±0.00</td>
<td>0.94±0.00</td>
</tr>
<tr>
<td>Vol. added to LD cell before FD (µl)</td>
<td>800</td>
<td>600</td>
<td>400</td>
</tr>
<tr>
<td>z average ± s.d. before FD (nm)</td>
<td>87.0±13.9</td>
<td>86.8±3.8</td>
<td>94.5±4.6</td>
</tr>
<tr>
<td>P.I. ± s.d. before FD (nm)</td>
<td>0.102±0.017</td>
<td>0.076±0.023</td>
<td>0.132±0.084</td>
</tr>
<tr>
<td>Soya PL blend concentration (mg/g) after reconstitution</td>
<td>30</td>
<td>60</td>
<td>90</td>
</tr>
<tr>
<td>Sucrose concentration (mg/g) after reconstitution</td>
<td>90</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>D-90 ± s.d. after reconstitution (µm)</td>
<td>9.89±0.81</td>
<td>7.66±1.28</td>
<td>3.94±0.86</td>
</tr>
<tr>
<td>Vol. added to LD cell after reconstitution (µl)</td>
<td>575</td>
<td>300</td>
<td>50</td>
</tr>
<tr>
<td>z average ± s.d. after reconstitution (nm)</td>
<td>86.9±2.2</td>
<td>88.3±1.5</td>
<td>109.9±2.1</td>
</tr>
<tr>
<td>P.I. ± s.d. after reconstitution</td>
<td>0.144±0.010</td>
<td>0.160±0.06</td>
<td>0.210±0.009</td>
</tr>
<tr>
<td>Appearance of reconstituted dispersions under LM</td>
<td>Fine</td>
<td>Fine</td>
<td>Moderately coarse</td>
</tr>
<tr>
<td>Characteristics of reconstituted dispersions</td>
<td>Fluid</td>
<td>Fluid</td>
<td>Fluid</td>
</tr>
</tbody>
</table>

Even though all the dispersions were fluid after rehydration, it was evident that some degree of liposome fusion/aggregation had occurred in all dispersions. The distinction between aggregation and fusion could not generally be made with these techniques. Therefore, any increase in liposome size and distribution will be attributed to fusion and aggregation. The extent of liposome fusion/aggregation varied according to the soya PL blend: sucrose ratio of the dispersion. Liposome dispersions with the lowest
concentration of lipid were stabilised by 90 mg/g of sucrose to a greater extent than liposome dispersions with a higher concentration of lipid. This fact was demonstrated when the size and size distribution of the dispersions were compared. The increase in P.I. for the dispersions with the lowest lipid concentration (30 mg/g) was smaller than the increase in P.I. for the dispersions with the higher lipid concentrations. The P.I. of the 30 mg/g dispersions rose from 0.10 to only 0.14 after freeze drying. In contrast, the dispersions with 90 mg/g of lipid rose from around 0.13 to 0.21. However, yet again the D-90 values contradicted this finding and indicated that the converse was true. The D-90 value of the dispersions containing 30 mg/g and 60 mg/g of lipid was 9.89 µm and 7.66 µm compared to 3.94 µm for the dispersions containing 90 mg/g of lipid. These D-90 values of the dispersions containing 30 mg/g and 60 mg/g of lipid were significantly lower than the D-90 value of the 90 mg/g lipid dispersions (p = 0.0001; p = 0.006 respectively). This demonstrated the inaccuracy of relying on one sizing measurement. Consideration of the volume added to the laser diffraction cell and light microscopy clearly confirmed that the dispersions with lower lipid concentrations had fewer larger liposomes present. The dispersions with a lipid concentration of 30 mg/g and 60 mg/g required far more sample volume to be added to the laser diffraction cell than the dispersions with 90 mg/g of lipid. This suggested that the two sets of dispersions with lower lipid concentrations had fewer larger liposomes than the set of dispersions with 90 mg/g of lipid. Similarly, light microscopy confirmed this finding: under a x400 magnification, the light microscope revealed only a few large liposomes in the dispersions containing 30 mg/g and 60 mg/g of lipid. In contrast, at the same magnification a greater number of large liposomes was present in the reconstituted dispersions containing a lipid concentration of 90 mg/g.

From the above results, on a mole for mole basis, it appeared that the protection provided by sucrose (Table 6.2) against fusion/aggregation was superior to the protection provided by glucose (Table 6.1). This parallels the findings of Crowe et al. (1987), who stated that the disaccharides are generally superior stabilisers than the monosaccharides. At equimolar levels, sucrose largely stabilised soya PL blend liposomes at a lipid concentration of 60 mg/g, whereas a considerable degree of liposome fusion/aggregation occurred in the presence of equimolar glucose. Due to the superior stabilising properties over glucose, the disaccharide sucrose was selected as the stabiliser for subsequent liposome formulations, unless otherwise stated.
6.5.2 Effect of varying lipid: sucrose concentrations whilst maintaining the lipid: sucrose ratio

The particle size of soya PL blend liposome dispersions before lyophilisation and after reconstitution, with 15, 30, 60 and 100 mg/g of lipid and 22.5, 45, 90 and 150 mg/g of sucrose respectively are shown in Table 6.3.

Table 6.3 Influence of lipid: sucrose concentration on the particle size of soya PL blend liposome dispersions before and after lyophilisation

<table>
<thead>
<tr>
<th>Soya PL blend concentration (mg/g) before FD</th>
<th>15</th>
<th>30</th>
<th>60</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose concentration (mg/g) before FD</td>
<td>22.5</td>
<td>45</td>
<td>90</td>
<td>150</td>
</tr>
<tr>
<td>D-90 ± s.d. before FD (µm)</td>
<td>0.94±0.00</td>
<td>0.94±0.00</td>
<td>0.94±0.00</td>
<td>3.59±0.27</td>
</tr>
<tr>
<td>Vol. added to LD cell before FD (µl)</td>
<td>&gt;2000</td>
<td>&gt;2000</td>
<td>&gt;2000</td>
<td>750</td>
</tr>
<tr>
<td>z average ± s.d. before FD (nm)</td>
<td>87.0±1.3</td>
<td>78.0±1.4</td>
<td>82.0±0.9</td>
<td>92.13±1.3</td>
</tr>
<tr>
<td>P.I. ± s.d. before FD</td>
<td>0.13±0.004</td>
<td>0.110±0.005</td>
<td>0.114±0.012</td>
<td>0.177±0.027</td>
</tr>
</tbody>
</table>

| Soya PL blend conc. (mg/g) after reconstitution | 60   | 60   | 60   | 60   |
| Sucrose conc. (mg/g) after reconstitution     | 90   | 90   | 90   | 90   |
| D-90 ± s.d. after reconstitution (µm)         | 6.09±0.58 | 6.55±1.55 | 9.66±3.74 | 18.27±2.96 |
| Vol. added to LD cell after reconstitution (µl) | 90   | 100  | 80   | 60   |
| z average ± s.d. after reconstitution (nm)    | 88.4±5.9 | 83.6±5.9 | 84.5±5.7 | 97.2±2.2 |
| P.I. ± s.d. after reconstitution              | 0.259±0.013 | 0.225±0.014 | 0.230±0.047 | 0.202±0.019 |

| Appearance of reconstituted dispersions under LM | Fine | Fine | Fine | Moderately coarse |
| Characteristics of reconstituted dispersions     | Fluid | Fluid | Fluid | Fluid |

The dispersions with a lipid concentration prior to lyophilisation of 100 mg/g showed the largest increase in liposome size after freeze drying and reconstituting to a lipid concentration of 60 mg/g. These reconstituted dispersions had an average D-90 value of over 18 µm, which was significantly greater than the average D-90 values of the other dispersions (p = 0.02 for 100 mg/g vs. 60 mg/g; p = 0.0009 for 100 mg/g vs. 30 mg/g; p = 0.0004 for 100 mg/g vs. 15 mg/g). The presence of larger liposomes in samples generated from the 100 mg/g lipid dispersions was also qualitatively confirmed by light microscopy. These results showed that freeze drying high concentrations of soya PL blend (100 mg/g) and sucrose (150 mg/g) produced dispersions with 10% of the liposomes (by volume) having a diameter greater than 18 µm. It seemed that freeze drying these dispersions may have increased the level of liposome fusion/aggregation. One possible explanation for this increase in size may have been attributed to the close proximity of neighbouring liposomes in concentrated dispersions. Even in the presence of saccharides, the wall to wall nature of the vesicles may have tended to induce liposome fusion/aggregation. The P.I. values of all four sets of dispersions increased to over 0.2 after freeze drying, indicating that lyophilisation increased the distribution of sizes as a result of fusion and/or aggregation.
Despite the fact that the lipid: sucrose weight ratios were identical in all reconstituted dispersions, the properties of the freeze dried cakes differed considerably. Freeze drying dispersions with lipid concentrations of 15 mg/g and 30 mg/g generated fragile cakes which crumbled into fluffy powders when gently disturbed. This intrinsic weakness was probably due to the large open structures of the cake, which were formed after the removal of the water. As a consequence of this large exposed surface area, the cake dispersed immediately upon the addition of deionised water. In contrast, lyophilisation of the dispersions with 100 mg/g lipid generated firmer cakes, which took an additional 90 seconds to rehydrate after one minute of gently inverting the vials. This slower rehydration may have resulted from the closer packing of the liposomes within the compact sucrose matrix. Lyophilisation of the dispersions containing 60 mg/g of lipid seemed to produce cakes with superior properties: these firm cakes remained intact after agitation and upon addition of deionised water rehydration was rapid.

6.5.3 Factors potentially affecting size of reconstituted liposomes

6.5.3.1 Half isotonic dispersions

The particle size of the “half isotonic” dispersion prior to freeze drying measured in 50 mg/g glucose is shown in Table 6.4.

| Soya PL blend conc. (mg/g) before FD | 60 |
| Sucrose concentration (mg/g) before FD | 45 |
| D-90 ± s.d. before FD (μm) | 0.94±0.00 |
| Vol. added to LD cell before FD (μl) | >2000 |
| z average ± s.d. before FD (nm) | 85.2±1.3 |
| P.I. ± s.d. before FD | 0.136±0.008 |

Prior to freezing and lyophilisation, the D-90 value (0.94 ± 0.00 μm) of this “half isotonic” dispersion was identical to the D-90 value (0.94 ± 0.00 μm) of isotonic dispersions (Table 6.2). This indicated that both dispersions were below the limit of detection of laser diffraction.

The z average of the dispersion was approximately 85 nm which was comparable to the z average of the isotonic dispersions (Table 6.2).
6.5.3.2 Influence of freezing history and gentle agitation

The particle size results for the reconstituted half isotonic dispersions frozen either in the freezer or liquid nitrogen prior to lyophilisation and dispersed either with or without agitation are shown in Table 6.5.

Table 6.5 Effect of the freezing history and inverting the rehydrating soya PL blend cakes on the liposome size (FS60 = Freezer frozen and rehydrated without shaking, FM60 = Freezer frozen and gently inverted during rehydration, NM60 = Nitrogen frozen and gently inverted during rehydration, NS60 = Nitrogen frozen and rehydrated without shaking)

<table>
<thead>
<tr>
<th>Conditions</th>
<th>FS60</th>
<th>FM60</th>
<th>NM60</th>
<th>NS60</th>
</tr>
</thead>
<tbody>
<tr>
<td>soya PL blend conc. (mg/g) after recon.</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>Sucrose conc. (mg/g) after recon.</td>
<td>45</td>
<td>45</td>
<td>45</td>
<td>45</td>
</tr>
<tr>
<td>D-90 ± s.d. after reconstitution (μm)</td>
<td>37.15±4.46</td>
<td>43.69±2.56</td>
<td>7.40±2.45</td>
<td>7.12±1.48</td>
</tr>
<tr>
<td>Vol. added to LD cell after recon. (μl)</td>
<td>100</td>
<td>90</td>
<td>200</td>
<td>175</td>
</tr>
<tr>
<td>z average ± s.d. after recon. (nm)</td>
<td>88.7±3.4</td>
<td>92.5±2.6</td>
<td>99.8±1.8</td>
<td>102.3±2.6</td>
</tr>
<tr>
<td>P.I. ± s.d. after reconstitution</td>
<td>0.235±0.026</td>
<td>0.252±0.015</td>
<td>0.251±0.014</td>
<td>0.236±0.015</td>
</tr>
<tr>
<td>Appearance of recon. dispersions under LM</td>
<td>Coarse</td>
<td>Coarse</td>
<td>Moderately coarse</td>
<td>Moderately coarse</td>
</tr>
<tr>
<td>Characteristics of recon. dispersions</td>
<td>Fluid</td>
<td>Fluid</td>
<td>Fluid</td>
<td>Fluid</td>
</tr>
</tbody>
</table>

After lyophilisation, the P.I. and D-90 value of all reconstituted samples increased (Table 6.5). The P.I. for all samples rose from 0.14 (Table 6.4) before lyophilisation to over 0.24 after lyophilisation. Similarly, the rise in the D-90 value of all the samples after freeze drying reflected a broadening in the size distributions. However, the most pronounced rise in D-90 value occurred with the dispersions frozen in the freezer. The D-90 value of these reconstituted dispersions (FS60 and FM60) exceeded 35 μm. The D-90 value of FM60 (freezer frozen and inverted during rehydration) and FS60 (freezer frozen and rehydrated without inverting) were significantly higher than the corresponding D-90 value of NM60 (liquid nitrogen frozen and inverted during rehydration) and NS60 (liquid nitrogen frozen and rehydrated without inverting) (p = 0.0002 for FM60 vs. NM60; p = 0.001 for FS60 vs. NS60). This indicated that some very large liposomes were present after reconstituting the lyophilised freezer frozen cakes. Indeed, compared to NM60 (Plate 6.3), the presence of many more discreet large liposomes in FM60 was confirmed under the magnification of light microscopy (Plate 6.4). Conversely, the z average value for FM60 and FS60 was significantly lower than the corresponding z average value for NM60 and NS60 (p = 0.003 for FM60 vs. NM60; p = 0.0003 for FS60 vs. NS60). A possible explanation for this finding is discussed in section 6.5.4.

Leaving the cakes to disperse without shaking did not significantly affect the z average or D-90 of the freezer frozen samples or nitrogen frozen samples. The z average of these
Chapter six-Freeze drying liposome dispersions

samples either subjected to inversions (denoted as FM60) or left to rehydrate without agitation (denoted as FS60) was not significantly different from one another providing the freezing regime prior to freeze drying was identical \((p = 0.12)\). Similarly, the D-90 value of FM60 and FS60 was not significantly different \((p = 0.26)\).

Plate 6.3 Typical light micrograph of a rehydrated soya PL blend dispersion which was frozen in liquid nitrogen prior to lyophilisation (NM60). Rehydration of the cake was carried out by gently inverting the cake and deionised water for rehydration for one minute \((\text{Bar} = 25 \mu\text{m})\), as described in section 6.5.3.2.

Plate 6.4 Typical light micrograph of a rehydrated soya PL blend liposome dispersions which was frozen in freezer just prior to lyophilisation (FM60). Rehydration of the cake was carried out by gently inverting the cake and deionised water for rehydration for one minute \((\text{Bar} = 25 \mu\text{m})\), as described in section 6.5.3.2.
6.5.3.3 Concentrating the dispersions by adding reduced amounts of water for rehydration to freeze dried cakes

The particle size of the freezer frozen and liquid nitrogen frozen dispersions reconstituted to 120 mg/g of lipid are shown in Table 6.6.

Table 6.6 Effect of freeze drying on the particle size of soya PL blend liposomes rehydrated with reduced amounts of deionised water (FS120 = Freezer frozen and left to rehydrate without agitation, NS120 = Liquid nitrogen frozen and left to rehydrate without agitation)

<table>
<thead>
<tr>
<th>Conditions</th>
<th>FS120</th>
<th>NS120</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soya PL blend conc. (mg/g) after reconstitution</td>
<td>120</td>
<td>120</td>
</tr>
<tr>
<td>Sucrose conc. (mg/g) after reconstitution</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>D-90 ± s.d. after reconstitution (μm)</td>
<td>38.28±4.75</td>
<td>6.71±0.60</td>
</tr>
<tr>
<td>Vol. added to LD cell after reconstitution (μl)</td>
<td>35</td>
<td>105</td>
</tr>
<tr>
<td>z average ± s.d. after reconstitution (nm)</td>
<td>92.6±5.8</td>
<td>107.9±6.3</td>
</tr>
<tr>
<td>P.I. ± s.d. after reconstitution</td>
<td>0.253±0.026</td>
<td>0.243±0.020</td>
</tr>
<tr>
<td>Appearance of reconstituted dispersions under LM</td>
<td>Coarse</td>
<td>Moderately coarse</td>
</tr>
<tr>
<td>Characteristics of reconstituted dispersions</td>
<td>Fluid</td>
<td>Fluid</td>
</tr>
</tbody>
</table>

The D-90 value of the rehydrated freezer frozen sample (FS120) was significantly higher than the D-90 value of the rehydrated liquid nitrogen frozen sample (NS120) (p = 0.001). However, the z average (92.6 nm) of the freezer frozen samples (FS120) was again significantly smaller than the z average (107.9 nm) of the liquid nitrogen frozen samples (NS120) after freeze drying (p = 0.012). The z average of these two sets of cakes (FS120 and NS120) rehydrated to 120 mg/g lipid was not significantly different from the corresponding z average of cakes (FS60 and NS60) rehydrated to 60 mg/g of lipid (Table 6.5) (p = 0.287 for FS120 vs. FS60; p = 0.175 for NS120 vs. NS60). This suggested that addition of the reduced weight of deionised water liberated the liposomes from the sugar matrix without greatly increasing fusion/aggregation. Therefore, reconstituting the final dispersion to either 60 mg/g or 120 mg/g of the soya PL blend did not seem to significantly affect the particle size, if sucrose was employed as the stabiliser.

6.5.4 Comparing freezer freezing with liquid nitrogen freezing

In view of the results indicating that the samples frozen in the freezer produced different sized liposomes compared to liquid nitrogen frozen samples, the effects of the freezing regime on the particle size of the liposome dispersions without lyophilisation were studied. The particle size of the half isotonic dispersion prior to freezing and post defrosting are shown in Table 6.7.
Chapter six-Freeze drying liposome dispersions

Table 6.7 Effect of freezing history on particle size of a half isotonic soya PL blend liposome dispersion after defrosting

<table>
<thead>
<tr>
<th></th>
<th>Dispersion before freezing</th>
<th>Liquid N2 frozen</th>
<th>-25 °C frozen</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-90 ± s.d. (μm)</td>
<td>0.94±0.00</td>
<td>3.25±0.31</td>
<td>0.94±0.00</td>
</tr>
<tr>
<td>Vol. added to cell (μL)</td>
<td>1000</td>
<td>670</td>
<td>1000</td>
</tr>
<tr>
<td>z average ± s.d. (nm)</td>
<td>84.0±4.4</td>
<td>84.5±0.49</td>
<td>73.6±1.88</td>
</tr>
<tr>
<td>P.I. ± s.d.</td>
<td>0.098±0.077</td>
<td>0.178±0.021</td>
<td>0.145±0.019</td>
</tr>
<tr>
<td>Appearance of reconstituted dispersions under LM</td>
<td>Very fine</td>
<td>Fine</td>
<td>Very fine</td>
</tr>
</tbody>
</table>

The results indicated that both forms of freezing affected the particle size of the dispersions in different ways. Firstly, the development of haziness after defrosting the liquid nitrogen samples visually reflected the fact that the number of larger liposomes had increased in these dispersions. Laser diffraction confirmed this increase in coarseness by measuring a rise in the average D-90 value of these dispersions from 0.94 μm before freezing to 3.25 μm after liquid nitrogen freezing and defrosting. Additionally, the volume of these samples added to the laser diffraction cell decreased compared to the dispersions prior to freezing. These laser diffraction results indicated that after defrosting liquid nitrogen frozen samples, fusion/aggregation between liposomes had occurred which broadened the size distribution of the dispersions. Furthermore, microscopic inspection of the dispersions under the light microscope revealed the presence of a few large liposomes. However, the only measurement which did not reflect an increase in the number of large particles was the z average. After defrosting the liquid nitrogen frozen dispersions, the z average remained at 84.5 nm which was virtually identical to the z average of the dispersions prior to freezing (84.0 nm).

In contrast, the z average of the freezer frozen samples was reduced by 10 nm after defrosting, and no large particles were detected under light microscopy or by laser diffraction.

When comparing the freezer frozen dispersions with the liquid nitrogen frozen dispersions, it was evident that the freezer frozen samples were finer than liquid nitrogen frozen samples. The average D-90 value of the freezer frozen dispersions was significantly lower than the average D-90 value of the nitrogen frozen dispersions (p = 0.009). Similarly, the z average of the freezer frozen dispersions were significantly lower than the z average of the nitrogen frozen dispersions (p = 0.001).

The reason for the size changes in the liposome dispersions may have been attributed to the type of ice crystal formation during freezing and/or defrosting. Slower freezing in
the freezer has been reported to produce larger crystals compared to the rapidly frozen liquid nitrogen samples (Carrington et al., 1996). These large ice crystals may have mechanically disrupted the bilayers perhaps generating smaller liposomes upon reannealing. Conversely, rapidly freezing the dispersions in liquid nitrogen would have produced finer crystals which were less likely to rupture the liposomes. However, a degree of fusion/aggregation between some liposomes must have taken place in liquid nitrogen frozen samples either during freezing or defrosting: liquid nitrogen frozen dispersions which had defrosted had larger sized liposomes compared to the pre-frozen dispersions as revealed by laser diffraction and light microscopy.

The results of the experiments investigating freezing did not directly correlate with the results of the freeze drying experiments in section 6.5.3.2. In both sets of experiments the mean z average of the dispersions frozen in the freezer was lower than the mean z average of the dispersions frozen in liquid nitrogen. However, the average D-90 value of the lyophilised freezer frozen samples was significantly higher than the average D-90 value of the lyophilised liquid nitrogen frozen samples (section 6.5.3.2). These results contrasted with the dispersions which were frozen in either the freezer or liquid nitrogen. Perhaps the reason for the increase in liposome size after reconstitution of the freezer frozen samples may be explained by the temperature of the samples during lyophilisation. Compared to the temperature of the liquid nitrogen frozen samples, the temperature of the freezer frozen samples would have probably been markedly higher during the primary drying phase. Hence the temperature of the cake may have risen above the T_g and may have resulted in a greater degree of fusion.

6.5.5 Lyophilising liposome dispersions incorporating glycerol and sucrose

The particle size data for the reconstituted dispersions containing six different levels of glycerol and sucrose are shown in Table 6.8.
### Chapter six-Freeze drying liposome dispersions

#### Table 6.8 Effect of freeze drying on the particle size of half isotonic soya PL blend liposome dispersions

<table>
<thead>
<tr>
<th>Glycerol: sucrose mol% ratio</th>
<th>100:0</th>
<th>90:10</th>
<th>75:25</th>
<th>50:50</th>
<th>25:75</th>
<th>10:90</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soya PL blend concentration before FD (mg/g)</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>Glycerol conc. mg/g prior to FD</td>
<td>13</td>
<td>11.7</td>
<td>9.8</td>
<td>6.5</td>
<td>3.3</td>
<td>1.3</td>
</tr>
<tr>
<td>Sucrose conc. mg/g prior to FD</td>
<td>0</td>
<td>4.5</td>
<td>11.3</td>
<td>22.5</td>
<td>33.8</td>
<td>40.5</td>
</tr>
<tr>
<td>D-90 ± s.d. before FD (μm)</td>
<td>0.94±0.00</td>
<td>0.94±0.00</td>
<td>0.94±0.00</td>
<td>0.94±0.00</td>
<td>0.94±0.00</td>
<td>0.94±0.00</td>
</tr>
<tr>
<td>z average ± s.d. before FD (nm)</td>
<td>85.1±1.6</td>
<td>87.4±0.7</td>
<td>80.6±2.2</td>
<td>84.0±3.1</td>
<td>81.4±2.4</td>
<td>87.2±3.2</td>
</tr>
<tr>
<td>P.I. ± s.d. before FD (nm)</td>
<td>0.082±0.010</td>
<td>0.106±0.060</td>
<td>0.129±0.039</td>
<td>0.158±0.021</td>
<td>0.082±0.007</td>
<td>0.145±0.028</td>
</tr>
<tr>
<td>Soya PL blend conc. after FD (mg/g)</td>
<td>120</td>
<td>120</td>
<td>120</td>
<td>120</td>
<td>120</td>
<td>120</td>
</tr>
<tr>
<td>Glycerol conc. (mg/g) after reconstitution</td>
<td>26</td>
<td>23.4</td>
<td>19.6</td>
<td>13</td>
<td>6.6</td>
<td>2.6</td>
</tr>
<tr>
<td>Sucrose conc. mg/g after reconstitution</td>
<td>0</td>
<td>9.0</td>
<td>22.6</td>
<td>45</td>
<td>67.6</td>
<td>81</td>
</tr>
<tr>
<td>D-90 ± s.d. after reconstitution (μm)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>5.46±0.70</td>
<td>5.22±0.70</td>
</tr>
<tr>
<td>Vol. added to LD cell after reconstitution (μl)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>40</td>
<td>80</td>
</tr>
<tr>
<td>z average ± s.d. after reconstitution (nm)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>156.2±4.8</td>
<td>127.0±4.5</td>
</tr>
<tr>
<td>P.I. ± s.d after reconstitution (nm)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.251±0.01</td>
<td>0.263±0.011</td>
</tr>
<tr>
<td>Appearance of recon. dispersions under LM</td>
<td>Very coarse</td>
<td>Very coarse</td>
<td>Very coarse</td>
<td>Very coarse</td>
<td>Moderately coarse</td>
<td>Moderately coarse</td>
</tr>
<tr>
<td>Characteristics of reconstituted dispersions</td>
<td>Gel</td>
<td>Gel</td>
<td>Gel</td>
<td>Gel</td>
<td>Fluid</td>
<td>Fluid</td>
</tr>
</tbody>
</table>

If the glycerol level was equal to or exceeded 50 mol% of the tonicity, the resultant freeze dried cakes collapsed, appeared transparent and blistered. This appearance seemed to be related to the amount of glycerol added prior to freeze drying. As the glycerol level was increased, the dried structure became more wax-like and transparent. Upon addition of deionised water these cakes slowly rehydrated to form gel-like dispersions, which were too coarse for meaningful data to be generated. The formation of this viscous structure suggested that massive fusion had occurred. The extensive liposome fusion which occurred in samples with high levels of glycerol may have been related to the low glass transition temperature of glycerol (-65 °C; Levine and Slade, 1988). During freeze drying and storage, the temperature of the cakes may have been higher than the Tg of the liposome samples with glycerol and as a direct consequence cake collapse may have occurred.

However, glycerol containing dispersions could be satisfactorily lyophilised to produce firm cakes if the glycerol: sucrose mol ratio was 25:75 mol% or 10:90 mol% of the tonicity. These two sets of cakes formed flowable fluids upon rehydration. Nevertheless,
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it was evident that even in these relatively superior samples, some fusion/aggregation between liposomes had occurred. The z average increased from 84.1 nm and 87.2 nm to 156 nm and 127 nm respectively, both the P.I.s rose to above 0.25 and light microscopy revealed many large particles greater than 10 μm in diameter in both dispersions. Therefore, it seemed evident that incorporating glycerol into the formulations did not aid liposome stabilisation during freeze drying.

6.5.6 Effect of lipid composition on size of reconstituted dispersions
6.5.6.1 Effect of blending soya PL with soya PL

The effect of blending soya PL with soya PC on the liposome size before and after freeze drying are shown in Table 6.9.

<table>
<thead>
<tr>
<th>SPC: SPL weight ratio</th>
<th>100:0</th>
<th>90:10</th>
<th>75:25</th>
<th>50:50</th>
<th>25:75</th>
<th>0:100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid conc. (mg/g) before FD</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>Sucrose conc. (mg/g) before FD</td>
<td>45</td>
<td>45</td>
<td>45</td>
<td>45</td>
<td>45</td>
<td>45</td>
</tr>
<tr>
<td>D-90 ± s.d. before FD (μm)</td>
<td>0.94±0.00</td>
<td>0.94±0.00</td>
<td>0.94±0.00</td>
<td>0.94±0.00</td>
<td>1.58±0.01</td>
<td>7.22±0.09</td>
</tr>
<tr>
<td>Vol. added to LD cell before FD (μl)</td>
<td>450</td>
<td>900</td>
<td>750</td>
<td>700</td>
<td>600</td>
<td>325</td>
</tr>
<tr>
<td>z average ± s.d. before FD (nm)</td>
<td>83.4±4.2</td>
<td>83.7±2.7</td>
<td>81.0±3.6</td>
<td>89.9±2.2</td>
<td>91.8±4.1</td>
<td>89.3±2.4</td>
</tr>
<tr>
<td>P.I.± s.d. before FD</td>
<td>0.107±0.037</td>
<td>0.120±0.014</td>
<td>0.142±0.023</td>
<td>0.113±0.011</td>
<td>0.124±0.020</td>
<td>0.143±0.034</td>
</tr>
<tr>
<td>Lipid conc. (mg/g) after recon.</td>
<td>120</td>
<td>120</td>
<td>120</td>
<td>120</td>
<td>120</td>
<td>120</td>
</tr>
<tr>
<td>Sucrose conc. (mg/g) after recon.</td>
<td>90</td>
<td>90</td>
<td>90</td>
<td>90</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>D-90 ± s.d. after recon. (μm)</td>
<td>4.20±2.17</td>
<td>5.68±1.91</td>
<td>8.65±2.92</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Vol. added to LD cell after recon. (μl)</td>
<td>5</td>
<td>75</td>
<td>70</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>z average ± s.d. after recon. (nm)</td>
<td>201.6±11.8</td>
<td>101.9±1.9</td>
<td>102.7±2.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P.I.± s.d. after recon.</td>
<td>0.272±0.016</td>
<td>0.225±0.020</td>
<td>0.239±0.010</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Appearance of recon. dispersions under LM</td>
<td>Very coarse</td>
<td>Moderately coarse</td>
<td>Moderately coarse</td>
<td>Coarse</td>
<td>Coarse</td>
<td>Coarse</td>
</tr>
<tr>
<td>Characteristics of recon. dispersions</td>
<td>Fluid</td>
<td>Fluid</td>
<td>Fluid</td>
<td>Gel</td>
<td>Gel</td>
<td>Gel</td>
</tr>
</tbody>
</table>

Prior to lyophilisation, most liposome dispersions had a z average of between 80 nm and 90 nm, a P.I. around 0.10 and the D-90 value of most dispersions was below the lower limit of laser diffraction detection. However, the two sets of dispersions with the two highest proportions of the soya PL had a D-90 value greater than 0.94 μm prior to lyophilisation. The D-90 value of the dispersions with soya PC: soya PL weight ratios of 25:75 and 0:100 was 1.58 μm and 7.22 μm respectively. The explanation for the larger
D-90 value of these two dispersions could perhaps be attributed to the composition of the liposome and the high lipid concentration. As explained in section 3.5.5.5.2, the presence of charged species in soya PL may have prevented the complete conversion of pro-liposomes at this high lipid concentration. Upon dilution in isotonic media of the laser diffraction cell, these remaining pro-liposomes would have converted into larger liposomes, because there was no osmotic gradient to generate finer particles. These large liposomes may have accounted for the raised D-90 value of the dispersions prior to freeze drying.

The level of soya PL blended with soya PC greatly influenced the size of the reconstituted freeze dried liposomes. Reconstitution of the freeze dried liposome cakes with soya PC: soya PL weight ratios of 50:50 or less resulted in the formation of gel-like masses after the addition of deionised water. However, reconstituting the freeze dried liposome cakes containing soya PC: soya PL weight ratios of 75:25 or more produced flowable dispersions. The z average of dispersions with soya PC: soya PL weight ratios of 75:25 and 90:10 increased from 84 nm and 81 nm before lyophilisation to 104 nm and 102 nm respectively after lyophilisation. However, the z average of the dispersions with soya PC: soya PL weight ratios of 75:25 (102.7 nm) and 90:10 (101.9 nm) were significantly lower than the z average of the dispersions produced entirely from soya PC (201.6 nm) (p = < 0.001 for 75:25 vs. 100:0; p = < 0.001 for 90:10 vs. 100:0). The improved stabilisation of these soya PL containing dispersions following lyophilisation and rehydration may perhaps be linked to the presence of glycolipids in the soya PL. These glycolipids (section 2.4.1) may have encouraged interaction between these membrane lipids and the saccharides by hydrogen bonding. This form of stabilisation has been discussed previously by Engel et al. (1994); Park and Huang (1992), although the glycolipids in Engel’s study (Engel et al., 1994) employed highly purified galactoside in higher amounts.

From the D-90 results, the dispersions with a soya PC: soya PL weight ratio of 100:0 were significantly smaller than the dispersions with a soya PC: soya PL weight ratio of 70:25 (p = 0.023). However, upon examination of the light micrographs of the two sets of dispersions containing soya PC: soya PL of 100:0 (Plates 6.5) and 75:25 (Plate 6.6), it was evident that the dispersions with a soya PC: soya PL weight ratio of 100:0 were considerably coarser. The apparent discrepancy in the D-90 value may be related to the
inability of the laser diffraction to detect smaller liposomes (section 3.3.8.4) which were present in the dispersions with soya PC: soya PL weight ratios of 75:25.

Plate 6.5 Typical light micrograph of a soya PC liposome dispersion reconstituted to a lipid concentration of 120 mg/g and a sucrose concentration of 90 mg/g (Bar = 25 μm), as described in section 6.5.6.1

Plate 6.6 Typical light micrograph of a liposome dispersion with soya PC: soya PL weight ratio of 75:25 reconstituted to a lipid concentration of 120 mg/g and a sucrose concentration of 90 mg/g (Bar = 25 μm), as described in section 6.5.6.1
6.5.6.2 Effect of blending soya PL with egg PC

The results of blending soya PL with egg PC on the liposome size before and after freeze drying are shown in Table 6.10.

Table 6.10 Effect of freeze drying on the particle size of egg PC liposomes blended with varying amounts of soya PL

<table>
<thead>
<tr>
<th>Egg PC: soya PL weight ratio</th>
<th>100:0</th>
<th>90:10</th>
<th>75:25</th>
<th>50:50</th>
<th>25:75</th>
<th>0:100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid conc. (mg/g) before FD</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>Sucrose conc. (mg/g) before FD</td>
<td>45</td>
<td>45</td>
<td>45</td>
<td>45</td>
<td>45</td>
<td>45</td>
</tr>
<tr>
<td>D-90 ± s.d. before FD (µm)</td>
<td>0.94±0.00</td>
<td>0.94±0.00</td>
<td>0.94±0.00</td>
<td>0.94±0.00</td>
<td>1.79±0.01</td>
<td>5.40±0.03</td>
</tr>
<tr>
<td>Vol. added to LD cell before FD (µl)</td>
<td>200</td>
<td>500</td>
<td>600</td>
<td>500</td>
<td>500</td>
<td>400</td>
</tr>
<tr>
<td>z average ± s.d. before FD (nm)</td>
<td>80.5±1.5</td>
<td>80.1±1.5</td>
<td>81.1±1.1</td>
<td>80.7±0.3</td>
<td>87.2±1.8</td>
<td>87.9±1.0</td>
</tr>
<tr>
<td>P.I. ± s.d. before FD</td>
<td>0.012±0.02</td>
<td>0.071±0.042</td>
<td>0.061±0.012</td>
<td>0.098±0.015</td>
<td>0.085±0.044</td>
<td>0.102±0.028</td>
</tr>
<tr>
<td>Lipid conc. (mg/g) after recon.</td>
<td>120</td>
<td>120</td>
<td>120</td>
<td>120</td>
<td>120</td>
<td>120</td>
</tr>
<tr>
<td>Sucrose conc. (mg/g) after recon.</td>
<td>90</td>
<td>90</td>
<td>90</td>
<td>90</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>D-90 ± s.d. after recon. (µm)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Vol. added to LD cell after recon. (µl)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>z average ± s.d. after recon. (nm)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P.I. ± s.d. after recon.</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Appearance of recon. dispersions under LM</td>
<td>Very coarse</td>
<td>Very coarse</td>
<td>Very coarse</td>
<td>Very coarse</td>
<td>Very coarse</td>
<td>Very coarse</td>
</tr>
<tr>
<td>Characteristics of recon. dispersions</td>
<td>Gel</td>
<td>Gel</td>
<td>Gel</td>
<td>Gel</td>
<td>Gel</td>
<td>Gel</td>
</tr>
</tbody>
</table>

After lyophilisation, all egg PC compositions with or without soya PL produced thick gels following subsequent rehydration. All samples looked very coarse under the light microscope. A light micrograph of a reconstituted dispersion with an egg PC: soya PL weight ratio of 100:0 is shown in Plate 6.7. Even lyophilised formulations with 75 parts or more of egg PC by weight produced fused gels after reconstitution. In comparison, equivalent dispersions containing 75 parts or more of soya PC by weight (Table 6.10) produced flowable dispersions. The reason for the fusion of egg PC samples upon rehydration was unclear. It may have been linked in some way to the degree of saturation of the fatty acids, which was the main difference between the egg PC and soya PC.

In view of the above results, it seemed that liposomes composed of soya PC blended with soya PL were more suited to the freeze drying at these concentrations than egg PC with or without soya PL.
Chapter six-Freeze drying liposome dispersions

Plate 6.7 Typical light micrograph of an egg PC liposome dispersion reconstituted to a lipid concentration of 120 mg/g and a sucrose concentration of 90 mg/g (Bar = 25 μm), as described in section 6.5.6.2.

6.5.6.3 Effect of blending EPG with soya PC

The size data for liposome dispersions containing various levels of EPG prior to and post lyophilisation are shown in Table 6.11.

Table 6.11 Effect of freeze drying on the particle size of soya PC liposomes incorporating various levels of EPG

<table>
<thead>
<tr>
<th>Soya PC: EPG weight ratio</th>
<th>100:0</th>
<th>99.5:0.5</th>
<th>99.0:1.0</th>
<th>97.5:2.5</th>
<th>95.0:5.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid conc. (mg/g) before FD</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>Sucrose conc. (mg/g) before FD</td>
<td>45</td>
<td>45</td>
<td>45</td>
<td>45</td>
<td>45</td>
</tr>
<tr>
<td>D-90 ± s.d. before FD (μm)</td>
<td>0.94</td>
<td>0.94</td>
<td>0.94</td>
<td>0.94</td>
<td>0.94</td>
</tr>
<tr>
<td>Vol. added to LD cell before FD (μl)</td>
<td>250</td>
<td>900</td>
<td>900</td>
<td>900</td>
<td>900</td>
</tr>
<tr>
<td>z average ± s.d. before FD (nm)</td>
<td>85.3±1.4</td>
<td>86.1±1.3</td>
<td>79.4±1.5</td>
<td>86.8±3.6</td>
<td>82.2±1.1</td>
</tr>
<tr>
<td>P.I. ± s.d. before FD (nm)</td>
<td>0.130±0.068</td>
<td>0.144±0.066</td>
<td>0.149±0.052</td>
<td>0.112±0.002</td>
<td>0.113±0.026</td>
</tr>
<tr>
<td>Lipid conc. (mg/g) after recon.</td>
<td>120</td>
<td>120</td>
<td>120</td>
<td>120</td>
<td>120</td>
</tr>
<tr>
<td>Sucrose conc. (mg/g) after recon.</td>
<td>90</td>
<td>90</td>
<td>90</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>D-90 ± s.d. after recon. (μm)</td>
<td>3.54±0.20</td>
<td>5.04±0.81</td>
<td>5.30±0.20</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Vol. added to LD cell after recon. (μl)</td>
<td>15</td>
<td>80</td>
<td>100</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>z average ± s.d. after recon. (nm)</td>
<td>185.8±12.8</td>
<td>108.6±11.2</td>
<td>116.9±9.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P.I. ± s.d. after reconstitution</td>
<td>0.270±0.025</td>
<td>0.215±0.02</td>
<td>0.245±0.016</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Appearance of recon. dispersions under LM</td>
<td>Very coarse</td>
<td>Coarse</td>
<td>Coarse</td>
<td>Very coarse</td>
<td>Very coarse</td>
</tr>
<tr>
<td>Characteristics of recon. dispersions</td>
<td>Fluid</td>
<td>Fluid</td>
<td>Thick fluid</td>
<td>Gel</td>
<td>Gel</td>
</tr>
</tbody>
</table>

The z average of the liposomes with or without EPG were within the range of 79 to 87 nm prior to lyophilisation, and the D-90 value of all dispersions was 0.94 μm. In contrast, after lyophilisation and reconstituting to a lipid concentration of 120 mg/g, the physical characteristics and the particle size of the dispersions differed distinctly. Gel-like masses were obtained upon rehydration if the soya PC: EPG weight ratios of the cake was equal to or less than 97.5:2.5. However, flowable dispersions were generated if
the soya PC: EPG weight ratio was equal to or greater than 99:1. The introduction of small levels of charge improved the particle size after reconstitution: the z average diameter of liposome dispersions with soya PC: EPG weight ratios of 99:1 and 99.5:0.5 increased by about only 30 nm to 110 nm after freeze drying. In contrast, without EPG, the z average of the soya PC liposomes increased by 100 nm after lyophilisation to over 180 nm.

6.5.7 Effect of including polymers in liposome dispersions

The particle size of the "half isotonic" dispersion before the addition of the polymers is shown in Table 6.12.

<table>
<thead>
<tr>
<th>Soya PL blend concentration (mg/g) before FD</th>
<th>Sucrose concentration (mg/g) before FD</th>
<th>D-90 ± s.d. (µm)</th>
<th>Vol. added to LD cell (µl)</th>
<th>z average ± s.d. (nm)</th>
<th>P.I. ± s.d</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>45</td>
<td>0.94</td>
<td>1000</td>
<td>81.3±3.6</td>
<td>0.110±0.045</td>
</tr>
</tbody>
</table>

Prior to the addition of polymer, the extruded dispersion was translucent and no large liposomes were visible under the light microscope at a x400 magnification.

The particle size of the dispersions after the addition of polymer and after freeze drying in the presence of polymer are shown in Table 6.13.

<table>
<thead>
<tr>
<th>Polymer MW (% w/w) before FD</th>
<th>Dextran 40,000 (1%)</th>
<th>Dextran 40,000 (2.5%)</th>
<th>Dextran 40,000 (5%)</th>
<th>PVP 700,000 (1%)</th>
<th>PVP 44,000 (1%)</th>
<th>PVP 44,000 (2.5%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soya PL blend conc. (mg/g) before FD</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>Sucrose concentration mg/g before FD</td>
<td>45</td>
<td>45</td>
<td>45</td>
<td>45</td>
<td>45</td>
<td>45</td>
</tr>
<tr>
<td>D-90 ± s.d. before FD (µm)</td>
<td>3.18±0.03</td>
<td>3.57±0.02</td>
<td>3.53±0.05</td>
<td>3.64±0.01</td>
<td>4.04±0.03</td>
<td>3.69±0.01</td>
</tr>
<tr>
<td>Vol. added to LD cell before FD (µl)</td>
<td>800</td>
<td>600</td>
<td>400</td>
<td>600</td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td>z average ± s.d. before FD (nm)</td>
<td>82.0±2.2</td>
<td>79.3±3.7</td>
<td>82.7±2.2</td>
<td>78.6±2.3</td>
<td>85.5±0.2</td>
<td>80.5±3.5</td>
</tr>
<tr>
<td>P.I. ± s.d. before FD</td>
<td>0.197±0.056</td>
<td>0.179±0.017</td>
<td>0.207±0.015</td>
<td>0.140±0.002</td>
<td>0.174±0.013</td>
<td>0.158±0.028</td>
</tr>
<tr>
<td>Soya PL blend conc. (mg/g) after recon.</td>
<td>120</td>
<td>120</td>
<td>120</td>
<td>120</td>
<td>120</td>
<td>120</td>
</tr>
<tr>
<td>Sucrose concentration (mg/g) after recon.</td>
<td>90</td>
<td>90</td>
<td>90</td>
<td>90</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>D-90 ± s.d. after recon. (µm)</td>
<td>7.97±2.04</td>
<td>-</td>
<td>-</td>
<td>7.10±0.66</td>
<td>7.79±1.79</td>
<td>-</td>
</tr>
<tr>
<td>Vol. added to LD cell after recon. (µl)</td>
<td>20</td>
<td>-</td>
<td>-</td>
<td>15</td>
<td>15</td>
<td>-</td>
</tr>
<tr>
<td>Appearance of recon. dispersions under LM</td>
<td>Very coarse</td>
<td>Very coarse</td>
<td>Very coarse</td>
<td>Very coarse</td>
<td>Very coarse</td>
<td>Very coarse</td>
</tr>
<tr>
<td>Characteristics of recon. dispersions</td>
<td>Fluid</td>
<td>Gel</td>
<td>Gel</td>
<td>Fluid</td>
<td>Fluid</td>
<td>Gel</td>
</tr>
</tbody>
</table>
The appearance of the clear liposome dispersions before freeze drying changed upon the addition of a polymer. Prior to lyophilisation, the inclusion of dextran at 2.5% w/w and 5% w/w in the formulations turned the dispersions somewhat cloudy and under the light microscope a small proportion of large liposomes was visible. This indicated that the addition of dextran at these levels induced some degree of fusion/aggregation. This development of cloudiness was unlikely to be due to the polymer not dissolving in the liposome dispersions, because the levels of the polymers used were much lower than the aqueous solubility of the polymers. This change was also reflected by the increase in P.I. value, z average and the raised D-90 values (Table 6.13). The P.I. increased from 0.11 (Table 6.12) to above 0.17 in all samples with dextran and the D-90 value had increased from 0.94 μm to above 3 μm. Similar results were obtained with all PVP samples, even at low concentrations of 1%. Therefore, even before freeze drying, these polymers adversely affected the quality of the dispersions.

The size characteristics of these liposome dispersions containing a polymer were dramatically affected after lyophilisation. Only the samples with 1% polymer could be measured by laser diffraction, because only these samples rehydrated satisfactorily to produce fluid dispersions. The D-90 of these samples with 1% polymer increased to above 7 μm, and perhaps more importantly the volumes of dispersions which had to be added to the laser diffraction cell had decreased greatly. The volumes decreased from 400-1000 μl prior to freeze drying down to 15 μl after freeze drying. None of the dispersions were sized by PCS, because the samples were too coarse for meaningful data to be generated. The addition of the polymers investigated here clearly did not have a beneficial impact on the liposomes during freeze drying. Although dextran and the other polymers have been used to improve the stability of pharmaceuticals by raising the T_g of cakes, in this case, their incorporation with liposomes was unfavourable and adversely affected the particle size. Since the addition of polymers did not improve the particle size of any of the dispersions, one can indirectly predict that the reason for the liposome fusion in the liposome dispersions containing saccharide was probably not related to a low T_g of the cake. If liposome fusion in these dispersions was solely attributable to low T_g, the addition of a high MW polymer should have reduced the degree of fusion (Crowe et al., 1994).
Chapter six-Freeze drying liposome dispersions

6.5.8 Freeze drying liposomes containing cyA

6.5.8.1 Effect of varying levels of cyA

6.5.8.1.1 CyA liposomes incorporating sucrose

The particle size data for the three cyA liposome dispersions incorporating sucrose are shown in Table 6.14.

Table 6.14 Effect of freeze drying on the particle size and association of cyA soya PL blend liposome dispersions incorporating sucrose

| Soya PL blend concentration before FD (mg/g) | 60 | 60 | 60 |
| CyA concentration before FD (mg/g)       | 1  | 1.3| 2.4|
| Sucrose concentration before FD (mg/g)   | 90 | 90 | 90 |
| D-90 ± s.d. before FD (μm)               | 0.94±0.00 | 0.94±0.00 | 0.94±0.00 |
| Vol. added to LD cell before FD (μl)     | >2000 | >2000 | >2000 |
| z average ± s.d. before FD (nm)         | 80.1±4.1 | 82.7±3.1 | 82.1±5.4 |
| P.I. ± s.d. before FD                   | 0.14±0.020 | 0.166±0.010 | 0.14±0.019 |

| Appearance of 100 nm filters under LM   | Clear | Clear | Clear |
| Soya PL blend concentration after recon. (mg/g) | 60 | 60 | 60 |
| CyA concentration after recon. (mg/g)     | 1    | 1.3 | 2.4 |
| Sucrose concentration after recon. (mg/g) | 90   | 90  | 90  |
| D-90 ± s.d. after reconstitution (μm)    | 8.55±1.06 | 9.57±1.66 | 8.76±0.55 |
| Vol. added to LD cell after recon. (μl)  | 150  | 210 | 275 |
| z average ± s.d. after recon. (nm)      | 84.6±2.7 | 81.3±4.2 | 80.6±3.3 |
| P.I. ± s.d. after recon.                 | 0.205±0.011 | 0.186±0.025 | 0.193±0.031 |

| Appearance of reconstituted dispersions under LM | Moderately coarse | Fine | Fine |
| Characteristics of reconstituted dispersions | Fluid | Fluid | Fluid |
| Ease of filtration                          | 1    | 1   | 1   |
| Filter weight increase (mg)                 | 0.107 | 0.143 | 0.079 |
| Appearance of filter under LM               | Clear | Clear | Clear |

The z average of these three cyA dispersions after freeze drying was indistinguishable from the z average of the corresponding cyA dispersions prior to freeze drying (Table 6.14). However, the increase in D-90 value and the decline of the volumes added to laser diffraction cell for the three sets of reconstituted cyA liposome dispersions indicated that a degree of fusion/aggregation had occurred after freeze drying. This was verified by light microscopy, which detected some larger liposomes in all the reconstituted freeze dried dispersions. However, the average D-90 value of the three sets of reconstituted liposome dispersions containing cyA was not significantly higher than the D-90 value (7.66±1.28 μm) of the corresponding reconstituted drug-free liposomes (Table 6.2) (p = 0.222 for 25:1 vs. drug-free dispersions; p = 0.166 for 45:1 vs. drug-free dispersions; p = 0.568 for 60:1 vs. drug-free dispersions). This indicated that the presence of cyA in the liposomes under these conditions did not seem to encourage the generation of larger liposomes after freeze drying compared to drug-free dispersions.
Since there did not appear to be any cyA precipitate on the polycarbonate filters used for the extrusion of the dispersions under the light microscope, this indicated that cyA was associated with the liposomes at all three cyA concentrations before freeze drying.

After reconstitution of the freeze dried cakes, the association of cyA did not seem to be compromised. The cyA remained associated with liposomes after reconstituting these freeze dried cyA sucrose liposome cakes. No cyA precipitation was seen under light microscopy after analytical filtration of the reconstituted cyA dispersions, and there were only small increases in filter weight after analytical filtration of the three sets of dispersions. The weight retained on the filter was less than 0.7% of 20 mg of cyA in the formulation.

### 6.5.8.1.2 CyA liposomes incorporating glucose

The particle size data for the three cyA dispersions containing 50 mg/g glucose is shown in Table 6.15:

| Soya PL blend concentration before FD (mg/g) | 60   | 60   | 60   |
| CyA concentration before FD (mg/g)          | 1    | 1.3  | 2.4  |
| Sucrose concentration before FD (mg/g)      | 50   | 50   | 50   |
| D-90 ± s.d. before FD (μm)                  | 0.94±0.00 | 0.94±0.00 | 0.94±0.00 |
| Vol. added to LD cell before FD (μl)        | >2000 | >2000 | >2000 |
| z average ± s.d. before FD (nm)             | 84.5±4.7 | 82.0±4.3 | 83.3±4.4 |
| P.I. ± s.d. before FD                       | 0.078±0.017 | 0.093±0.020 | 0.150±0.08 |

| Soya PL blend concentration after recon. (mg/g) | 60   | 60   | 60   |
| CyA concentration after recon. (mg/g)           | 1    | 1.3  | 2.4  |
| Sucrose concentration after recon. (mg/g)       | 50   | 50   | 50   |
| D-90 ± s.d. after reconstitution (μm)           | 22.1±0.87 | 18.6±3.6 | 19.0±0.32 |
| Vol. added to LD cell after reconstitution (μl) | 65   | 50   | 25   |
| z average ± s.d. after reconstitution (nm)      | 133.0±4.0 | 137.9±3.6 | 188.0±21.9 |
| P.I. ± s.d. after reconstitution                | 0.268±0.026 | 0.273±0.011 | 0.289±0.056 |

| Appearance of reconstituted dispersions under LM | Coarse | Coarse | Coarse |
| Characteristics of recon. dispersions           | Fluid  | Fluid  | Fluid  |
| Ease of filtration of reconstituted dispersions  | 1      | 1      | 1      |
| Filter weight increase (mg)                     | 0.106  | 0.086  | 0.127  |
| Appearance of filter under LM                   | Clear  | Clear  | Clear  |

The increases in z average, P.I. and D-90 value (Table 6.15) of the three cyA formulations indicated that the glucose level (50 mg/g) was insufficient to stabilise these three cyA liposome dispersions (soya PL blend concentration 60 mg/g) during freeze drying. This finding was perhaps unsurprising, since it was previously found that drug-free liposomes of identical lipid and glucose composition had fused considerably (Table 6.1). This again demonstrated the inferior stabilising properties of glucose compared to sucrose on a mole: mole basis.
After reconstituting the lyophilised cakes, the z average of the soya PL blend liposome dispersions containing 1 part of cyA for every 25 parts of lipid, had increased more than the z average of the two other lower concentrations of cyA (p = 0.005 for dispersions 25:1 vs. 45:1; p = 0.007 for dispersions 25:1 vs. 60:1). These liposomes containing cyA at a lipid: drug weight ratio of 60:2.4 doubled in diameter from 83 nm prior to lyophilisation to over 180 nm after lyophilisation. Similarly, the smaller volume of this sample added to the laser diffraction cell indicated that these dispersions with a higher cyA content may have contained a greater number of larger particles after freeze drying. These results suggested that the higher cyA content may have encouraged liposome fusion/aggregation in these dispersions. Nevertheless, despite this greater degree of fusion/aggregation, there was no precipitation of cyA in these dispersions nor in the sets of the other two dispersions containing glucose: after analytical filtration, a minimal increase in filter weight was noted and perhaps more importantly, the light microscope did not reveal precipitation. Therefore, under the conditions tested, the selection of either glucose or sucrose did not influence the association of cyA before or after freeze drying. After freeze drying, cyA remained associated with the liposomes in the presence of either of these two sugars.

6.5.8.2 Effect of varying lipid composition

The particle size of the cyA dispersions with two different lipid compositions prior to and post freeze drying is shown in Table 6.16.

<table>
<thead>
<tr>
<th>Lipid composition</th>
<th>99.5% Soya PC 0.5% EPG</th>
<th>90% soya PC 10% soya PL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid conc. (mg/g) before FD</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>CyA conc. (mg/g) before FD</td>
<td>2.4</td>
<td>2.4</td>
</tr>
<tr>
<td>Sucrose conc. (mg/g) before FD</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>D-90 ± s.d. before FD (μm)</td>
<td>0.94±0.00</td>
<td>0.94±0.00</td>
</tr>
<tr>
<td>Vol. added to LD cell before FD (μl)</td>
<td>&gt;2000</td>
<td>&gt;2000</td>
</tr>
<tr>
<td>z average ± s.d. before FD (nm)</td>
<td>77.2±3.4</td>
<td>79.8±2.8</td>
</tr>
<tr>
<td>P.I. ± s.d. before FD</td>
<td>0.124±0.033</td>
<td>0.087±0.034</td>
</tr>
<tr>
<td>Appearance of 100 nm filter under LM</td>
<td>Clear</td>
<td>Clear</td>
</tr>
<tr>
<td>Lipid conc. (mg/g) after recon.</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>CyA conc. (mg/g) after recon.</td>
<td>2.4</td>
<td>2.4</td>
</tr>
<tr>
<td>Sucrose conc. (mg/g) after recon.</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>D-90 ± s.d. after reconstitution (μm)</td>
<td>8.55±1.15</td>
<td>8.74±1.38</td>
</tr>
<tr>
<td>Vol. added to LD cell after reconstitution (μl)</td>
<td>85</td>
<td>150</td>
</tr>
<tr>
<td>z average ± s.d. after reconstitution (nm)</td>
<td>81.5±3.2</td>
<td>83.5±2.1</td>
</tr>
<tr>
<td>P.I. ± s.d. after reconstitution</td>
<td>0.220±0.014</td>
<td>0.202±0.020</td>
</tr>
<tr>
<td>Appearance of reconstituted dispersions under LM</td>
<td>Moderately coarse</td>
<td>Fine</td>
</tr>
<tr>
<td>Characteristics of recon. dispersions</td>
<td>Fluid</td>
<td>Fluid</td>
</tr>
<tr>
<td>Ease of filtration of recon. dispersions</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Increase in filter mass (mg)</td>
<td>0.103</td>
<td>0.124</td>
</tr>
<tr>
<td>Appearance of filter under LM</td>
<td>Clear</td>
<td>Clear</td>
</tr>
</tbody>
</table>
Chapter six-Freeze drying liposome dispersions

Compared to the reconstituted cyA EPG dispersions, the reconstituted cyA soya PL blend dispersions seemed somewhat finer under the light microscope, although no significant differences were detected using D-90 and z average measurements (p = 0.410 for D-90 values; p = 0.891 for z average measurements).

After lyophilisation, both sets of dispersions generated compact porous cakes, which rapidly dispersed to form fluid cyA liposome dispersions. The cyA remained associated with the liposomes before and after reconstituting the freeze dried cake, irrespective of the lipid composition. No cyA precipitation was found on the surfaces of either the filters used to reduce the size of the liposomes prior to freeze drying or the filters used for the analytical filtration of the three dispersions after lyophilisation.

6.5.8.3 Effect of raising lipid concentration and cyA concentration

The particle size data for two cyA dispersions containing 3 mg/g and 2.4 mg/g of cyA prior to freeze drying and 3 mg/g and 4.8 mg/g of cyA respectively post freeze drying are shown in Table 6.17.

<table>
<thead>
<tr>
<th>Soya PL blend conc. (mg/g) before FD</th>
<th>60</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>CyA conc. (mg/g) before FD</td>
<td>3.0</td>
<td>2.4</td>
</tr>
<tr>
<td>Sucrose conc. (mg/g) before FD</td>
<td>90</td>
<td>45</td>
</tr>
<tr>
<td>D-90 ± s.d. before FD (μm)</td>
<td>0.94±0.00</td>
<td>0.94±0.00</td>
</tr>
<tr>
<td>Vol. added to LD cell before FD (μl)</td>
<td>&gt;2000</td>
<td>&gt;2000</td>
</tr>
<tr>
<td>z average ± s.d. before FD (nm)</td>
<td>78.7±4.7</td>
<td>79.0±3.0</td>
</tr>
<tr>
<td>P.I. ± s.d. before FD</td>
<td>0.088±0.027</td>
<td>0.087±0.012</td>
</tr>
<tr>
<td>Appearance of 100 nm filter under LM</td>
<td>Clear</td>
<td>Clear</td>
</tr>
</tbody>
</table>

| Soya PL blend conc. (mg/g) after recon. | 60 | 120 |
| CyA conc. (mg/g) after recon.          | 3.0 | 4.8 |
| Sucrose conc. (mg/g) after recon.      | 90 | 90 |
| D-90 ± s.d. after reconstitution (μm)  | 16.6±4.74 | - |
| Vol. added to LD cell after reconstitution (μl) | 90 | - |
| z average ± s.d. after reconstitution (nm) | 90.8±2.0 | - |
| P.I. ± s.d. after reconstitution       | 0.216±0.013 | - |
| Appearance of reconstituted dispersions under LM | Moderately coarse | Very coarse |
| Characteristics of recon. dispersions  | Fluid | Gel |
| Ease of filtration                    | I   | II  |
| Increase in filter mass (mg)          | 0.114 | 0.169 |
| Appearance of filter under LM         | Clear | Clear |

The cakes reconstituted to 120 mg/g of lipid did not rapidly rehydrate to form fluid dispersions. Even after gently inverting the vial for one minute, these particular cakes took an additional 10 minutes to disperse and rehydrate into thick gel-like structures. Due to their viscous nature, analytical filtration of these gels could only be carried out by diluting the gels three fold with filtered deionised water. The presence of cyA in
these dispersions clearly encouraged fusion. When cyA was omitted from these dispersions, the cakes could be rehydrated into fluid liquids (Table 6.6). This indicated that at high lipid: sucrose weight ratios, cyA encouraged liposome fusion under the adopted freeze drying conditions. However, yet again after analytical filtration of the cyA dispersions with 120 mg/g of lipid, no cyA precipitation was seen under the light microscope and the weight increase was less than 1% of cyA. Similarly, if cyA was incorporated into liposomes (60 mg/g lipid) at a lipid: cyA weight ratio of 20:1, there was a significant increase in D-90 value (p = 0.02) and a large reduction in the volume of sample added to the laser diffraction cell, compared to the drug-free dispersions (Table 6.2). These results could not be attributed to cyA precipitation, because no precipitation was observed on the filter and the filter weight had not increased greatly after analytical filtration. Furthermore, the coarse appearance of these dispersions with 60 mg/g of lipid under the light microscope confirmed that the increase in z average and D-90 value was indeed attributable to large liposomes. These results indicated that at a cyA concentration of 3 mg/g, the fusion/aggregation of 60 mg/g of soya PL blend liposomes was encouraged even in the presence of sucrose as a stabiliser.

6.6 Conclusions

The production of freeze dried liposomes for intravenous administration is generally more involved than the production of traditional freeze dried injectables. The presence of liposomes in a formulation introduces considerations into the freeze drying process which may not otherwise need to be addressed. Specific factors, such as the liposome size after reconstitution and the association of the drug with the liposome, must be monitored and controlled. Both the formulation factors and the lyophilisation process influenced the particle size of the reconstituted liposomes in this study. Various formulation factors, such as the inclusion of a saccharide, a low lipid: saccharide ratio and careful selection of lipid composition aided liposome stabilisation during freeze drying. Specifically, the inclusion of the sucrose at soya PL blend: sucrose concentrations of 30:90 mg/g or 60:90 mg/g produced reconstituted dispersions with a z average identical to the z average of the original dispersions. However, the D-90 value of these dispersions increased compared to the D-90 value prior to freeze drying. This suggests that after
rehydrating the lyophilised cakes, some degree of fusion and/or aggregation was occurring in these dispersions.

The incorporation of either glycerol or polymer into the liposome formulations prior to freeze drying considerably increased the z average of the reconstituted dispersions (120 mg/g lipid).

The freezing stage of the lyophilisation process affected the particle size of the dispersions in this study. Freezer freezing without vacuum drying decreased the z average of the dispersions and did not raise the D-90 value. In contrast, nitrogen freezing without vacuum drying increased the average D-90 value (3.25 μm) but did not affect the z average size of the liposome dispersions. However, when these frozen dispersions were lyophilised and rehydrated to a soya PL blend: sucrose concentrations of 120:90 mg/g, a somewhat different picture emerged: the reconstituted freezer frozen samples had a considerably higher D-90 value (38 μm) compared to the D-90 value of the liquid nitrogen frozen samples (6.71 μm). This suggested that the type of freezing affected the proportion of larger sized liposomes and demonstrated the need for careful control during the freezing stage.

Maintaining the association of cyA with liposomes after freeze drying seemed less problematic than maintaining the particle size of the liposomes after freeze drying. After reconstituting lyophilised cakes containing either glucose or sucrose, cyA remained fully associated with all the liposome dispersions- even in cyA dispersions which had appeared to have extensively fused and/or aggregated. No cyA precipitation was detected on the surface of any of the filters used for the analytical filtration of the reconstituted cyA dispersions. However, the association of cyA with liposomes increased the size of liposomes in some dispersions. Rehydrating dispersions to soya PL blend: sucrose: cyA concentrations of 120:90:4.8 mg/g resulted in the formation of a fused gel. Similarly, rehydrating dispersions to final lipid: sucrose: cyA concentrations of 60:90:3 mg/g tended to fuse/aggregate the liposomes to a greater extent compared to the rehydrated lyophilised drug-free liposomes.
Chapter seven
Final considerations
Chapter seven- Final considerations

7. Final considerations

Since the first description of liposomes in the mid 1960s, many investigators have studied their various potential applications. After approximately 30 years of liposome research, Ambisome® was launched. This was the first liposome pharmaceutical product to be licensed in the UK. Despite the clinical and commercial success of this novel liposome amphotericin B formulation, only a couple of liposome based pharmaceuticals have been subsequently approved for human use in the UK. Both of these liposome products are high priced cytotoxic parenteral products, which alter the pharmacokinetics of the active component. As yet no commercial preparation has employed liposomes as a formulation aid.

This study has explored one potential application of liposomes as a formulation aid and focuses upon the use of liposomes as a solubiliser of lipophilic hydrophobes for intravenous administration. Using unsaturated lipids, the “fluid” liposomes generated for solubilising hydrophobes are fragile and readily cleared from the bloodstream, therefore these types of liposomes are unsuitable for entrapment and prolonged targeting of hydrophilic drugs. All liposomes used in these studies were generated by adding excess water to pro-liposomes, described by Perret et al. (1993).

In chapter two, TLC and $^{31}$P-NMR were employed to examine four phospholipid compositions extracted from naturally occurring egg yolk and soya bean. Phosphatidylcholine was quantitatively established as the major phospholipid in all four compositions, however, there were other phospholipids present in the four lipid samples. Furthermore, in the soya PL sample there were several non-phosphorus containing lipids, revealed by TLC, which were not quantitatively analysed. Further studies evaluating these non-phosphorus containing lipids, e.g. the glycolipids, using techniques such as HPLC with refractive index detectors may be informative. The quantification of the glycolipids identified in soya PL may help clarify why blending soya PL with soya PC helped maintain the size of the freeze dried liposomes after rehydration.

To avoid any potential variation due to differences in lipid composition, the same batch of each of the four main lipids was employed throughout the studies. Future studies could focus upon the composition and variation between batches. In particular, the consistency of the soya phospholipid composition should be investigated. Variability in
the soya PL composition may be especially important if it is blended with soya PC to obtain a minimum particle size. In this situation, the levels of charge in the soya blend would have to fall within relatively narrow limits. Variations in the content of charged species may directly impact on the particle size of the formulation by increasing the particle size of the liposomes in the dispersions.

In chapter three, the particle size of liposome dispersions was assessed using a variety of sizing techniques, including light microscopy, freeze fracture electron microscopy, PCS, laser diffraction and Coulter Counter. The particle sizing techniques revealed that the direct addition of a pro-liposome to water to form a liposome dispersion resulted in the formation of relatively coarse dispersions. The particle size characteristics of these coarse dispersions, converted in one stage, was highly dependent on both the lipid composition and the amount of energy introduced during one stage conversion. Replacing soya PC in the pro-liposome with soya PL generated dispersions with a smaller z average value and reduced the proportion of large liposomes. However, the inclusion of high levels of soya PL in the pro-liposome produced viscous dispersions at lipid concentrations of 60 mg/g, which would have precluded the intravenous use of such dispersions. Furthermore, the size characteristics of the dispersions converted in one stage were dependent upon the level of energy input during the one stage conversion of the pro-liposome into a liposome dispersion: dispersions converted by vigorously handshaking for one minute were significantly smaller than dispersions left to convert without any agitation.

The average size of the liposomes and the size and number of large liposomes could be significantly reduced if a two stage conversion was adopted. Two stage conversion involved hydrating the pro-liposome with a small amount of water, prior to the addition to bulk water. This procedure also reduced the variability in the number of large liposomes if the pro-liposome was not vigorously shaken. Indeed, in some experiments the presence of larger sized liposomes could not be accurately determined in dispersions with only a few large liposomes. Due to the smaller number of larger sized liposomes in these dispersions, it may be more appropriate to count the number of liposomes with diameters greater than 500 nm and 1 μm. Further electrical zone sensing studies, employing a smaller capillary, could perhaps be carried out to evaluate the number of particles per unit volume in these finer dispersions. Future studies should try to further
minimise the amount of extraneous contaminants adulterating these liposome dispersions, which may interfere with the particle size count of the fine dispersions. This could be achieved by aseptically filtering and aseptically producing the pro-liposome to reduce the level of particulates in the subsequent liposome dispersion. Ideally, Coulter Counter measurements should also be made in a low contamination environment.

In order to generate fine dispersions using two stage conversion, two criteria had to be met: firstly, the appropriate amount of water for hydration had to be added to the appropriate amount of pro-liposome and secondly, the water for hydration and pro-liposome had to be thoroughly mixed. In this study, this mixing was achieved by passing the two components through two Eppendorf syringes twenty times. This particular method for two stage conversion would clearly be unsuitable in a clinical setting. Any method of mixing used in a clinical setting would have to consistently mix the two sterile fluid components in an aseptic manner to ensure the pro-liposome was evenly hydrated, without generating excessive amounts of particulates.

The stability of the four lipids examined in chapter two was investigated in chapter four. Each of the four lipids was assessed in solid form and two pro-liposome formulations under stress conditions. Using traditional stability assays; detecting conjugated dienes and trienes, TBARS and peroxides, the egg PL and soya PL seemed noticeably more resistant to oxidation than the corresponding PCs. In contrast, the peroxide level of the soya PC increased the most over the 84 week period. Of the three forms examined the solid lipid appeared to be somewhat more stable than the two pro-liposome formulations under stress storage conditions. The physical stability of the soya PL and egg PL in all three formulations changed considerably at the accelerated storage temperature. After storage of the formulations at 55 °C for 84 days all PL formulations turned brown. This browning was probably attributable to the formation of Maillard browning products. These reaction products may have also conferred some antioxidant properties to the soya PL and egg PL lipids, thereby lowering the oxidation values throughout the accelerated stability testing.

The amount of LPC formation in the four different lipids in three formulations was studied under accelerated storage conditions at 55 °C. The degree of LPC formation seemed to be related to the type of phospholipid employed. The egg PC and soya PC hydrolysed to a greater extent than the corresponding egg PL and soya PL. The toxicity
of the liposomes generated from pro-liposomes containing lipids with varying amounts of LPC should be examined. Any haemolysis induced by the hydrolysis products including LPC and fatty acids may be enhanced by the presence of ethanol in the formulation.

From the particle size investigations it was determined that blending 10 parts by weight of soya PL with 90 parts by weight of soya PC reduced the particle size of the liposomes converted in two stages (chapter three). This soya PL was examined for its oxidative stability at 40 °C, 20 °C and 5 °C. Blending soya PL seemed to improve the oxidative stability of the phospholipid in a glycerol pro-liposome formulation. Stored at 40 °C, neither the oxidative stability values nor the physical stability seemed to change greatly over the 112 day test period.

Further studies using HPLC-chemiluminesce may be useful to complement the results of the traditional oxidation assays. This sensitive technique may enable the peroxide content of individual lipid classes to be determined. Future studies may also consider the antioxidant effect of tocopherols incorporated into pro-liposome formulations.

In chapter five, pro-liposomes dissolving a model hydrophobic drug, cyA (cyclosporin A), were used to generate liposome dispersions containing cyA. The ability of liposomes to associate with cyA, was studied by passing the cyA dispersion through polycarbonate filters to ensure no precipitate was present. An increase in weight or presence of precipitate under the light microscope or SEM indicated that the association was incomplete. The effect of adopting either a one stage or two stage conversion, described in chapter three, on cyA association was studied. The degree of cyA association was found to be highly dependent upon achieving the correct balance of lipid, ethanol and cyA in the pro-liposome. None of the dispersions converted in one stage completely associated cyA: after analytically filtering the dispersions converted in one stage, some precipitation was recovered from all dispersions containing cyA. CyA association could be improved by adding a small volume of water to a finely balanced pro-liposome, prior to the addition to bulk water. However, either delayed mixing of the pro-liposome and water for hydration or addition of an inappropriate amount of water for hydration resulted in the formation of precipitate in the liposome dispersions. Therefore, not only did insufficient mixing impact on the liposome dispersion by increasing the particle size of the liposome dispersions (chapter three), but delayed mixing also compromised cyA
association. The necessity for a carefully controlled hydration stage may prevent pro-liposomes from being employed clinically to generate liposome dispersions just prior to administration. Failure to manipulate the pro-liposome in an appropriate manner could generate a dispersion which falls outside of the size and association parameters, which is unacceptable for clinical use.

However, if the levels of components and hydration were carefully controlled, liposome dispersions with a lipid concentration of 50 mg/g could carry 2 mg/g of cyA. Therefore, approximately 150 g of dispersion would be required to solubilise the daily required dose (300 mg) of cyA for an adult. This relatively large amount of cyA dispersion is not ideal for clinical use, because it makes aseptic handling of the final dispersion difficult. The large amount of dispersion is as a consequence of two factors namely: the high lipid: cyA weight ratio required to solubilise the hydrophobe and the relatively large dose of cyA. Therefore, perhaps future studies could assess the suitability of this pro-liposome system for solubilising other hydrophobic lipophiles which are either administered in lower doses, such as tacrolimus and bryostatin or have a greater affinity for the phospholipid bilayer.

Future solubilisation work with cyA could investigate the composition of the precipitate. HPLC analysis of the cyA content and lipid analysis could perhaps be used to quantify the composition of the precipitate. Furthermore, HPLC could be used to quantitatively determine the amount of cyA retained on the filter.

Lyophilisation of liposome dispersions was investigated as an alternative liposome presentation. It is envisaged that lyophilised cakes could be manufactured by freeze drying a liposome dispersion containing cyA generated from a cyA pro-liposome. The resultant cakes would be reconstituted just prior to administration. Compared to the pro-liposome system used to generate dispersions prior to use, an advantage of a lyophilised presentation would be the absence of ethanol after reconstituting the cake. Although ethanol is commonly used in the solubilisation and formulation of many hydrophobic lipophiles, it may induce adverse reactions, particularly if it is concurrently administered with some biologically active compounds, for example metronidizole. Furthermore, the generation of a liposome dispersion by adding water to a lyophilised cake is considerably less troublesome than the two stage conversion of the pro-liposome.
The freeze drying studies investigated various factors which could have affected the particle size of the reconstituted liposomes. Factors such as; the selection of the saccharide, lipid: saccharide ratio and concentrations, freezing conditions, lipid composition and incorporation of cyA on the size of the reconstituted liposomes were studied.

Generating reconstituted dispersions with high lipid concentrations was rather more difficult than lower lipid concentrations. If the concentration of saccharide was the same in both sets of dispersions, the dispersions with a higher lipid concentration tended to generate coarser dispersions after reconstitution than the dispersions with lower lipid concentrations. Coupled with the fact that higher cyA: lipid weight ratios seemed to increase the size of the liposomes, this meant that generating liposome dispersions with high cyA concentrations was problematic in this study.

Although PCS indicated that the average diameter of the liposomes in some reconstituted dispersions had not increased after reconstituting the freeze dried cakes, the laser diffraction and light microscopy always detected large structures in the rehydrated dispersions. Without exception, the size measurements of all reconstituted dispersions appeared to have increased to varying extents. Future work could investigate the possibility of using filters to either reduce the size of the large liposomes or remove the large liposomes prior to infusion of reconstituted dispersions.

Future studies could investigate the difference between fusion and aggregation by employing membrane probes, which could establish the degree of lipid intermixing. Other means of reducing the liposome size prior to freeze drying could also be explored: high pressure homogenisation or microfluidisation may provide alternative methods of reducing the liposome particle size to below 100 nm. Particularly if large volumes of small unilamellar liposomes are desired.

Studies optimising the freeze drying cycle could be carried out. Ideally, for such studies the temperature of the primary stage of drying should be controlled using a freeze drier with adjustable temperature during primary and secondary drying. This would enable the effect of temperature during freeze drying on the quality of the reconstituted lyophilised liposomes to be examined. Finally, storage studies on the lyophilised cakes could investigate changes in particle size and cyA association which occur with time. However, for these types of studies, it would be beneficial for the cakes to be
immediately sealed under a vacuum after freeze drying to prevent exposing the cakes to the moisture from the air.
References


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Appendices
Appendix I

Graph showing standard curve for peroxide value

\[ y = 0.0081x - 0.023 \]

\[ R^2 = 0.9895 \]

Appendix II

Standard curve for TBAR value

\[ y = 0.0327x - 0.0372 \]

\[ R^2 = 0.9985 \]