Massey University

# Structure and properties of liposomes prepared from milk phospholipids

**A thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Food Technology**


**By**

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# Abstract

The isolation of milk fat globule membrane (MFGM) material from buttermilk on a commercial scale has provided a new ingredient rich in phospholipids and sphingolipids. The aim of this project was to explore the possibility of producing liposomes from MFGM-derived phospholipid matieral (Phospholac) and to compare the properties of these liposomes with those produced from commercial soy phospholipid fractions (SigP3644 and Ultralec). The technique used for liposome production was to be suitable for use in the food industry.

All three phospholipid fractions were primarily composed of phosphatidyl choline and phosphatidyl ethanolamine, but the dairy-derived Phospholac also contained approximately a third sphingomyelin. It also had a more highly saturated fatty acid profile, and contained a significantly higher proportion of protein than the soy-derived fractions.

The phospholipid fractions were dispersed in an aqueous system and cycled through a Microfluidizer® (a high-pressure homogeniser) to successfully produce liposomes. These were then characterised using a wide range of techniques.

The hydrodynamic diameter of the liposomes, measured using Photon Correlation Spectroscopy, ranged from an average of ~95 nm for the Phospholac dispersion to ~80 nm for the SigP3644 and Ultralec samples. All three dispersions had a very wide particle size distribution. Electron microscopy showed that all three dispersions appeared to be primarily unilamellar, but there was a small percentage of multilamellar and multivesicular liposomes. The unilamellar nature of the dispersions was further supported by the small-angle X-ray diffraction images and $^{31}$P-NMR results.

The SigP3644 dispersion had a much higher permeability than either the Phospholac or Ultralec sample, with minimal difference between the Phospholac and Ultralec samples at either 20 or 40 °C. Differential scanning calorimetry (DSC) found that SigP3644 and Ultralec had phase transition temperatures below 0 °C, while Phospholac dispersions showed a very broad transition with a centre between 28 and 30 °C. However, these differences did not appear to relate to the membrane permeability at its phase transition temperature. The Phospholac and Ultralec bilayers were approximately 20% thicker than SigP3644 membranes, with no significant change in thickness between 20 and 40 °C.

The liposomes produced from the Phospholac fraction showed considerably improved stability under a variety of environmental conditions than those produced from soy phospholipids. The Phospholac dispersions were able to withstand more severe processing treatments, were stable for longer periods at higher storage temperatures, and were less affected by changes in pH and in ionic concentration. It is thought that these differences are due to the high sphingomyelin concentration and more saturated fatty-acid profile of the dairy-derived fraction.

There were noticeable differences in entrapment characteristics of the fractions. It was found that the entrapment efficiency of hydrophobic compounds was directly proportional to the solubility of the compound in the solvent phase used for dispersion. Hydrophilic entrapment was also investigated, but the rapid diffusion of the small hydrophilic molecules through the liposome membrane prevented quantification of the entrapment efficiency. To produce liposome dispersions suitable for the encapsulation of hydrophilic material, further work must be completed to reduce the membrane permeability.

Differences in the properties of the liposome dispersions appear to be related to the composition differences between the phospholipid fractions, and it may be possible to exploit the unique composition of the MFGM phospholipid material in the delivery of bioactives in functional foods.

# Acknowledgements

I have been very fortunate with the support and assistance that I have received throughout the course of this research project. Many people have freely and graciously helped with my work and impacted positively on my life during the last 4 years, and I take this opportunity to convey my sincere thanks to them.

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Thank you to my friends and flatmates, who have been tolerant when I snapped at them after yet another mechanical failure, tried to understand when I headed to bed early again because I was just too tired, and were willing to talk for hours about anything other than my thesis. Hopefully by the time any of you read this, you will never again have to tentatively enquire whether we are still not talking about the PhD!

I am extremely lucky to belong to a family who consider education a natural and important part of life. I have often turned to my parents for guidance and reassurance during my research and the preparation of this thesis, and am extremely grateful for their sympathetic, practical advice. They were always there to remind me that if it was easy, everyone would have a PhD; but never wavered in their total confidence that I would (eventually) have one myself.

I also wish to thank two of my best friends: Adrian, who helped me get through the highs and lows of the first years of my thesis and who has continued to have unconditional faith in me; and Timothy, for his patience, encouragement and for ensuring I never forget that there is more to life than work. My friendship with both of them has added to my enjoyment and understanding of life and of people, and I hope we will continue to challenge and support each other in the years ahead.

Finally, I'd like to share two quotes which have kept me company throughout this part of my life. They have helped me keep everything in perspective, especially during the preparation of this thesis, emphasising that things do not necessarily have to work in order to be worthwhile, and reminding me that with determination, perseverance and sheer hard work almost anything can be achieved.

*"Make everything as simple as possible, but not simpler."*

Albert Einstein

*"I have not failed. I've just found 10,000 ways that won't work."*

Thomas Alva Edison

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# Chapter 1: Introduction

Over 95% of the lipid in milk is present in the form of tiny spherical droplets of triglycerides, each surrounded by a membrane consisting of phospholipids, glycosphingolipids and proteins. This membrane is referred to as the milk fat globule membrane (MFGM), and it stabilises the hydrophobic lipid phase within the aqueous milk environment.

During the butter manufacturing process, the MFGM is disrupted and the triglycerides are released. To minimise contact with the aqueous phase, the triglycerides aggregate, producing lumps of butter. The majority of the amphiphilic, MFGM material remains in the dilute aqueous phase commonly referred to as buttermilk. In previous years, the buttermilk was generally regarded as a valueless waste product, and disposed of accordingly. However, recent research has shown that dietary phospholipids and sphingolipids may contribute to improved health and well-being. The relatively high concentration of phospholipids and sphingolipids in buttermilk has provided incentive to develop methods suitable for their extraction and purification. These techniques range from traditional methods using solvent extraction through to emerging technologies such as microfiltration and supercritical fluid extraction. At time of writing, the Fonterra Co-operative Group Ltd (New Zealand) was the only company known to extract and purify MGFM phospholipid fractions from buttermilk on a commercial basis. These products are primarily used in food products and cosmetics for their nutritional and health-promoting qualities.

In the pharmaceutical and cosmetic industries, highly purified phospholipids extracted from soy oil or egg yolks may be used to produce liposomes. Liposomes are spherical structures consisting of one or more phospholipid bilayers enclosing an aqueous core. They may used for the entrapment and controlled release of drugs or nutraceuticals, as model membranes or cells, and even for specialist techniques such as gene delivery. Sphingolipids have sometimes been used in liposome production, and they are reported to have functional advantages over the more common phospholipids.

There are many potential applications for liposomes in the food industry, ranging from the protection of sensitive ingredients to increasing the efficacy of food additives. However, the

high cost of the purified soy and egg phospholipids combined with problems finding a production method suitable for use in the food industry has limited the use of liposomes in foods. There is growing consumer concern regarding genetic modification of food sources, and soybeans are widely regarded as one of the most commonly affected products. Sphingolipids are traditionally extracted from bovine brain, and are not only very expensive but are unsuitable for use in foods for vegetarians. Issues relating to BSE and Creutzfeldt Jakob disease also make it undesirable to use material extracted from bovine brain in food systems.

The use of dairy phospholipid fractions would avoid many of the negative issues surrounding many of the other sources of phospholipids and sphingolipids, and may also provide some labelling benefits. The high levels of sphingolipids may provide nutritional benefit for the consumer, as well as improved liposome functionality.

The overall aim of this project was to determine whether the phospholipid component of MFGM was able to be used to prepare liposomes. The technique used for liposome production was to be suitable for use in the food industry, and therefore must avoid the use of organic solvents and be easily scaled up to produce large quantities in a relatively small amount of time. If possible, the liposomes were to be produced from the commercially-produced dairy phospholipid fractions without any additional purification steps.

Once a technique that could successfully produce liposomes from the dairy phospholipids was identified, the second phase of the work was to physically and chemically characterise the dairy-based liposomes, and compare them with those produced from commercial soy phospholipids. The possibility of using the liposomes for entrapment of model compounds was also to be briefly explored.

# Chapter 2: Literature review

## 2.1 Introduction

This thesis aimed to produce liposomes from milk fat globule membrane phospholipids, commercially extracted from butter milk. The following literature review covers physical and chemical characteristics of the milk fat globule membrane, structure of phospholipids, the differences between phospholipid sources and the nutritional benefits reported for dietary phospholipids. The review also outlines the formation and structure of liposomes, as well as liposome preparation methods and techniques for liposome characterisation. Potential applications for liposomes in the food industry are also discussed.

## 2.2 Milk fat globule membrane (MFGM)

Milk is an oil-in-water emulsion, containing droplets of triglycerides stabilised by a complex bilayer membrane. This membrane (called the milk fat globule membrane or MFGM) is composed of phospholipids, glycosphingolipids and proteins. The approximate gross composition of the MFGM is given in Table 2-1, with the polar lipid composition shown in Table 2-2. The origin, formation and secretion of milk fat globules has been extensively studied, and the reader is referred to the recent reviews by Heid and Keenan (2005), Evers (2004) and Keenan and Mather (2002) for detailed information on this area.

MFGM may be released from dispersions of milk fat globules by agitation, freezing and thawing, exposure to ultrasound, or the addition of mild detergents or polar aprotic solvents (Keenan and Mather, 2002). The MFGM may then be isolated through centrifugal sedimentation. The agitation during the churning of butter causes the rupture of the MFGM, allowing the release and aggregation of the triglycerides to form butter. Most of the MFGM remains in the aqueous (buttermilk) phase, with a potential yield of approximately 0.25 mg of phospholipid per mg of protein (Corredig *et al.*, 2003).

**Table 2-1 Gross composition of milk fat globule membranes**

| Constituent group | Amount |
|---|---|
| Proteins | 25-60% by weight |
| Total lipids | 0.5-1.1 mg $mg^{-1}$ protein |
| Neutral lipids | 0.25-0.88 mg $mg^{-1}$ protein |
| Phospholipids | 0.13-0.34 mg $mg^{-1}$ protein |
| Glycosphingolipids | 0.0013 mg $mg^{-1}$ protein |

(from Keenan and Mather, 2002)

**Table 2-2 Polar lipid composition of MFGM**

| Lipid type | Percentage present |
|---|---|
| Sphingomyelin | 22 |
| Phosphatidyl choline | 36 |
| Phosphatidyl ethanolamine | 27 |
| Phosphatidyl inositol | 11 |
| Phosphatidyl serine | 4 |
| Lysophosphatidyl choline | 2 |

(from Keenan and Mather, 2002)

The butter manufacturing process involves a number of stages, with an example of a cultured butter process shown in Figure 2-1, and is usually performed by a continuous buttermaker.

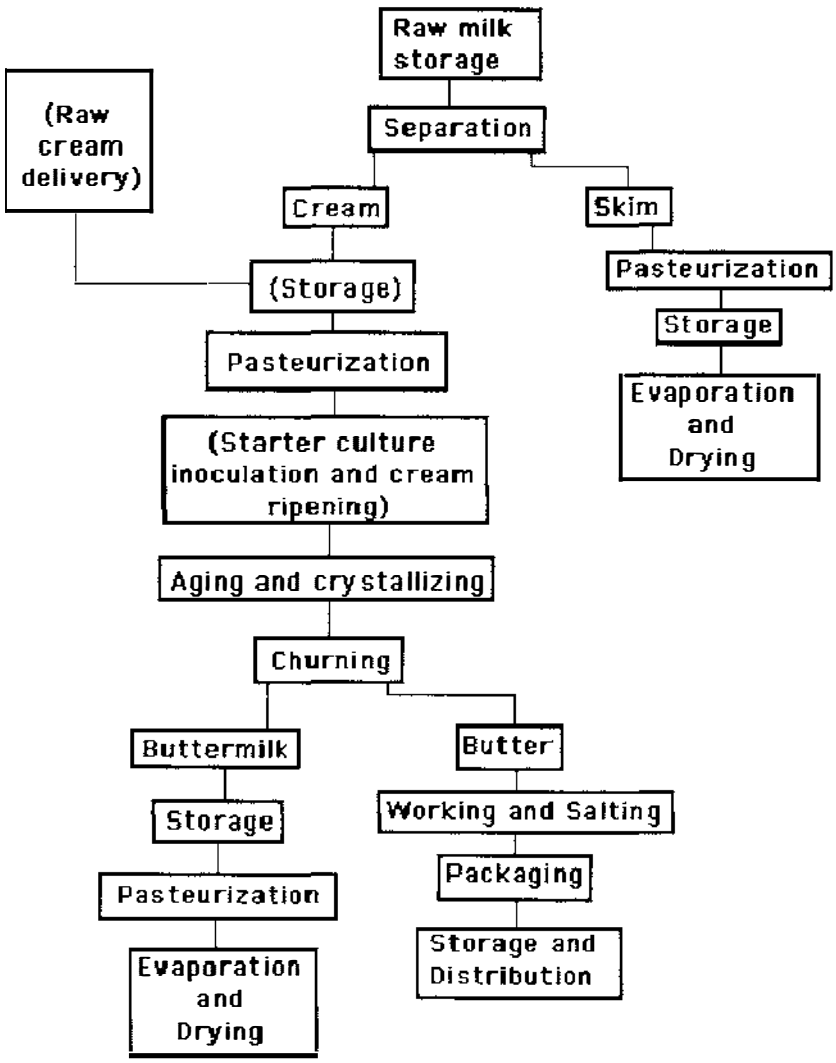



**Figure 2-1 Schematic showing general steps in cultured butter manufacture.**

The cream is separated from the milk and pasteurised. If ripening is desired for the production of cultured butter, mixed cultures of *S. cremoris*, *S. lactis diacetyl lactis* and *Leuconostocs* are used and the cream is ripened to pH 5.5 at 21 °C and then pH 4.6 at 13 °C. The cream is then subjected to a program of controlled cooling designed to give the fat the required crystalline structure. This aging process usually takes 12 - 15 hours. The cream is then pumped to the churn or continuous buttermaker via a plate heat exchanger to bring it to the required temperature. In the churning process, the cream is violently agitated to break down the fat globules, causing the fat to coagulate into butter grains, while the fat content of the remaining liquid, the buttermilk, decreases. In traditional churning, the machine stops when the grains have reached a certain size, whereupon the buttermilk is drained off. With the continuous buttermaker the draining of the buttermilk is also continuous. The butter is then salted and worked to ensure an even distribution of salt throughout a continuous fat

phase containing a finely dispersed water phase. The finished butter is discharged into the packaging unit, and from there to cold storage.

Whole milk contains approximately 0.035% phospholipid, of which 35% is in the milk serum and 65% is in the MFGM. Buttermilk produced from 40% cream contains 0.13% phospholipid, almost three times the level of whole milk (Walstra *et al.*, 1999). Walstra *et al.* (1999) stated that buttermilk may be used in certain food products, but it was usually desirable to minimise production due to insufficient demand in the food industry. Buttermilk may be included in foods to add flavour, as a source of calcium and protein, improve emulsification or foaming properties, or help colour development due to Maillard browning and improve shelf-life by binding water in baked goods.

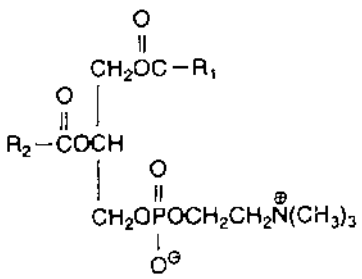
The possibility of obtaining a valuable product from what was previously regarded as a waste stream is obviously appealing from a financial perspective, and there are a number of recent papers discussing possible techniques for achieving this. Corredig *et al.* (2003) outlined a method for concentrating the polar MFGM lipids using microfiltration, while Astaire *et al.* (2003) used a combination of microfiltration and supercritical fluid extraction. However, at time of writing, Fonterra Co-operative Ltd (New Zealand) was the only company known to extract and purify MGFM phospholipid fractions from buttermilk on a commercial basis. Details of their technique were not available due to commercial sensitivity, but it was known that some solvent-based purification steps are used in the process.

## *2.3 Phospholipids*

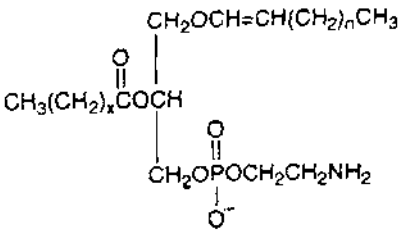
### *2.3.1 Basic structure*

Phospholipids are amphipathic lipid compounds, and along with glycolipids and proteins are the building blocks of biological membranes. They are derived from either glycerol, a three-carbon alcohol, or sphingosine, a more complex alcohol.

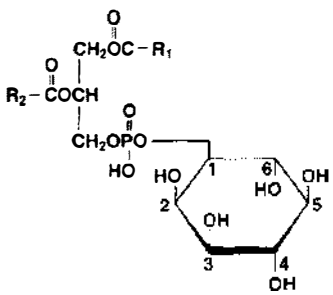
Glycerophospholipids are found in both plant and animal membranes. The most common natural glycerophospholipids are phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), phosphatidyl inositol (PI) and phosphatidyl serine (PS) (Figure 2-2).



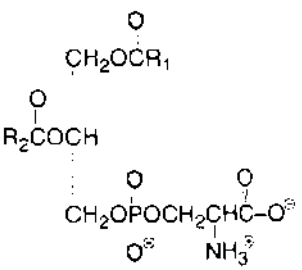
**Phosphatidyl choline**



**Phosphatidyl ethanolamine**



**Phosphatidyl inositol**



**Phosphatidyl serine**

**Figure 2-2 Basic structure of the common glycerophospholipids, phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl inositol and phosphatidyl serine.**

As can be seen from the structures of these molecules, phospholipids are surface active compounds, containing both hydrophobic (acyl residues, N-acyl sphingosine) and hydrophilic (phosphoric acid) sections.

All phospholipid molecules have at least one fatty acid chain. Fatty acid biosynthesis involves building up the acyl chain in two-carbon units, so fatty acids from natural sources almost always have even numbers of carbons in the chain. Nearly all double bonds in natural fatty acids are in the *cis* configuration. Most natural phospholipids have different fatty acids attached in the *sn*-1 and *sn*-2 position of the glycerol residue, with the inside chain usually being unsaturated and the outer chain saturated. Chains in the *sn*-2 position are usually longer than those in *sn*-1. Phospholipids from natural sources contain a mixture of fatty acid chains of different length and varying degrees of unsaturation. Plant sources have high levels of unsaturation, while animal sources have higher proportions of fully saturated chains (New, 1990b).

Phospholipids are not soluble in water in the traditional sense (Gibbs *et al.*, 1999), but when dispersed in water the molecules aggregate to reduce the unfavourable interactions between

the bulk aqueous phase and the hydrocarbon chains. The shape of the specific molecules is a major factor in determining which of a variety of different structures is most likely to be formed (Figure 2-3). The hydrophilic and hydrophobic parts of PC are approximately equal in size, giving it a tubular shape. This fits well into planar sheets, while the polar head and single chain of lysophospholipids result in a conical shape that easily forms a spherical micellar structure.

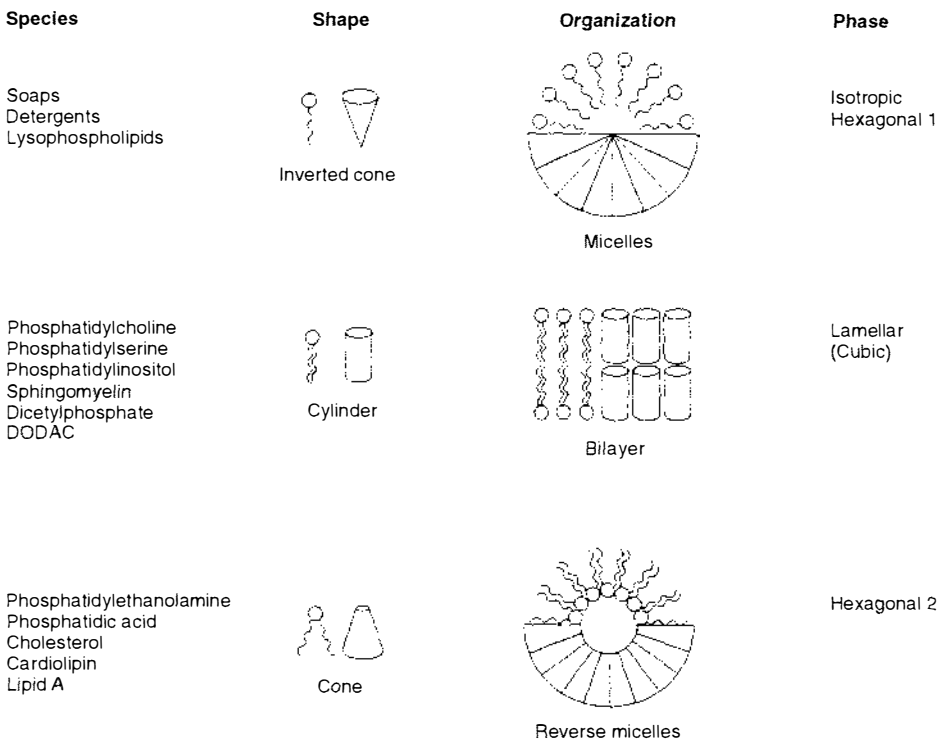



**Figure 2-3 The effect of molecular shape on the structure of the amphiphilic aggregate (From Lasic, 1998).**

### 2.3.1.1 Phosphatidyl choline

Phosphatidyl choline (PC) has a glycerol bridge linking a pair of hydrophobic acyl hydrocarbon chains and a hydrophilic polar headgroup (phosphocholine). Relatively crude extracts of PC may sometimes be referred to as lecithin. PC is the most abundant phospholipid, and can be derived from a number of both natural and synthetic sources, most commonly egg yolk and soya beans. PC tends to have a low relative cost, is chemically inert, and has no charge (New, 1990b).

Lysophosphatidylcholine (LPC) has a single fatty acid attached in the *sn*-1 position and is formed when the *sn*-2 fatty acid is hydrolysed by the enzyme phospholipase $A_2$. It is found

in small amounts in most tissues as part of the natural de-acylation/re-acylation cycle that controls the overall molecular composition of the tissue. Careless handling during the extraction of the lipids may also activate the phospholipase and cause the formation of LPC.

PC derived from soy oil, egg yolk, bovine brain or heart, and *Escherichia coli* is available commercially, as are a variety of synthetic forms (Sigma Aldrich, 2000-2001).

#### 2.3.1.2 Phosphatidyl ethanolamine

Another common phospholipid is the zwitterionic phosphatidyl ethanolamine (PE), the major structural phospholipid in brain tissue. It has an unsubstituted ammonium group that is protonated at neutral pH (New, 1990b). This phospholipid has a tendency to form reversed hexagonal structures rather than the linear bilayers of PC (Figure 2-3), which can create stability problems at high PE concentrations.

Commercial isolates derived from soy oil, egg yolk, sheep or bovine brain, and *Escherichia coli*, as well as synthetic forms are available (Sigma Aldrich, 2000-2001).

#### 2.3.1.3 Other phosphatidic acid derivatives

Phosphatidyl inositol (PI) and phosphatidyl serine (PS) may also be found in some lecithin fractions. Both PI and PS are commercially extracted from soy oil or bovine brain, with PI also commercially derived from bovine liver (Sigma Aldrich, 2000-2001).

#### 2.3.1.4 Sphingolipids

Sphingolipids are mainly found in the cell membranes of animals and higher-order plants, and can be split into two groups. Sphingophospholipids are phospholipids with a sphingosine backbone, while sphingoglycolipids are sugar-containing lipids with a sphingosine backbone, where one or more sugars are linked to the primary hydroxyl group.

In sphingomyelin (SM), the replacement of ester groups by ether linkages increases the resistance of such lipids to hydrolysis without significantly affecting the physical properties of the membranes (Figure 2-4). The molecules are cylinder-like (Barenholz and Thompson, 1999), and favour the formation of a bilayer structure in an aqueous environment (Figure 2-3).

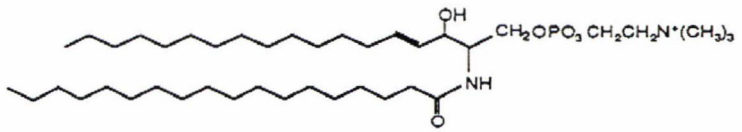



**Figure 2-4 Basic structure of sphingomyelin**

Most commercially-available sphingolipids are extracted from egg yolk and bovine brain (Sigma Aldrich, 2000-2001), although sphingolipids extracted from milk are now available from specialist lipid companies such as Avanti Polar Lipids (Alabama, USA).

### *2.3.2 Differences between phospholipid sources*

There are a large number of different sources for extracting phospholipids for use in liposomes. The most common natural sources include soya oil, egg yolk, bovine brain, and *Escherichia coli*. Synthetic forms are also available for many phospholipid classes. Each source has a different fatty acid profile, as shown in Table 2-3.

**Table 2-3 Percentage fatty acid composition of phosphatidylcholines extracted from different sources.**

| Type of fatty acid | | Fatty acid composition (%) | | |
|---|---|---|---|---|
| | | **Egg PC** (Weiner, 1995) | **Milk PC** (Cerbulis *et al.*, 1983) | **Soy oil PC** (Weiner, 1995) |
| 14:0 | Myristic | | 2.2 | |
| 16:0 | Palmitic | 32 | 36.9 | 12 |
| 16:1 | Palmitoleic | 1.5 | | <0.2 |
| 18:0 | Stearic | 16 | 9 | 2.3 |
| 18:1 | Oleic | 26 | 46.3 | 10 |
| 18:2 | Linoleic | 13 | 5.6 | 68 |
| 18:3 | Linolenic | <0.3 | <0.5 | 5 |
| 20:4 | Arachidonic | 4.8 | | <0.1 |
| 22:6 | Docosahexaenoic | 4.0 | | <0.1 |

#### 2.3.2.1 Soya beans

Phospholipids extracted from soya beans are the most common in both the food and pharmaceutical industries. Soya oil is produced in large quantities for numerous applications, and as crude lecithin is by-product of the oil processing, there is a ready supply of cheap raw material. This is usually reflected in the low relative cost of soy phospholipids, which can be purchased for less than NZ$10/kg for orders of 20-25kg. According to the American Lecithin Company (Connecticut, USA), the primary phospholipid components of soy lecithin are 13-18% phosphatidylcholine (PC), 10-15% phosphatidylethanolamine (PE), 10-15% phosphatidylinositol (PI), and 5-12% phosphatidic acid (PA). These will of course vary with the type of processing and degree of purification. Highly purified or specially processed soy phospholipids can fetch prices over NZ$500/g (Sigma Aldrich, 2000-2001).

The fatty acids in phospholipids of soya origin tend to be unsaturated, predominantly oleic acid (18:1) and linoleic acid (18:2) (Table 2-3).

#### 2.3.2.2 Milk

Whole bovine milk contains between 3.5 and 5% total lipid. Of this, approximately 98% of the total lipid is triacylglyercol, with only 0.5 to 1% phospholipid (Checkley *et al*, 2000).

At time of writing, Fonterra Co-operative Ltd (New Zealand) is the only company in the world isolating and purifying dairy phospholipid fractions from buttermilk on a commercial basis. A range of different fractions varying in purity are available, and these are summarised in Table 2-4. In 2002, prices for the dairy fractions ranged from NZ$50-400/kg depending on purity of the fraction and quantity ordered.

**Table 2-4 Composition of dairy phospholipid fractions commercially available as supplied by Fonterra Co-operative Ltd (New Zealand) (%).**

| Component | Phospholac 500 (%) | Phospholac 600 (%) | Gangolac 600 (%) |
|---|---|---|---|
| Lipid | 87.3 | 83.5 | 26.8 |
| - PC | 14.3 | 36.8 | 5.0 |
| - PS | 4.6 | 5.2 | 4.2 |
| - PE | 7.6 | 9.6 | 2.1 |
| - Sphingomyelin | 7.4 | 18.2 | 2.0 |
| - Gangliosides | (not given) | (not given) | 1.2 |
| Lactose | 5.1 | 6.2 | 62.6 |
| Protein | (not given) | (not given) | 9.3 |
| Moisture | 3.2 | 2.6 | 3.6 |
| Ash | (not given) | 11.5 | 7.2 |

#### 2.3.2.3 Egg

Phospholipids obtained from egg yolks are usually expensive, presumably due to the increased price and reduced availability of the raw material compared with other phospholipid sources. Prices range from NZ$1000/kg to NZ$3000/g (Sigma Aldrich 2000-2001). The fatty acids in egg yolk are mainly oleic and linoleic acid (Pheko *et al.*, 1998) (Table 2-3).

#### 2.3.2.4 Others

Phosphatidyl choline extracted from bovine brain is also available, and is priced at around NZ$200 for 100mg (Sigma Aldrich, 2000-2001).

### *2.3.3 Phospholipids and human health*

Apart from the functional advantages of including liposomes in food systems, there are also nutritional and other health benefits that come from the presence of the phospholipids.

Phospholipids have been shown to have benefits including liver protection (Koopman *et al.*, 1985) and memory improvement (Crook *et al.*, 1992, 1991). Choline (a major component of phosphatidylcholine) is an essential nutrient, and its deficiency can lead to more rapid exhaustion of muscles. Phosphatidylcholine may also assist in cholesterol reduction.

Sphingolipids are required for cellular signaling, and have been shown to be involved in the control of cell proliferation, apoptosis, inflammation and cancer. A comprehensive review of sphingolipid structure and physiological function is provided by Huwiler *et al.* (2000). Sphingomyelin inhibits intestinal absorption of cholesterol and fat in rats, with milk sphingomyelin more effective than egg sphingomyelin (Peel, 1999).

## *2.4 Liposomes*

The word 'liposome' derives from two Greek words, *lipo* - fat, and *soma* – structure. It refers to a spherical-shell structure consisting of a phospholipid bilayer (or two or more such bilayers separated by liquid regions) enclosing a liquid core. During the formation of the liposome, hydrophobic material may be incorporated in the lipid membrane while hydrophilic molecules present in the aqueous phase may become trapped inside the liposome. Thus liposomes can encapsulate both hydrophilic and hydrophobic compounds within a single structure.

The main differences between a liposome and an emulsion droplet are shown in Figure 2-5. An emulsion forms when small droplets are stabilised in an immiscible phase (often oil-in-water or water-in-oil) by a single layer of amphiphilic material. To form a stable emulsion the droplets are usually around 1-10 micron or 1,000-10,000 nm in diameter. Liposomes have the same aqueous phase on both sides of the phospholipid bilayer, and tend to be approximately 50-500 nm in diameter.

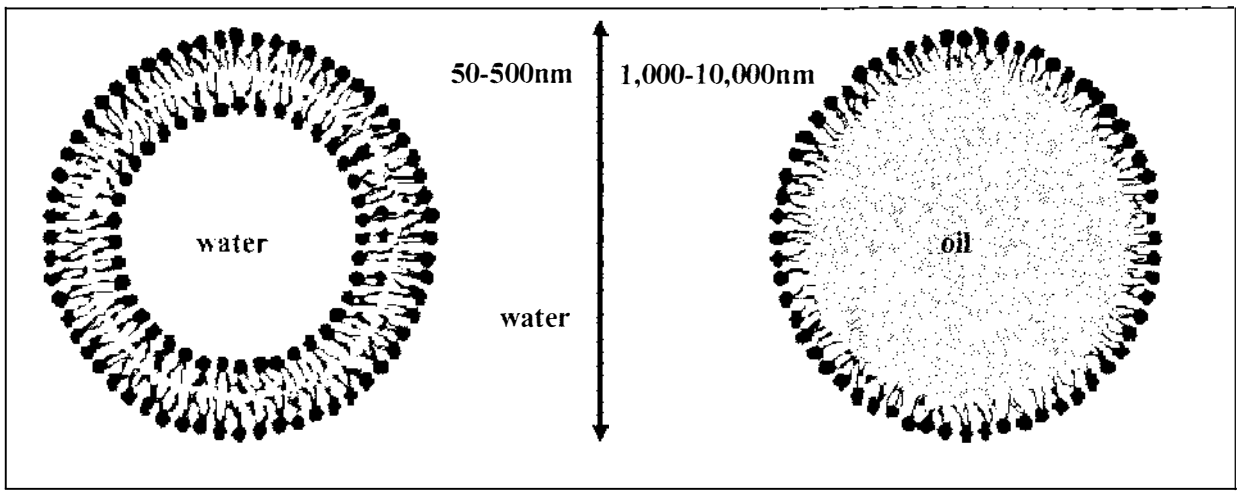



**Figure 2-5 Schematic of a liposome (left) and an emulsion droplet (right).**

Liposomes protect their contents from the external environment while still allowing small molecules to pass in and out of the membrane (Gibbs *et al.*, 1999). They imitate living cells, and have been used to model the structure and function of biological membranes. They are biologically compatible with the skin and body, and so have been used in a wide variety of pharmaceutical and cosmetic systems. Liposomes can also be used to delay the release of the encapsulated material (Arnaud, 1995a). The most common method of release is where the entrapped material slowly diffuses out through the phospholipid membranes as the concentration in the external phase decreases.

Liposomes are much smaller than other types of microencapsulation systems used in foods, and so can achieve much higher levels of dispersion (Kirby, 1991). Despite their much smaller size, they are apparently more stable than most other capsules.

### *2.4.1 Liposome formation*

When phosphatidyl choline is dispersed in water, the molecules aggregate and form a bilayer sheet arrangement to minimise contact between the hydrophobic fatty acid chains and the hydrophilic aqueous environment. However, there are still exposed hydrophobic fatty acids at the edges of the sheet. The unfavourable interactions between the fatty acid and the water are completely eliminated when the edges fold together to form closed sealed vesicles (New, 1990b). While this arrangement is at the minimum thermodynamic energy for the system (Martin, 1990), sufficient energy must be introduced to overcome the energy barrier

preventing the spontaneous formation of liposomes. The major steps in liposome formation are shown schematically in Figure 2-6.

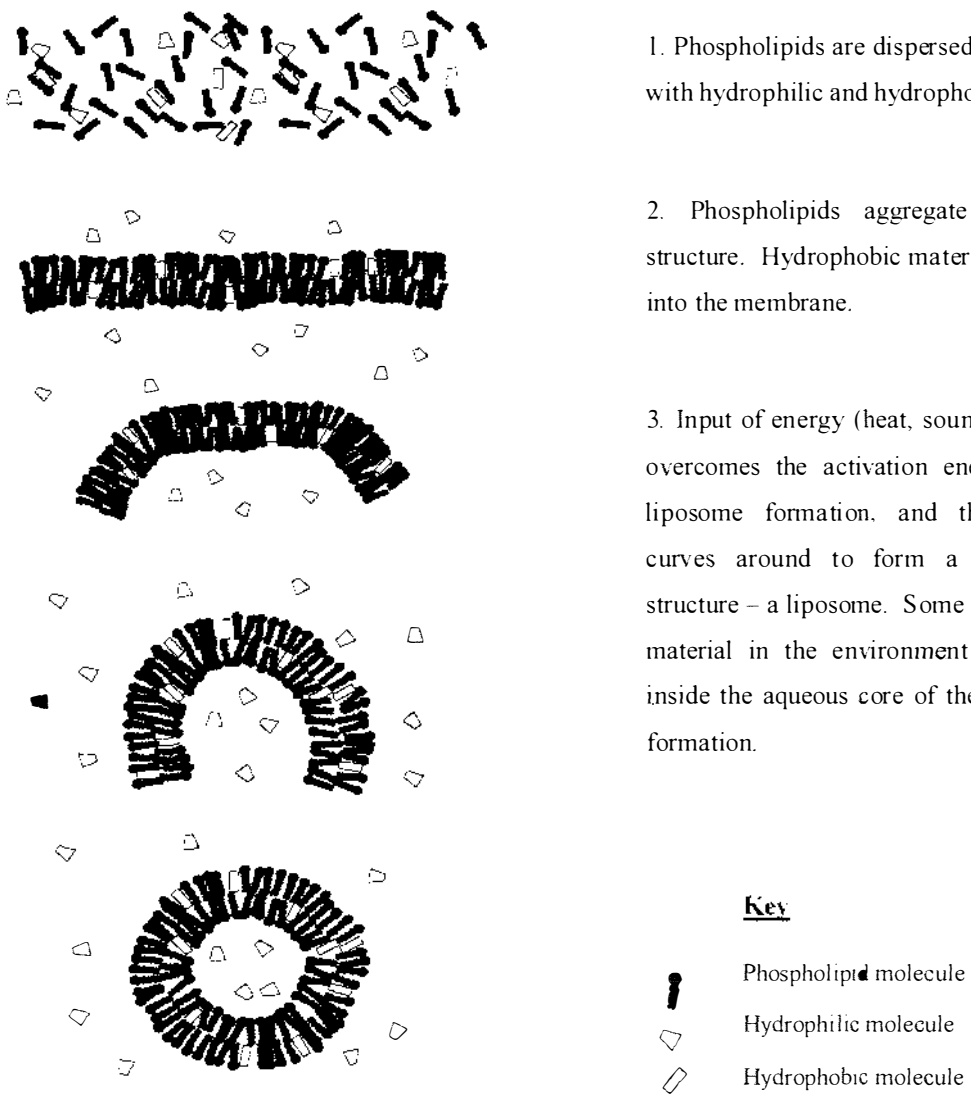



**Figure 2-6 Schematic representation of the basic steps in liposome formation**

The thermodynamic stability of any closed system near equilibrium is determined by the Gibbs free energy (G) of the system, given by the equation: $\Delta G = \Delta H$ (enthalpy) - $T\Delta S$ (entropy). If a negative value of G exists between two states, the proposed reaction is favourable. Liposomes have the lowest possible enthalpy value for the system, but all the molecules are lined up in an orderly fashion, so the system has a lower entropy value than emulsions or random structures. This means the reaction is temperature-dependent, and at ambient temperatures the $\Delta G$ for formation of liposomes is always negative. However, for the reaction to proceed, the system needs to overcome the required activation energy. This energy can be provided by a variety of sources, including mechanical, heat, and sound. The

type of energy used may influence liposome characteristics such as size and lamellarity (Kim and Baianu, 1991).

The size of the phospholipid molecule headgroup will influence its position in the liposome membrane, with those with smaller headgroups (PI, PA, PS) preferentially occupying the inner bilayer, and PC on the outer shell (New, 1990b). Different phospholipids will give the liposome different charges, with PE giving the surface a positive charge, and PS or diacetyl phosphate a negative charge (Jackson and Lee, 1991).

The formation of large numbers of tiny vesicles results in a dramatic increase in surface area. Arnaud (1998) estimates that 1g of phospholipid made into liposomes would give an external surface area of about 200 $m^2$.

### *2.4.2 Phase transition*

The ordering of the phospholipids within a liposome bilayer membrane depends on temperature. Regions with an ordered, gel-like structure and regions with a fluid, liquid-crystal character are both present, with the equilibrium between the two shifting its position over a temperature range that is determined by the types of phospholipids and fatty acids present. Higher temperatures favour the more permeable fluid phase; lower temperatures favour the more dense gel phase. The temperature at which equal proportions of the two phases coexist is known as the critical temperature or phase transition temperature ($T_c$) (Lian and Ho, 2001). Different phospholipids exhibit different $T_c$s. The $T_c$ of a heterogeneous membrane is usually equal to the weighted average of the $T_c$s of its component phospholipids. As the liquid-crystal phase allows phospholipid molecules more freedom of movement, the membrane fluidity is dependent on temperature.

The phase transition temperature of a liposome is the basis for explaining a wide variety of characteristics and behaviours, including membrane permeability. Membrane permeability is at its highest at the $T_c$. This is because at points where the gel-like and liquid-crystal-like phases meet, the membrane molecules are unable to maintain the structured arrangements typical of either phase, leading to numerous defects in their packing along the phase boundaries. It is much easier for entrapped material to diffuse through these less structured areas than through areas composed entirely of one phase. Therefore, the release of entrapped

material may be controlled by carefully selecting a membrane composition that has a $T_c$ corresponding to the desired release temperature. For example, a flavour might be entrapped inside liposomes and the liposomes incorporated in a chilled product. When the food is consumed, the flavour needs to be released quickly while it is in the mouth. Using a liposome with a $T_c$ close to body temperature will help maintain stability in chilled conditions, but allow immediate release in the mouth.

The specific phospholipids used in the liposome membrane are fundamental in determining phase transition temperature (section 2.3), although there is evidence that the type of phospholipid vesicle formed can affect $T_c$ (section 2.4.4.5). The phase transition of a membrane is also influenced by the presence of non-phospholipid molecules such as sterols.

In general, phospholipids from animal sources contain a higher proportion of saturated fatty acids than those from plant sources. This is reflected in the phase transition temperatures (Table 2-5). Egg yolk phospholipids tend to have $T_c$s higher than plant phospholipids but lower than those from mammalian membranes (Gibbs *et al.*, 1999). Mammalian phospholipids produce membranes with $T_c$s between 0 and 40 °C while membranes made of unmodified soy phospholipids have $T_c$s below 0°C and those from egg yolk lecithin usually have $T_c$s between -15 to -7 °C (Reineccius, 1995b).

**Table 2-5 Phase transition temperatures (°C) of fully hydrated phospholipids from various biological sources.**

| Source | Phase transition temperature (°C) | | | | |
|---|---|---|---|---|---|
| | PC | PE | PS | PG | PA |
| Egg yolk | -10 ± 5 | 10 ± 2 | | | 18 |
| Egg yolk (hydrogenated) | 46 | | | | |
| Soybean | -15 ± 5 | -5 | | | |
| Soybean (hydrogenated) | 51 | | | | |
| Bovine brain or spinal chord | | | 13 ± 3 | | |
| *E. coli* | | 30 ± 8 | | 36 ± 2 | |

From Cevc (1993)

### 2.4.3 Effect of sterols on liposome characteristics

Sterols are amphipathic molecules that are important components of most natural membranes. They do not form bilayer structures by themselves, but can be incorporated into membranes in very high concentrations. Cholesterol is the most common sterol associated with liposome manufacture, with a 1:1 molar ratio of cholesterol to phosphatidyl choline commonly used, and ratios as high as 2:1 reported (Chapman, 1984; New, 1990b). Chapman (1984) expresses doubt regarding concentrations higher than this, suggesting that the additional cholesterol may be dispersed as fine crystals. Almost all liposomes used for pharmaceutical purposes have quite a large proportion of cholesterol in the liposome bilayer.

When included in a phospholipid membrane, the polar hydroxyl group of the sterol orients itself towards the aqueous phase, and the aliphatic, cyclic section of the molecule aligns parallel to the acyl chains in the centre of the bilayer (Reineccius, 1995b). Sterol molecules are very rigid and reduce the freedom of motion of the upper section of neighbouring phospholipid chains (New, 1990b). This contributes to the stability of the liposome and reduces the permeability of the membrane (Reineccius, 1995b). The resulting increased rigidity of the membrane and tighter packing of the phospholipid molecules reduces the membrane permeability both at and above $T_c$ (Lian and Ho, 2001). By including approximately 50 mol% cholesterol the increase in permeability usually observed at the $T_c$ may be avoided. This effect may be useful in some potential food applications where the $T_c$ of the otherwise most suitable phospholipid mixture has an undesirable value. However, care must be taken to ensure cholesterol concentrations are sufficient, as Lian and Ho (2001) reported that the incorporation of cholesterol at low concentrations may actually increase membrane permeability.

Despite the potential benefits of adding cholesterol to liposomes, this is not considered a viable possibility in the food industry. Cholesterol is an expensive additive (approximately $1000/kg (Sigma Aldrich, 2000-2001)), and would dramatically increase the cost of liposome production, especially at the high concentrations used in most studies. Consumer concern about dietary cholesterol and heart disease would also be an issue, regardless of the fact that the amount of cholesterol added through use of liposomes as food components would be insignificant (Reineccius, 1995b).

### 2.4.4 Liposome structure

There are a number of different liposome structures that may be formed, varying both in terms of overall liposome size and the number of concentric bilayers contained within each vesicle (Figure 2-7). These differences can affect a number of liposome characteristics, including stability and the rate of release of entrapped material. Details on methods for determining lamellarity of liposomes are given in section 2.6.4.

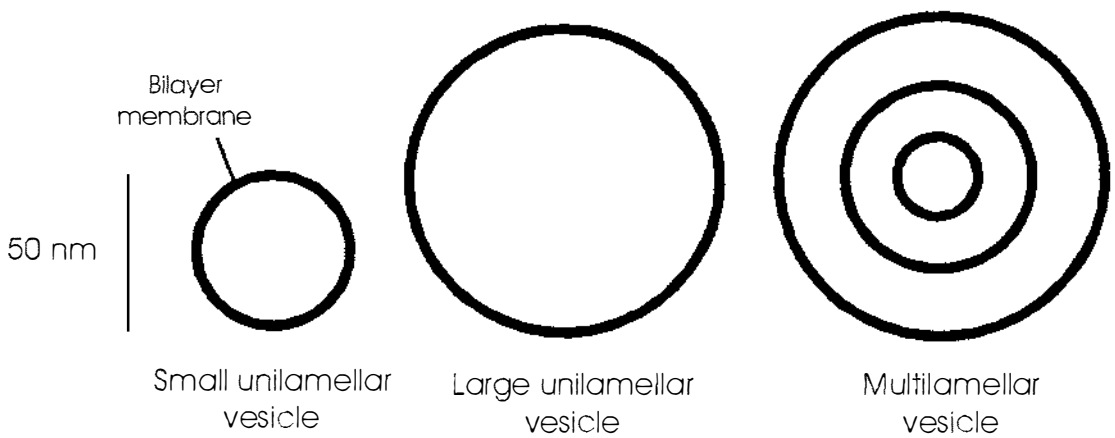



**Figure 2-7 Diagram showing the structural characteristics of the three most common types of liposome.**

#### 2.4.4.1 Small unilamellar vesicles (SUVs)

Small unilamellar vesicles (SUVs) have diameters between 0.02 and 0.05 μm (Table 2-6) and only a single bilayer membrane. The theoretical minimum diameter possible for a liposome is 0.02-0.025 μm (Reineccius, 1995b), limited by the surface curvature (resulting in crowding of the phospholipid headgroups) (New, 1995b) and the thickness of the phospholipid bilayer (about 4nm) (Watwe and Bellare, 1995). SUV populations tend to be well-characterised, homogenous in size and with consistent properties (New, 1995c). However, the single bilayer does tend to be quite permeable, particularly to water-soluble molecules.

With SUVs, the entrapped volume varies as the cube of the radius. This results in a rapid decrease in entrapped volume as the vesicles become smaller, leading to low entrapment efficiency. Because SUVs entrap less hydrophilic phase per mole of wall material, they are more useful for lipophilic active materials. Their use is not recommended if the wall material is expensive and it is the aqueous phase that is desired. (Reineccius, 1995b).

**Table 2-6 Size limits for small unilamellar vesicles (SUVs).**

| Reference | Lower size limit (micron) | Upper size limit (micron) |
|---|---|---|
| Arnaud (1995b) | 0.02 | 0.05 |
| Martin (1990) | 0.02 | 0.05 |
| New (1990b) | 0.025 | 0.05 |
| Reineccius (1995b) | 0.025 | 0.04 |
| Watwe and Bellare (1995) | 0.02 | 0.05 |
| Weiner (1995) | 0.025 | 0.05 |

Martin (1990) stated that liposomes were most thermodynamically stable when the packing density of the phospholipids present in each monolayer of the bilayer was roughly the same. Very small liposomes may develop packing differentials due to the extreme curvature of the membrane. New (1990b) reported that in liposomes with diameters of approximately 25nm, packing geometry dictated that over 70% of the phospholipids were in the outer layer. This could lead to physical instability such as coalescence and fusion (Martin, 1990; New, 1990c,b; Reineccius, 1995b). There are contradicting reports regarding the effect of temperature on the rate of fusion, as Reineccius (1995b) commented that fusion was most likely to occur at or above transition temperature, while New (1990c) asserted that SUVs are particularly susceptible to fusion below the phase transition temperature. Both authors agreed that fusion increased at the phase transition temperature, so if storage conditions are close to the transition temperature, the inclusion of sufficient cholesterol to reduce or remove the transition is often used to increase liposome stability. SUVs are particularly susceptible to degradation in the presence of biological molecules, so they are useful if the rapid release of lipid soluble materials is required (New, 1995b). An example of such an application is where the entire contents of the liposomes are required in a short sharp burst – like the delivery of proteins for a vaccination.

The standard preparation method for SUVs is through the sonication of MLVs (section 2.4.4.3) (Chapman, 1984; Jackson and Lee, 1991; Kim and Baianu, 1991) , or the extrusion of a MLV solution through a French pressure cell (Hamilton and Guo, 1984) or membrane (Schneider *et al.*, 1994; Turanek, 1994) . Chapman (1984) also mentioned that the injection of an ethanolic solution of phospholipid through a small-bore needle into a rapidly agitated aqueous solution would produce SUVs similar to those formed during sonication. In

general, the production of SUVs requires a high energy input, which is not only expensive but also increases the risk of lipid peroxidation and hydrolysis (New, 1995c).

New (1990c) commented that SUVs were not able to be produced on commercial scale - bath sonication is not powerful enough to deal rapidly with high lipid concentrations, and ethanol injection results in dilution of the lipids in large volumes of aqueous medium. He recommended probe sonication and the French Pressure Cell technique for small-scale use.

#### 2.4.4.2 Large unilamellar vesicles (LUVs)

Large unilamellar vesicles can be produced by the slow injection of ether solution and lipids into a phosphate buffer (Jackson and Lee, 1991) or the fusion of acid phospholipid SUVs in the presence of calcium (New, 1995b). Table 2-7 shows size ranges given in the literature for LUVs.

**Table 2-7 Size limits for large unilamellar vesicles (LUVs).**

| Reference | Lower size limit (micron) | Upper size limit (micron) |
|---|---|---|
| Arnaud (1995b) | 0.15 | 0.5 |
| Chandran *et al.* (1997) | 0.1 | None given |
| Reineccius (1995b) | 10 | None given |
| Watwe and Bellare (1995) | 0.06 | None given |
| Weiner (1995) | 0.1 | 0.5 |

LUVs have the highest aqueous phase to phospholipid ratio, and are therefore very efficient at entrapping large volumes of hydrophilic material (New, 1990c; Arnaud, 1995c; Reineccius, 1995b). Mechanical instability can be a problem due to the single membrane, and retention of water-soluble and low molecular weight solutes may also be an issue (New, 1990c; Arnaud, 1995c). Reineccius (1993b) stated that the fragility of LUVs may prevent them from surviving a food processing operation. However, New (1990c) reported that LUVs were very good for active loading (see section 2.7).

#### 2.4.4.3 Multilamellar vesicles (MLVs)

Multi-lamellar vesicles (MLVs) are the most heterogeneous of the common types of liposome. The term covers a wide range of particle sizes (Table 2-8), with the defining

characteristic being the presence of more than one membrane. The standard preparation procedure is *via* the rotary evaporation of a chloroform solution of phospholipid, cholesterol and other hydrophobic compounds to give a thin phospholipid film. Addition of water and hydrophilic compounds causes bilayer sheets of the lipid to separate from the bulk and form liposomes with alternating membrane and hydrophophilic phase (Jackson and Lee, 1991; Picon *et al.*, 1994).

**Table 2-8 Size limits for multilamellar vesicles (MLVs).**

| Reference | Lower size limit (micron) | Upper size limit (micron) |
|---|---|---|
| Arnaud (1995b) | 0.15 | 0.5 |
| Reineccius (1995b) | 0.1 | 1 |
| Watwe and Bellare (1995) | 0.1 | 6 |
| Weiner (1995) | 0.05 | 10 |

MLVs are mechanically stable upon storage for long periods (New, 1995c). New (1990b) commented that MLVs were thought to give much more gradual and sustained release of material than unilamellar liposomes. The concentric rings gradually degrade, slowly releasing the entrapped material. The high proportion of phospholipid material in the liposome means MLVs are very efficient at entrapping hydrophobic actives. MLVs may not be the most efficient means of carrying hydrophilic actives, as having more than one membrane gives less entrapment volume for given size. The minimum lipid for aqueous space ratio is achieved with unilamellar vesicles (Reineccius, 1995b).

#### 2.4.4.4 Other structures

There are a number of other types of liposome. Some of these are only mentioned in the work of a single author, while others appear more frequently. Many types of liposome may have at least two names, one based on structure and the other on the method of production.

The most common of these additional terms is intermediate unilamellar vesicle (IUV), which Reineccius (1995b) defined as having a diameter of approximately 100nm. New (1990b) also used this term for liposomes of this size, commenting that while the term is not often used in the literature, it is useful for convenience and to avoid confusion. Arnaud (1995b) mentioned oligo-lamellar liposomes, defined as those that are generally smaller than MLVs with fewer lamellae. They are reported to combine high entrapped volume with good

retention capacity. Watwe and Bellare (1995) also used this term, but did not include any details on this sort of liposome. Other shapes and structures encountered include giant unilamellar, multivesicular, stable pausilamellar, helical, and cochleate (Watwe and Bellare, 1995).

The naming system based on the production method is quite self-explanatory. For example, liposomes made through the dehydration-rehydration method are sometimes referred to as dehydration-rehydration vesicles (Picon *et al.*, 1994). or dried-reconstituted vesicles (New, 1995c), both abbreviated to DRV. Those produced in a Microfluidizer may sometimes be called micro-emulsification liposomes (MEL), and those made by reverse-phase evaporation are often called reverse-phase evaporation vesicles (REV) (New, 1995c). There are many other similar abbreviations used in the literature, but the term is usually defined in the specific reference so there is no need for a complete list to be given here.

#### 2.4.4.5 Effect of liposome type on phase transition

It has been reported that the type of phospholipid vesicle may also influence $T_c$. Hays *et al* (2001) stated that for the same phospholipid composition, the phase transition temperature of sonicated dielaidoyl-phosphatidylcholine (DEPC) vesicles was approximately 5 °C, compared with 10 °C for LUVs, and 9 °C for MLVs.

### *2.4.5 Liposome composition*

PC tends to be the material of choice for liposomes as the hydrophilic and hydrophobic parts of the molecule are of equal size. This creates a tubular shaped molecule, which easily aggregates into planar sheets (Figure 2-3). Other phospholipids that do not tend to form liposomes on their own may be added to modify the net charge. Phosphatidylsterine, phosphatidylglycerol, phosphatidylinositol and phosphatidic acid all promote electrostatic repulsion, preventing aggregation or fusion (Martin, 1990; Weiner, 1995). The inclusion of charged phospholipids will also increase the distance between concentric membranes in MLVs, thus increasing entrapped volume (Martin, 1990). Levels of between 5-20 mol % of charged species have been suggested (Martin, 1990; Weiner, 1995). For natural PCs, the thickness of the bilayer membrane that is formed is approximately 4-5nm (Watwe and Bellare, 1995).

SM has a more highly ordered gel phase than phosphatidyl choline, which may be explained by the hydrogen bond interactions permitted by the amide linkage and hydroxyl groups in SM. This high degree of order is likely to be responsible for tendency of SM membranes to demonstrate higher stability and be more tightly packed than lecithin bilayers, resulting in a lower permeability to solutes, greater resistance to lysis by bile salts, and reduced membrane fluidity (New, 1990b; Barenholz and Thompson, 1999). SMs tend to be much more saturated than other naturally-occurring phospholipids, and may also have longer acyl chains. This results in a higher phase transition temperature than for most other membrane phospholipids, and for SMs from biological membranes the phase transition temperature is usually around 37 °C (Barenholz and Thompson, 1999). PE molecules are able to form hydrogen bonds with neighbouring molecules, and saturated

PEs have transition temperatures approximately 20°C higher than their PC analogues. At low pH, the PE molecule becomes more protonated, reducing hydrogen bonding and resulting in transition temperatures similar to PC (New, 1995b). Charge neutralisation means that liposomes with high levels of PE tend to aggregate in the presence of $Ca^{2+}$ and $Mg^{2+}$ ions (van Nieuwenhuyzen and Szuhaj, 1998). PC-enriched fractions are insensitive to $Ca^{2+}$ and $Mg^{2+}$ ions, and are therefore suitable for systems with hard water and in the presence of milk proteins (van Nieuwenhuyzen and Szuhaj, 1998).

Negatively charged phospholipids such as PS can bind strongly to cations, particularly divalent cations such as magnesium and calcium. This binding reduces the electrostatic charge on the molecule, and if the membrane has a high proportion of these phospholipids, may cause it to condense. This increases the packing density in the gel phase and therefore raises the transition temperature (New, 1995b). The reduction in electrostatic repulsion also can result in the aggregation of liposomes. New (1990c) reported that the presence of negatively charged lipids in the solution tended to reduce liposome size, while cholesterol increased the average diameter. Liu *et al.* (2001) used second harmonic generation (SHG) to study both the adsorption of a positively charged organic dye onto liposomes of different lipid compositions, and the transport kinetics of the dye across the liposome bilayer. SHG shines a beam of monochromatic light onto a surface, and lack of symmetry at the surface can lead to the generation of light at a frequency twice that of the incident light (i.e. the second harmonic). This allows surface phenomena including molecular adsorption, aggregation and orientation to be studied. As expected, dye adsorption increased linearly

with the fraction of negatively charged lipids in the bilayer. The transport rate constant for crossing the bilayer also increased linearly with the fraction of negatively charged lipid present.

The most stable liposomes are made of PC, cholesterol and a negatively charged phospholipid in the ratio 0.9:1:1 (New, 1995c; Reineccius, 1995b). Frezard (1999) stated that a zwitterionic or non-ionic lipid was usually used as the basic lipid for liposome production, with charged phospholipids added to modify the net surface charge as desired. New (1990b) commented that in many cases sphingomyelin may be preferable to lecithin, particularly for immunological applications, but this choice was usually restricted by cost. Saturated fatty acids are more rigid than their unsaturated counterparts, and can be used in combination with or independently from cholesterol and tocopherols to decrease permeability and increase stability (Jackson and Lee, 1991). Saturated fatty acids are also less likely to cause membrane degradation through peroxidation and the release of free radicals (Reineccius, 1995b).

Maximum stability is not always desirable, and liposomes that rapidly release their contents are required for some applications (Reineccius, 1995b). For example, a flavour might be encapsulated inside a liposome and incorporated inside a chilled product. When the food is consumed, the flavour needs to be released quickly while it is in the mouth, as there are no tastebuds further along the digestive tract. Using a liposome with a phase transition temperature close to body temperature will help maintain stability in chilled conditions, but with a release of entrapped material when the food is heated to body temperature during consumption (New, 1995b).

The bilayer membrane is held together by non-covalent interactions, allowing it to carry a wide variety of lipophilic compounds. The inclusion of these 'carried' molecules at up to approximately 10 wt. % will not usually cause significant disruption to the membrane (New, 1995c; Reineccius, 1995b), although New (1990b) mentioned that membrane fluidity and permeability may be affected. Compounds which interact with the membrane in a favourable manner may be included at higher concentrations (e.g. fatty acids or $\alpha$–tocopherol) while other materials will readily disrupt the membrane at much lower levels (e.g. some polyene antibiotics) (New, 1995c). Table 2-9 summarises the effects that changes in the phospholipids present can have on the liposome membrane.

**Table 2-9 Attributes of head and fatty acyl groups of commonly used phospholipids.**

| Domain | Effect on liposome membrane | Functional attribute on lipid bilayer |
|---|---|---|
| *Two tail-group fatty acid chains (C14-18 in length)* | | |
| - increase degree of saturation | Increase rigidity, decrease fluidity | Elevate $T_c$ |
| - increase chain length in both chains | Increase thickness of bilayer | Elevate $T_c$ |
| - varying saturation and length between the two fatty acid chains | Decrease order of membrane packing (i.e. more disorder) | Lower $T_c$ (compared to phospholipid with two identical fatty acyl tails) |
| *Head group* | | |
| - choline: $-CH_2-CH_2-N(CH_3)_3$ | Some surface hydration | Neutral charge |
| - ethanolamine: $-CH_2-CH_2-NH_3$ | Minimum degree of surface hydration | Neutral charge |
| - serine: $-CH_2-CH(COO^-)-NH_3$ | Some surface hydration | Negative charge |
| - glycerol: $-CH_2-C(OH)CH_2$●H | Some surface hydration | Negative charge |
| -PEG (ethanolamine): $-CH_2-CH_2-$ NH-PEG | Enhanced surface hydration and steric effect | Negative charge |

Taken from Lian and Ho (2001)

Yoshio *et al.* (2001) observed that membrane fluidity was significantly higher in liposomes composed of unsaturated phospholipids than in those made of saturated phospholipids. As nearly all double bonds in natural unsaturated fatty acids are in the *cis* configuration (New, 1995b), the unsaturated phospholipids do not pack as closely as the saturated ones. Greater packing impedes freedom of movement, thereby reducing permeability and increasing membrane stability. Increased packing density in the gel phase raises the phase transition temperature (New, 1995b).

Unmodified soy phospholipids have phase transition temperatures below 0°C (Table 2-5). This means that the membrane composed of these lipids would be in the liquid state at all temperatures from refrigeration through to body temperature. Liposomes are most stable below their phase transition temperature (section 2.4.2). Liposomes for use in drug delivery

usually require a long shelf-life and cannot be administered in frozen form, so hydrogenated phospholipids with much higher $T_c$'s are commonly used.

## *2.5 Liposome preparation methods*

There is a great deal of variation between techniques in terms of liposome size distribution, entrapment efficiency and stability. Each technique has certain advantages and disadvantages that determine its suitability or otherwise for a specific application. As a number of excellent reviews have been published that provide preparation details for the more common currently used preparation techniques and resulting liposome characteristics (New, 1990a,b,c; Kim and Baianu, 1991; Hauser, 1993a; Reineccius, 1995b; Watwe and Bellare, 1995; Betageri and Kulkarni, 1999; Frezard, 1999; Zeisig and Cämmerer, 2001) these matters will not be discussed in more detail here.

Phospholipid membranes form spontaneously as a result of unfavourable interactions between phospholipids and water, and thus liposome-like structures can be produced relatively easily. The difficulty in making liposomes is in getting these membranes to form vesicles of the right size and structure which entrap the desired materials with high efficiency and retain these materials for the required length of time (New, 1995c). Watwe and Bellare (1995) stated that the ideal preparation method should be simple, standardised, reproducible and cost effective. The yield should be homogenous and stable, and the size of liposomes able to be easily controlled.

A number of techniques are based on the standard preparation method for MLVs given in section 2.4.4.3. For example, the dehydration-rehydration method sonicates MLVs to produce SUVs, the active material is added to the liposome solution and the mixture freeze-dried and rehydrated, causing encapsulation to occur (Picon *et al.*, 1994). The French pressure cell method takes the MLV solution and passes it through a small aperture under high pressure (20,000-40,000 psi), disrupting the structure of the liposomes and causing them to reform as SUVs (Hamilton and Guo, 1984).

Small variations in preparation techniques can make large differences in the characteristics of the liposomes. Weiner (1995) outlined a specific method involving the agitation of a thin lipid film and water. The duration and intensity of agitation was varied, and it was reported

that 20 hours of gentle shaking resulted in 50% more entrapment than 2 hours vigourous shaking, although with a similar average liposome size. A few minutes with a vortex mixer produced liposomes with a smaller average size and further reduction in entrapment efficiency.

The use of solvents in the preparation of liposomes is almost universal. Many techniques also use detergents, alcohols, or buffers - compounds that are usually undesirable in food products. There are a variety of dialysis methods, chromatography techniques, or dilution and concentration cycles that may be used to reduce the amount of these materials present in the liposome solutions, but some residues always remain (New, 1995c).

Sonication is a common tool used in the preparation of SUVs from MLVs made through the standard thin-film method. Sonication causes ultrasound-driven mechanical vibrations, resulting in cavitation of the larger MLVs. The force associated with the implosion of vapour bubbles reduces the size of the vesicles (Maa and Hsu, 1999). The high localised energy densities that occur during sonication can damage heat sensitive materials (Maa and Hsu, 1999) and it can be difficult to obtain reproducible results on the large scale. A relatively new technique that may avoid these problems involves the use of a high-pressure homogeniser such as the Microfluidizer®.

### *2.5.1 Extrusion*

Extrusion techniques usually involve passing a solution of MLVs formed via thin film rehydration through a small orifice or a membrane with defined pore size. The solution is subjected to extremely high shear forces during this processing, breaking up the structures formed in the original mixture, and providing sufficient energy to allow the molecules to realign and form liposomes.

Turanek (1994) used a FPLC system to pass the MLV dispersion through filters with pore sizes of between 100-400 nm. Hydrophilic trapping efficiencies of 56-80% are reported, although freeze-thaw processing prior to extrusion gave an even higher efficiency. Northern Lipids (Vancouver, Canada), a manufacturer of extruders and accessories for liposome production, reported that 80% of aqueous solute is entrapped when a 400 mg/ml lipid

dispersion is passed through 200 nm pore size filters. However, New (1990c) stated an entrapment efficiency of only 30% for extrusion techniques.

Advantages of extrusion include the avoidance of sonication, the ability to control the size distribution by the choice of filter pore size, the use of a wide range of lipid concentrations (0-400 mg/ml lipid), and the reported high entrapment efficiencies. However, there are also a number of disadvantages to this method. In general, extrusion techniques seem very slow, working with small volumes and low flowrates (Turanek, 1994). The commercial extrusion system offered by Northern Lipids has barrel sizes of between 1.5 and 800 ml. They state that the maximum batch size possible with this equipment is 50 litres, but there is no information on the time taken to produce this quantity. New (1990c) reported that the filters clog easily, and recommended that the use of extruders for the production of liposomes be limited to laboratory scale. However, Northern Lipids claim that it is a valid technique for use on a commercial scale.

### *2.5.2 Microfluidization*

Jackson and Lee (1991) stated that the large-scale production of liposomes was limited by poor encapsulation efficiencies, the lack of a continuous production process, and the use of organic solvents. They concluded that the solution to this problem may be the use of a Microfluidization technique. Reineccus (2001) also claimed that Microfluidization is the only commercially-feasible method of liposome production. The Microfluidizer® can rapidly produce a large volume of liposomes in a continuous and reproducible manner (Chen *et al.*, 2001a), and there is no use of sonication, detergents, solvents or alcohols (Kim and Baianu, 1991). The liposome population produced appears to be relatively stable, without rapid aggregation or fusion (Kim and Baianu, 1991). The Microfluidizer® can handle lipid concentrations of 20% or more by weight, much higher than in most other methods. Increasing the phospholipid concentration increases the liposome concentration. As the Microfluidizer® produces liposomes with an approximately constant entrapped volume per liposome, increasing the liposome concentration will result in a higher proportion of the aqueous phase being entrapped. This enables the Microfluidizer® to obtain high entrapment efficiencies for hydrophilic materials (New, 1990b). However, although Microfluidization

appears to work very well for the production of small liposomes, it is not suitable for producing a unimodal preparation of large vesicles (Sachse, 1998).

In Microfluidization, the phospholipid and the material to be entrapped are dispersed in a liquid phase. This may be water, an aqueous buffer solution, or a solvent depending on the solubilities of the components. The solution is pressurised in continuous flow, and split into two streams which are then forced together at high velocities (> 500 m/s). The resulting release of kinetic energy provides the required activation energy and allows the formation of liposomes (Kim and Baianu, 1991). There is some debate over the type of liposomes produced by Microfluidization. Jackson and Lee (1991) stated that liposomes were SUVs, while New (1990c), Arnaud (1995), and Chen *et al.* (2001a) reported that the Microfluidizer® produced MLVs. The review by Chandran *et al.* (1997) reported that when large positively charged MLVs were passed through a Microfluidizer®, the different layers 'peeled' off to form LUVs.

The size distribution of the liposomes produced can be controlled by varying the pressure used and the number of times the dispersion is recirculated through the Microfluidizer® (Jackson and Lee, 1991). Increasing the number of circuits through the chamber reduces the liposome size and narrows the size distribution. In general, diameters of between 50 and 200 nm with a relatively homogenous size distribution can be expected (Weiner, 1995), although Martin (1990) suggested that the distribution may be polymodal.

Vemuri *et al.* (1990) reported that the size of the multilamellar vesicles produced decreased drastically from 0.64 to 0.16 μm after 3 passes through the interaction chamber, and that an additional 3 passes did not reduce the size any further. The polydispersity (measure of the size distribution spread) decreased slightly with each pass. Percentage encapsulation was proportional to vesicle diameter, and therefore was seen to decrease with an increasing number of passes through the Microfluidizer®. It was also found that the larger interaction chamber in the commercial scale Microfluidizer® produced larger vesicles at a given pressure than the laboratory scale model. Following the correlation between vesicle size and encapsulation efficiency, higher efficiencies were obtained on the production-scale model.

A report by Barnadas-Rodriguez and Sabés (2001) supports the above results, but also considers other variables within the system. The mean liposome diameter was found to decrease with increasing inlet pressure, number of cycles and ethanol concentration. Mean

liposome diameter could be increased by raising the ionic strength in the range 22-155 mM. The authors provide equations for calculating liposome diameter based on significant variables, but due to the differences in preparation methods, it is unlikely that these will apply to the aqueous system used in this thesis. The concentration of phospholipid in the solution did not appear to have any significant effect on the size or size distribution of the liposomes. Not surprisingly, the entrapped volume of liposomes followed the same pattern of response to the variables as liposome diameter.

Some concerns have been expressed about the use of a Microfluidizer® in liposome production. Martin (1990) and Weiner (1995) stated that there could be problems with heat regulation and the degradation of lipids caused by high shear. During processing, there is an increase in temperature of 1.7°C for every 1000 psi (6800 kPa) of pressure. This would correspond to a 34°C temperature rise for a pressure of 20,000 psi (13,600 kPa). A cooling unit can be used at the discharge end of the system to limit the time at high temperatures, but there is currently no way to avoid the increase in temperature during processing. However, Barnadas-Rodríguez and Sabés (2001) stated that, within the ranges of variables they studied, the Microfluidization process did not damage the phosphoplipids as measured by their oxidation index.

### *2.5.3 Pro-liposome*

Another production technique that is gaining prominence is the so-called pro-liposome method. This involves the addition of excess water to a phospholipid, ethanol and water dispersion with mixing (Arnaud, 1995c). Hydrophobic compounds to be entrapped are dispersed with the phospholipid and ethanol phase before any hydrophilic material to be entrapped is added along with the extra water. No solvents (apart from the ethanol) or high-energy processes are required, and high entrapment efficiencies are reported (Perrett *et al.*, 1991; Arnaud, 1995c; Dufour *et al.*, 1996; Laloy *et al.*, 1998).

This technique has been patented, so full details of the methodology are given in US patent 5004611. The article 'Pro-liposomes for the food industry' gives a comprehensive overview both of the principles behind the pro-liposome technique and of its practical application (Arnaud, 1995c). The following is a summary of this article, but it must be noted that

Arnaud was an employee of Lucas Meyer (Illinois, USA), the company that commercially manufactures the pro-liposome system.

The pro-liposome technique is based on the addition of water to an appropriate mixture of ingredients, leading to the spontaneous formation of liposomes. This mixture consists of a carefully chosen blend of phospholipids in a combination of solvents, usually aqueous ethanol and glycerol. The system does not contain sufficient water to allow liposome formation, and the phospholipids are mainly arranged in the form of stacked bilayer sheets. When excess water is added, the high concentration of small hydrophilic molecules between the bilayers means that more water is drawn in due to osmotic forces. As more and more water is absorbed, the stacked lipid bilayers spontaneously re-organise, forming vesicles that entrap any active ingredients dissolved or suspended in the aqueous phase.

Lipid soluble actives are blended into the pro-liposome mixture prior to the addition of water. The most efficient entrapment of water-soluble ingredients is via a two-stage dilution process. The addition of a just sufficient volume of a concentrated suspension/solution of the active ingredient to cause liposome formation will give a slurry of loaded vesicles. This is then diluted with further water as desired. Reported entrapment efficiencies for most hydrophilic substances are between 40-60%, but it is claimed that with careful formulation can be as high as 70-80%. As for most techniques, entrapment efficiencies for lipophilic substances are dependent on their partition properties, but can approach 100%. It is recommended that the amount of lipid-soluble ingredient does not exceed 25% of the phospholipid content, and that high concentrations of very hydrophobic substances such as oils should be incorporated into liposomes in the form of separate stabilised droplets.

The only equipment required to produce large-scale batches of liposomes using this method is standard mixing apparatus, however there are obvious complications due to patent issues, and it is likely that the pro-liposome mixture would have to be purchased from Lucas Meyer for approximately NZ$400/kg.

## *2.6 Liposome characterisation*

The composition and physical structure of the liposomes have a major effect on their behaviour, as does the phase transition, permeability, and stability of the liposomes. Other

measurements such as the entrapped aqueous volume per unit of phospholipid provide useful information for predicting the entrapment efficiency of the population. The concentration of the phospholipids and of the entrapped compounds are also important characteristics of the system, and are required to calculate yields, costs, and optimise processes. This section briefly discusses some of the methods for characterising the composition of the initial phospholipid preparation, the liposome solution, and the purified liposomes.

### *2.6.1 Phospholipid content*

The classes of phospholipid present in a particular phospholipid fraction will influence the characteristics of any liposomes produced from that fraction. There are a number of methods that may be used to determine the phospholipid content of a fraction, both in terms of identifying the classes of phospholipid and measuring the total amount of phospholipid material present.

#### 2.6.1.1 Phospholipid concentration

The determination of the phospholipid concentration of a sample is usually done indirectly through the measurement of phosphorous content. Most of the phospholipid classes used in liposomes contain one mol of phosphorus per mol phospholipid, so the phospholipid concentration can be derived directly from the phosphorous measurement. Phosphorous content is usually measured using either the Bartlett assay or the Stewart assay. The Stewart assay involves the measuring the absorbance of complexes formed between the phospholipids and ammonium ferrothiocyanate (Stewart, 1980), while the Bartlett assay measures the light absorbance at 830 μm of acid-digested phosphate eluting from a chromatographic column.

Commercially available test kits for measuring choline are available (Chemicals USA, Inc., Richmond, Virginia, USA) but these are only useful if the phospholipid is purified PC. Total amount of phosphorus present can also be determined by $^{31}$P-NMR (Menses and Glonek, 1988; Metz and Dunphy, 1996).

#### 2.6.1.2 Phospholipid class

Thin-layer chromatography (TLC) can be used to identify which phospholipids are present in a fraction. Grit *et al.* (1993) reviewed various TLC methods, and observed that one of the most common solvent mixtures for separating phospholipids by TLC consists of chloroform, methanol and water in varying proportions. Iodine vapour was widely used to visualize the phospholipid bands, but the poor response of saturated phospholipids resulted in accuracy problems. Obtaining a quantitative measurement of the amount of each phospholipid present requires scraping off of the phospholipid spots followed by phosphate determination, or measuring the colour density of the spots using a densitometer. These methods are susceptible to human error, but can be relatively straight-forward for pure samples of single phospholipid types and for commercial soy fractions. However, quantitative detection using TLC can be difficult for dairy fractions as standards are not as readily available.

For quantitative determination of PC levels, high performance liquid chromatography (HPLC) techniques are usually more accurate, providing improved resolution for the minor components Grit *et al.* (1993). Phospholipid head group and the nature of the fatty acid chains may both influence elution times, and complex mixtures such as those found in most natural phospholipid sources can produce a multitude of overlapping peaks. Grit *et al.* (1993) presented a large number of examples of HPLC conditions used for phospholipid analysis. They noted that hydrophilic silica gel columns were generally used for separation of phospholipid classes, with the eluent solution and pH being both important considerations. However, the method suffers from complications in finding a suitable detector for the lipids. UV detection was often difficult due to relatively weak phospholipid absorption of UV-vis electromagnetic radiation and its sensitivity to double bonds present in the system. Refractive index was not sensitive enough for most uses, and fluorescence and infrared detectors could only be used for certain lipids (Sotirhos *et al.*, 1986).

The use of Nuclear Magnetic Resonance (NMR) spectroscopy is becoming more common for quantifying the specific types of phospholipid present in a sample. The resonance frequency of phosphorus depends on the structure of the particular molecule, meaning that each phospholipid type gives a slightly different signal (Sotirhos *et al.*, 1986). The size of the signal reflects the concentration of that particular phospholipid class, so quantitative results can be obtained by comparison with the resonance frequency and signal height of standards.

Enzymatic cleavage tests have also been used for phospholipid analysis, but tend to be used only on PC (Gurantz *et al.*, 1981; Gober *et al.*, 1993; Grohganz *et al.*, 2003). In general, these techniques use phospholipase D to release choline, choline oxidase to oxidize the choline and hydrogen peroxide, and then measure the formation of a red quinone dye by peroxidase.

### *2.6.2 Size distribution of liposomes*

Due to their small size it is usually necessary to use electron microscopy to attain definite confirmation that liposomes are present in a sample, but size distribution is also a useful indirect method of monitoring liposome presence. In addition, the size distribution of liposomes is among the most important properties for determining their behaviour and characteristics. There are many different methods that can be used to obtain this information, each with different advantages and limitations.

#### 2.6.2.1 Photon correlation spectrometry (PCS)

Light scattering techniques are usually relatively easy and fast, and are based on the principle of the time dependence of intensity fluctuations in scattered laser light, due to Brownian motion of particles in suspension (New, 1995a). Small particles in the solution will diffuse more rapidly than larger particles, and so the rate of fluctuation of the scattered light will vary accordingly. Use of a correlation technique to analyse the intensity fluctuations will determine the diffusion coefficient for the particles, which can be substituted into the Stokes-Einstein equation to allow the calculation of their equivalent hydrodynamic radius (Arnaud, 1995a). This method (often referred to as photon correlation spectrometry or PCS) is used widely throughout the literature (Masson, 1989; Vemuri *et al.*, 1990; Malmsten *et al.*, 1994; Schneider *et al.*, 1994; Turanek *et al.*, 1997; Maa and Hsu, 1999; Copland *et al.*, 2000; Chen *et al.*, 2001a; Hays *et al.*, 2001; Valenti *et al.*, 2001).

#### 2.6.2.2 Electron microscopy

Light microscopy may be useful for the upper end of size spectrum, but is not suitable for the size range typical of liposome dispersions. However, electron microscopy is a commonly used technique which can provide some valuable information.

Negative staining electron microscopy using molybate or phosphotungstate as the stain allows accurate determination of size at the very low end of the size spectrum, and can give useful pictures of individual vesicles to determine the number of phospholipid bilayers. However, it is time consuming, and requires patience and skill to obtain a reliable estimation of size distribution. Despite this, Weiner (1995) stated that this was the method of choice below 5 micron. Freeze fracture electron microscopy is very useful for looking at the morphological structure of the liposomes (Arnaud, 1995a; Weiner, 1995), but is much more expensive and difficult than the negative staining process.

An important consideration in the use of electron microscopy is that the critical procedures of cryofixation or chemical fixation and staining may produce artifacts. Hauser (1993a) reports that while negative staining may be quick and simple, it has been shown that negatively charged PS dispersions have particular problems with artifact production. It is suggested that cryofixation methods, including freeze-fracturing and freeze-etching are much more likely to preserve the original structure of the liposomes than any staining procedure, especially for charged vesicles.

#### 2.6.2.3 Turbidity and right-angle light scattering

Some systems have rapidly changing particle size distributions, such as those undergoing aggregation and fusion. The methods discussed so far are not able to follow changes in real time, but investigations using right angle light scattering and turbidity have shown potential for tracking these events. Dynamic light scattering was used to provide the mean cumulant radii of a variety of liposomes, with right angle light scattering intensity and optical density at 436 nm then measured and plotted against vesicle radius. It was found that liposomes with a radius of 100 nm could be accurately modeled by homogenous spheres (Rayleigh-Gans-Debye theory). The mean refractive indices were estimated by the volume fractions of lipids in vesicles (Matsuzaki *et al.*, 2000).

### *2.6.3 Entrapped volume*

The entrapped volume for a liposome population provides an indirect measurement of the hydrophilic entrapment efficiency for that dispersion. Entrapped volume is generally proportional to the cube of the liposome radius, however, the entrapped volume is reduced as

the liposomes become smaller and the membrane phospholipids occupy more of the internal space. For a given amount of lipid, large liposomes entrap far greater aqueous volume than small ones (New, 1995b). The method of liposome preparation can also affect both the location of actives and the entrapment efficiency (Weiner, 1995).

Perkins *et al.* (1993) provided a comprehensive review of techniques for determining entrapped volume. The most common method was through the calculation of the total quantity of entrapped solute and the assumption that the concentration of solute in the aqueous medium in the interior of the liposomes was the same as the original solution. However, this assumes that there is no movement of either solutes or water into or out of the liposome. This may not be valid if the entrapped material diffuses through the bilayer at a noticeable rate, or if there are differences in osmotic pressure across the bilayer. Use of a non-membrane-permeable solute improves the accuracy of this method, but problems arise if the solute is not evenly distributed throughout the system. Such a situation may occur when MLVs are produced by rehydrating a lipid film or from freeze/thaw cycles. Unfortunately, because this technique has been so widely accepted as providing accurate results, there has only been limited development of alternative methods (Perkins *et al.*, 1993).

Arnaud (1995a) stated that the best method for the determination of entrapped volume was the inulin-inaccessible space technique. Inulin is a large polysaccharide molecule that is unable to diffuse through the liposome membrane. If a known weight of inulin is added to identical volumes of both a liposome suspension and the buffer without the liposomes, the concentration of inulin in the external phase of the suspension will be higher than that in the buffer. Centrifugation of the suspension will result in the sedimentation of the liposomes, and the concentration of the inulin in the supernatent can be measured. A known number of counts of $^{3}$H-labelled inulin can also be used. Perkins *et al.* (1993) outlined a similar method where an ESR spin probe was added to the liposome dispersion, and the concentration of the probe in the total volume compared to the concentration in the supernatant after the liposomes have been sedimented.

It is also possible to directly measure the actual amount of water entrapped. This is achieved by the replacement of the extraliposomal fluid by a spectroscopically inert fluid such as deuterium oxide, and measuring the water signal for the system. This may be done by NMR techniques (New, 1995a).

Values are commonly reported as millilitres or microlitres of aqueous space entrapped per gram or microgram of phospholipid. Arnaud (1995a) gives typical entrapped volumes of approximately 2 ml/g for SUVs, 2-8 ml/g for MLVs, and 6-15 ml/g for LUVs, while Hauser (1993a) reports values of 1-5 ml/g for MLVs and a maximum of close to 40 ml/g for LUVs.

### *2.6.4 Lamellarity*

Liposome lamellarity has a significant effect on encapsulation efficiency and the rate of diffusion of encapsulated material out of the interior spaces of the liposome. While the typical MLV is often portrayed as an onion-like structure with neatly stacked bilayers at regular intervals, this ideal is often not the case. The lamellar within MLV may be at irregular intervals, and are often arranged in "liposome within liposome" type structures (Perkins *et al.*, 1993).

New (1990a) gave a technique for predicting the lamellarity of a population of intermediate or large liposomes where membrane thickness is small compared with the particle diameter. For unilamellar vesicles, the entrapped volume (E) can be estimated by Equation 2-1, where $A$ is the area of membrane occupied by one mole of lipid and $r$ is the average radius of the vesicles. The calculated E can then be compared with the estimation of entrapped volume obtained by experimental methods (section 2.6.3). If the experimental value is less than the calculated one, either there is a significant proportion of SUVs present or some of the vesicles are multilamellar.

$$E = {}^{1}/_{3}Ar \qquad \textbf{Eq. 2-1}$$

Another method provided by New (1990c) involved measuring the percentage of phospholipid molecules on the surface of the outermost membrane. This can be measured chemically or spectroscopically. If a small quantity of PE is included in the phospholipid mix during liposome production, the exposed PE molecules can be derivatized in the liposomes by reaction with trinitrobenzene sulphonic acid (TNBS). Control samples containing disrupted liposomes allow all PE molecules to interact with the TNBS, and comparison of the optical density of the two solutions provides an indication of the

percentage of the PE molecules on the liposome surface. A value of close to 50% suggests LUV, lower values indicate SUVs or MLVs.

The spectroscopic method measures the $^{31}$P-NMR signal of the phospholipid headgroups both before and after the addition of manganese ions to the solution (New, 1990a; Perkins *et al.*, 1993). Manganese ions interact with the surface phosphorus, causing the resonance signal to broaden beyond detection. The remainder of the peak height is due to the phosphorus inside the liposome, and the lamellarity can be estimated by Equation 2-2, where 'lipidout' refers to the phospholipids on the exterior of the liposome bilayer. Lanthanide ions (such as $Pr^{3+}$) may be substituted for the manganese, shifting the resonance signal for the exterior phosphorus away from the signal from the internal phosphorus (Perkins *et al.*, 1993). Matsuzaki *et al.* (2000) used a similar technique to estimated liposome lamellarity based on fluorescence quenching of N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)dipalmitoyl-L-alpha-phosphatidyl-ethanolamine (NBD-PE) labeled liposomes by sodium dithionite. Changes in the NBD fluorescence were followed using a spectrofluorometer, and the fraction of the NBD-lipid exposed to the external aqueous phase could be deduced.

$$\textbf{Average number of bilayers in the liposome population} = {}^{1}/_{(2 \times \text{lipidout})} \qquad \textbf{Eq. 2-2}$$

These methods may underestimate the lamellarity if the ions permeate the liposome membrane, which may be determined by monitoring the signal as a function of time. Equation 2-2 will also underestimate the lamellarity of small multilamellar liposomes, as the interior bilayers have significantly smaller surface areas than the external membrane.

Froehlich *et al.* (2001) investigated the accuracy of lamellarity determination using $^{31}$P-NMR in combination with chemical shift reagents. There have been a number of publications using this method, but it was felt that very few details about the required conditions throughout the measurements were known. The influence of various parameters including different buffers with changing ion concentrations, varying pH and different shift reagents at increasing concentrations was investigated. Cryo-electronmicroscopy was used as the reference method. It was concluded that $^{31}$P-NMR might not result in the correct

determination of liposome lamellarity, depending on the experimental settings and the shape of the liposomes.

An alternative technique is the use of small-angle X-ray diffraction (Moody, 1993; Huang *et al.*, 1999; Csiszár *et al.*, 2003; Matuoka *et al.*, 2003; Fonollosa *et al.*, 2004). MLVs produce a characteristic small-angle X-ray diffraction pattern that consists of a series of sharp peaks in the low-angle region in the ratio 1:½:⅓:¼ etc, and a broad, diffuse relection in the wide-angle region. The small angle X-ray diffraction pattern for dilute dispersions of unilamellar vesicles has only a broad scattering peak in the low-angle region. Examples of the diffraction patterns for SUVs and MLVs are shown in Figure 2-8.

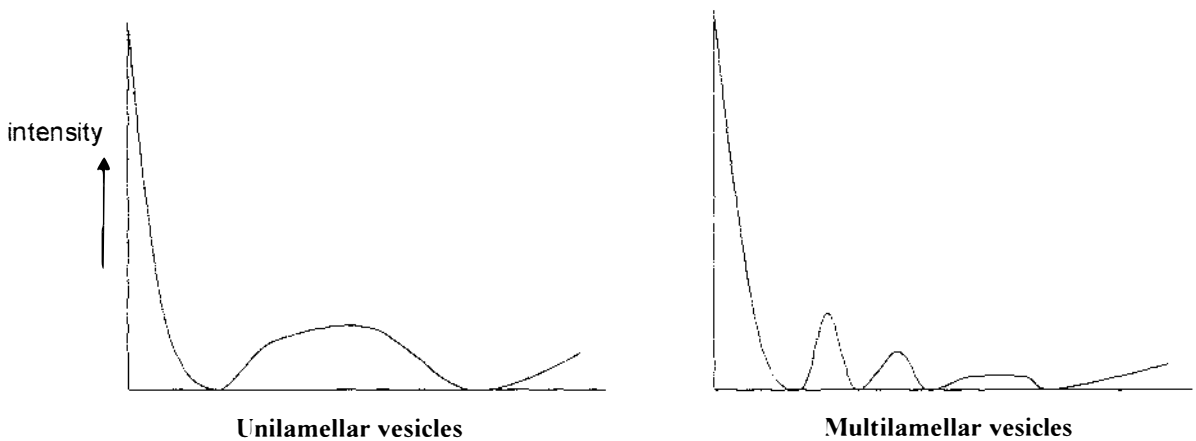



**Figure 2-8 Example of small-angle X-ray scattering curves for liposomes.**

Although these methods may be useful for determining whether a liposome population is uni- or multi-lamellar, none of these methods can quantify the average number of bilayers contained within a vesicle in a population of MLVs. Electron microscopy is the only method capable of providing this level of information. Perkins *et al.* (1993) stated that the negative-stain used is able to permeate the membrane and allow lamellarity of a liposome dispersion to be estimated in a qualitative or semi-quantitative manner.

### 2.6.5 *Relationship between liposome size, shape, entrapped volume and lamellarity*

The size, shape, entrapped volume and lamellarity of a liposome dispersion are all interdependent. However, it is impossible to use just one of these to infer the others. This is demonstrated in Figure 2-9. In order to fully characterise a population of liposomes, as many of these parameters as possible should be determined on an individual basis.

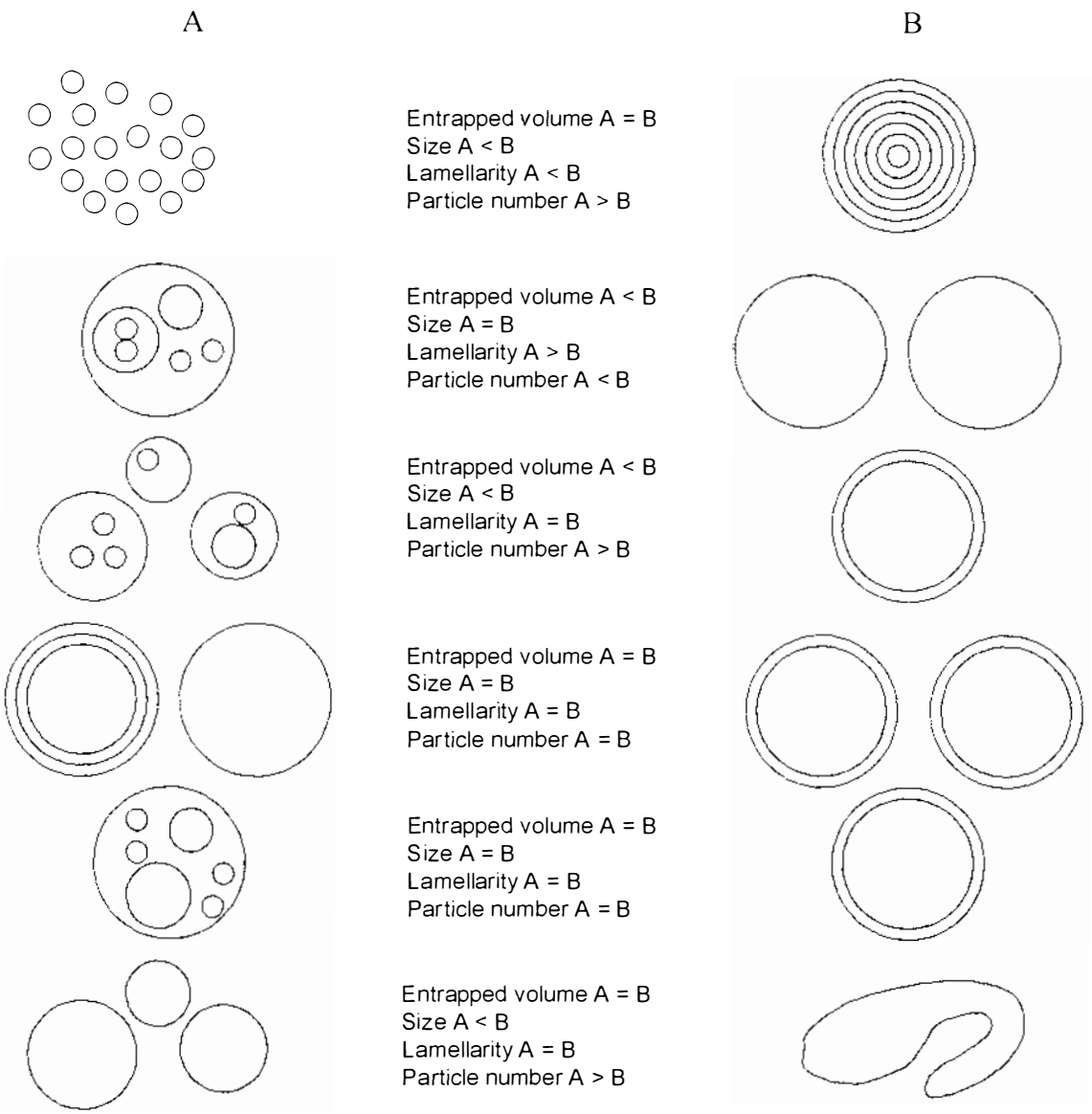



**Figure 2-9 Examples of entrapped (captured) volume, size, lamellarity and particle number for a variety of liposome systems. From Perkins *et al.* (1993).**

### 2.6.6 Phase transition temperature

As outlined in section 2.4.2, the phase transition temperature ($T_c$) of a liposome dispersion will influence the membrane stability and permeability. The $T_c$ is a function of the types of phospholipids and their fatty acid profile, although the presence of other molecules in the membrane (such as sterols) may also affect the $T_c$.

The $T_c$ is determined using differential scanning calorimetry (DSC), which measures the excess heat capacity of a system as a function of temperature (Biltonen and Lichtenberg, 1993). When the membrane undergoes the transition from the gel phase to the liquid-crystal phase, there is a peak in the heat capacity corresponding to the $T_c$. The peak is sharp and well-defined if the phase transition occurs over a small temperature range, indicating a relatively pure phospholipid sample. However, more heterogeneous samples may have a much broader peak as the different phospholipid molecules present go through the phase transition over a range of temperatures.

### 2.6.7 Permeability

Liposome membranes are semi permeable, allowing the transfer of some molecules and ions with relative ease whilst offering a substantial barrier to others. The bilayer permeability is a measure of the flux or rate at which a solute diffuses from an internal aqueous compartment through the bilayer and out into the solute (Frezard, 1999). The relative permeability will vary depending on the molecule in question, the environmental conditions, the composition of the membrane and inter/extra liposomal fluid, and the processing/storage history of the solution. Membrane permeability is highest at $T_c$, and lower in the gel than fluid phase.

Molecules with a high solubility in both organic and aqueous media (including many flavour compounds (Reineccius, 1995b)) may pass in and out of the liposome without interference. Large polar solutes such as proteins will slowly diffuse through the membrane (Gibbs *et al.*, 1999), while smaller polar solutes like glucose will diffuse slightly more quickly (New, 1995b). Smaller molecules with a neutral charge (water, urea) cross the membrane quite rapidly, but charged ions vary considerably in their behaviour (New, 1990b; Gibbs *et al.*, 1999). According to New (1990b), protons and hydroxyl ions tend to permeate reasonably quickly, sodium and potassium ions very slowly, and anions such as chloride and nitrite at a

moderate pace. Membranes are less permeable to multivalent ions than monovalent ions due to their increased charge and size. Frezard (1999) reported the following general sequence of hydrophilic solute permeability: water > small non-electrolytes > anions > cations ~= large non-electrolytes > large poly-electrolytes.

The composition of the bilayer and the lipids used will determine the phase transition temperature of the membrane (section 2.4.2). The permeability of the membrane to protons and water molecules increases at the phase transition temperature, and remains high above this point, whereas sodium ions (and most other solutes) diffuse more slowly above and below the transition temperature than at the actual transition point (New, 1995b). Based on this observation, New (1990b) suggested that there are different mechanisms for transfer across the membrane for different molecules.

Packing irregularities such as point, line, or grain boundary defects in the membrane may also allow small molecules to pass through more easily. New (1990b) described in some detail the mechanisms behind these irregularities, mainly the transformation of sections of the membrane from *trans* to *gauche* configuration. This change expands the surface area of the membrane, reducing the bilayer thickness. The *trans/gauche* transformation becomes more favourable at higher temperatures, resulting in an increase in the number and duration of defects. Because of this, there is a temperature dependency to permeability, especially to small ions and polar molecules. Permeability is highest at the phase transition temperature, as the gel and liquid-crystal phases co-exist in different parts of the same membrane, and packing defects such as grain boundaries are found at each interface of the two phases.

The fatty acid composition of the phospholipid fractions will influence liposome permeability, with the degree of fatty acid saturation affecting the phase transition temperature and therefore membrane permeability at different temperatures. The use of phospholipids with longer chain fatty acids will increase the thickness of the bilayer and decrease the rate of diffusion through the membrane for all solutes (New, 1995b).

It has been shown that the membrane permeability of sodium ions decreases with increasing unsaturation, while glucose permeability increases. New (1990b) proposed the following explanation. Small metal ions are able to travel along the transient kinks formed by single bond rotation in the fatty acid chain. Double bonds cannot rotate freely, so hinder progress of the ion. Glucose is a much bigger molecule, and cannot fit through the small gaps created

by these rotations, instead taking advantage of the looser packing of unsaturated fatty acids for increased permeability.

Concentration gradients across the bilayer can generate osmotic pressure, leading to the accumulation of water molecules (being the fastest diffusing species) on one side. If a concentrated solution is encapsulated inside a liposome and the extraliposomal fluid is a dilute solution, the liposome will take on water and swell up, considerably increasing the surface area of the vesicle as well as the spacing between adjacent phospholipid molecules. This increases membrane permeability for solutes of molecular weights up to that of glucose, but the leakage of larger molecules such as sucrose is not usually affected. In some cases, the pressure generated can cause the rupture of the liposome (New, 1995b).

Interaction between the bilayer and compounds introduced to the liposome solution can also affect membrane permeability. Inclusion of molecules such as sterols which stabilize the membrane will usually reduce membrane permeability, while molecules which destabilize the membrane tend to increase it. Weiner (1995) mentioned that hydrolysis and oxidation reaction products can cause dramatic changes in the permeability properties of liposomes.

### *2.6.8 Liposome stability*

There are two main areas of importance in liposome stability – stability during storage and/or processing, and stability in the body. For pharmaceutical applications, the latter is extremely important, as the release of drugs in the blood system needs to be very well defined. However, for the majority of food applications it is really only the stability prior to ingestion that is of concern.

Liposome dispersions must demonstrate adequate stability both in terms of chemical and physical changes. The high dependence of liposome behaviour on composition means that it is difficult to predict whether an individual liposome system will retain structure and function under different stresses. The stability of any liposome system under conditions of varying pH or temperature, or in the presence of degradative substances, will ultimately depend on the composition of the phospholipid mixture used and the inclusion of protective compounds, such as antioxidants and thermoprotective sugars.

2.6.8.1 Chemical degradation

Martin (1990) stated that phospholipids from natural sources degrade in 2 major ways – through the peroxidation of double bonds which may be present in acyl chains, and the hydrolysis of ester bonds linking fatty acids to the glycerol moiety.

The hydrolysis of sn-2 fatty acids usually occurs first, following first-order kinetics. The rate of reaction is very slow at low temperatures, but becomes significant above 40°C. Storage temperatures are usually easier to control than processing temperatures, where a certain length of time at a given temperature may be required. However, this time is usually a few hours at most, and Martin (1990) asserted that this should not cause much concern. The rate of hydrolysis is also affected by pH, rising either side of a minimum at a pH of 6.5 (Martin, 1990). Other actions to minimise hydrolysis include using pure solvents and removing as much of the water in the system as possible (Gibbs *et al.*, 1999). The latter suggestion is for solvent-based systems, and is obviously not appropriate for water-based ones.

One of the most important mechanisms for minimizing the degradation of liposomes through peroxidation is the use of high quality lipids which have been correctly prepared and stored (Gibbs *et al.*, 1999; Reineccius, 1995b). Avoiding exposure to light and oxygen will also reduce the oxidation rate, as these both promote oxidation. When oxygen is in excess, the rate of oxidation is independent of oxygen concentration, but at very low oxygen levels the rate is approximately proportional to oxygen concentration (Nawar, 1996). The effect of oxygen concentration is also influenced by temperature and surface area, with the rate of oxidation increasing with temperature. The flushing of packaging with an inert atmosphere would reduce the rate of oxidation, as would storage in light resistant containers (Weiner, 1995) and limiting exposure to high temperatures.

The inclusion of a water and/or lipid soluble antioxidant will help slow oxidation by acting as a hydrogen donor or a free radical acceptor, thus inhibiting the oxidation chain reaction. Antioxidants often work synergistically with other antioxidants, one that reacts directly with the oxidation products and one that regenerates the first compound. An example of such a system is α-tocopherol and ascorbic acid (Nawar, 1996).

Saturated fatty acids are less susceptible to oxidation than unsaturated fatty acids (Reineccius, 1995b). At room temperature, saturated fatty acids remain virtually unchanged

even when oxidative rancidity of unsaturated fatty acids becomes detectable (Nawar, 1996). The use of saturated lipids, or hydrogenating unsaturated fatty acids, will reduce the rate of oxidation, but will also affect the properties of the liposome (see section 2.3.2). *Cis* acids oxidize more readily than their *trans* isomers, and conjugated double bonds are more reactive than nonconjugated (Nawar, 1996).

Peroxidation rates may be significantly increased in the presence of transition metal ions. Even at concentrations as low as 0.1 ppm they can decrease the induction period and increase the rate of oxidation. A number of mechanisms for their action have been suggested, and these are outlined by Nawar (1996). The inclusion of a chelator such as EDTA may be worthwhile, particularly if the materials used contain free metal ions as contaminants (Martin, 1990; Weiner, 1995; Gibbs *et al*., 1999).

Degradation of lipids can be monitored in a number of ways. Thin-layer chromatography (TLC) can be used to provide information on the purity and concentration of the lipids present in the sample. Phospholipids that have undergone extensive degradation will be observed as a smear fanning out from the point of origin, compared with the single spot for the pure material. Another method involves the use of HPLC to follow the production of lysolecithin, the major hydrolysis product of lecithin. UV absorbance at 230nm can measure the formation of conjugated dienes during the free radical chain initiation phase of oxidation. Extensive phospholipid degradation may be signaled by a third peak at 270nm due to the formation of conjugated trienes. Separate tests are used to measure the levels of the two types of peroxides which can be formed during oxidation reactions - hydroperoxides and cyclic peroxides (or 'endoperoxides'). The latter are detected by reaction of their breakdown product at elevated temperatures (malondialdehyde) with thiobarbituric acid (TBA) giving a red chromophore that absorbs at 532 nm. The method for detecting hydroperoxides is based on their susceptibility to reduction by iodide. Gas-liquid chromatography may be used to detect the decrease in acyl chain length that occurs during the final stages of lipid oxidation.

Grit *et al.* (1993) and Herman and Groves (1992) determined hydrolysis rates of PC and PE in aqueous media at various temperatures. Lipid hydrolysis was lowest at pH 6.5, but the rate of hydrolysis varied depending on the concentration of other phospholipid components.

### 2.6.8.2 Physical stability

#### 2.6.8.2.1 Symptoms of physical instability

In addition to the chemical reactions discussed in the above section, there are also physical mechanisms for liposome degradation. Defects in lattice structure at time of manufacture can result in leakage or fusion of liposomes. Some structural defects can be corrected by annealing, where the liposomes are held at a temperature slightly greater than the phase transition temperature for a period before being cooled to below the transition point. Annealing works by maintaining the bilayer in its liquid crystal phase, where the membrane has an increased fluidity. This allows the phospholipids to continue moving towards the lowest energy state, a perfectly ordered lattice without any defects. However, even annealed liposomes will undergo some fusion or aggregation over time (Reineccius, 1995b). Fusion may also occur amongst populations of very small liposomes (less than 40nm in diameter (Gibbs *et al.*, 1999) as a means of relieving the stress caused by high membrane curvature.

Techniques based on size changes cannot distinguish between aggregation and actual fusion. Jones and Cossins (1990) gave details on a method which involves the mixing of two sets of liposomes, each containing a specific marker. Interaction of the two markers causes changes in the fluorescence of the system, which can be used to monitor fusion. The markers chosen will determine whether they are entrapped within the bilayer or within the aqueous interior, so different markers may be used to ascertain whether the fusion is occurring in the hydrophobic or aqueous compartments.

#### 2.6.8.2.2 Long-term stability

Because liposome dispersions are at the minimum energy level for the system, liposomes are inherently stable units and many liposome dispersions will be stable for long periods under a variety of temperatures and other conditions. Nevertheless, it would seem reasonable to assume that storage in a dark environment at low temperatures, with air exclusion, would minimise oxidative degradation.

To ensure long term stability, freezing, spray-drying and freeze-drying may be considered. With all of these techniques, care must be taken to avoid dehydration-induced phase transition and membrane fusion. Aqueous dispersions of SUVs produced from egg PC aggregate and fuse to produce large MLVs when subjected to these treatments (Hauser,

1993a). New (1990b) stated "techniques which prevent membrane fracture upon freezing or drying have not yet been fully perfected", observing that the use of highly concentrated sugar solutions on both sides of the membrane may reduce membrane disruption. Frezard (1999) commented that the cryoprotective effect of sugars on liposome integrity has been extensively studied, and that sugars have been found to preserve the reactivity of entrapped active materials. Cullis *et al.* (1987), Kirby (1991) and Gibbs *et al.* (1999) have all reported that liposomes can be successfully stored by freeze-drying, and Crowe and Crowe (1993) stated that freeze-dried liposomes could be obtained that retain 100% of their original contents upon rehydration. However, the use of a cryoprotectant seems to be essential.

Among the most commonly used cryoprotectants are mono- and disaccharides. It is thought that these materials protect liposome systems during drying and freezing by forming a stable glassy matrix, preventing phase transitions and crystallisation which would usually damage the membrane (Chen *et al.*, 2001b). Hydrogen bonding between some sugars and polar groups on the lipids may also help prevent fusion (Anchordoquy *et al.*, 1987). Trehalose appears to be the most successful sugar in preserving the vesicle structure and preventing fusion during freeze-drying (Chen *et al.*, 2001b), but Anchordoquy *et al.* (1987) found that both sucrose and trehalose seem to be equally as effective during freeze-thaw processes. Crowe and Crowe (1993) commented that although similar levels of stabilization could be obtained using either trehalose or sucrose, a higher concentration of sucrose is required to achieve this. Lasic (1993) stated that glucose, lactose or mannitol tend to be used for freezing while sucrose is used for freeze-drying. Komatsu *et al.* (2001) reported that the effectiveness of cryoprotective sugars such as maltose was affected by the fatty acid composition of the phospholipids, and therefore the effectiveness of a certain sugar would depend on the specific composition of a liposome dispersion. Extensive reviews on the freezing and drying of membrane systems compiled by Wolfe and Bryant (1999), Oliver *et al.* (1998) and Crowe and Crowe (1993) are recommended for a more in-depth coverage of this topic.

Hauser (1993b) examined the stability of SUVs during spray-drying, and reported that the addition of 10% sucrose appeared to protect the liposome integrity as measured by ESR signal intensity. Approximately 90% of $K_3Fe(CN)_6$ and $^3H$-labeled raffinose that was entrapped prior to spray-drying remained inside the liposomes upon rehydration.

Liposomes with no exterior charge will undergo aggregation and sedimentation due to Van der Waals forces between vesicles. The inclusion of a charged phospholipid such as phosphatidic acid or phosphatidyl glycerol will help minimise this, even at levels as low as 5% (Reineccius, 1995b). Calcium and magnesium cause neutralisation of negative electrostatic charges, and can result in the aggregation, fusion or precipitation of negatively charged liposomes (Lichtenberg and Barenholz, 1988). Larger liposomes with high concentrations of phosphatidyl serine (PS) are likely to be particularly susceptible to this kind of destabilization, with the rate of fusion increasing with increasing temperature and membrane fluidity, and being especially rapid at the transition temperature (New, 1995b). Metal chelators may be used to help minimize the neutralisation of the charge by metals present. Lasic (1998) reported that decreasing the ionic strength of the medium could also reduce problems with the aggregation of liposomes.

There are a number of reviews that claim that holding liposome dispersions at temperatures above the phase transition temperature helps avoid liposome aggregation and fusion (Lichtenberg and Barenholz, 1988; Reineccius, 1995b; Gibbs *et al.*, 1999). This seems surprising given the higher stability of the gel phase compared with the liquid-crystal phase. However, to date, only one review has been found that contradicts this by reporting that liposome fusion decreased at lower temperatures (New, 1990c).

While liposome composition is likely to have a significant affect on the stability of the liposomes at different pH values, there are reports of liposomes being stable across the range from pH 4 (Madden *et al.*, 1990) to pH 9 (Deamer *et al.*, 1972).

There is relatively little literature regarding the stability of liposomes during heat processing. However, Zuidam *et al.* (1993) reported that liposome structure and lipid oxidation values were not affected by autoclaving (121 °C for 15 minutes), and Arnaud (1995a) also stated that heat sterilisation could be used to prevent microbial growth.

#### 2.6.8.2.3 Retention of entrapped material

One of the most important stability measurements is the ability of the liposomes to retain the entrapped material for as long as required – be that a few days or several years. It is also important to consider the chemical stability of the any entrapped material. The stability of both the liposome and any active material can be measured directly or indirectly through some corresponding characteristic. Changes in the size distribution of a liposome population

are often used as a measure of liposome stability over time. Valenti *et al.* (2001) monitored the leakage of entrapped essential oil and changes in the average size distribution of a liposome suspension stored at refrigerator temperatures for over a year. During this time, there was no leakage or change in size distribution, and negative staining electron microscopy confirmed that the original liposome structure was still present.

Hays *et al.* (2001) investigated the factors that influenced leakage of carboxyfluorescein (CF) from liposomes during chilling. As expected, maximum leakage occurred at the phase transition temperature. Leakage at the phase transition temperature was increased through the addition of defect-forming additives such as a second phospholipid or a surfactant, but was not affected above or below that temperature. Small unilamellar vesicles leaked much more rapidly than large unilamellar vesicles. It was also found that increasing the speed of temperature changes reduced leakage, and that increasing the pH of the external buffer to pH 7 and above decreased leakage of carboxyfluorescein. Liposomes in an aqueous buffer with a pH greater than 9 showed minimal leakage, while those at pH 7 and 6 retained approximately 85% and 70% of entrapped CF respectively. However, it was noted that the effect of pH is likely to be restricted to ionizable solutes.

Leakage at the phase transition temperature can be reduced or prevented through the use of antifreeze proteins. Glyco protein type AFGP is reported to be highly effective at preventing leakage from liposomes composed of a variety of phospholipids. Care must be taken when selecting potential antifreeze proteins, as some have been shown to increase liposome leakage (Wu and Fletcher, 2000). Other compounds that have demonstrated some ability to maintain liposome stability at phase transition temperature include albumin (Wu and Fletcher, 2000) and sterols.

## *2.7 Encapsulation*

As the phospholipid bilayer sheet is formed, hydrophobic or amphipathic material in the environment is caught up in the membrane. The folding of this membrane to give the liposome traps a portion of the aqueous environment in the centre of the vesicle, including any hydrophilic molecules present. The precise location of entrapped material will depend on the physiochemical characteristics both of the molecule itself and that of the lipids

forming the bilayer, and is determined by the partition coefficient of the molecule between the aqueous and lipid environment (Weiner, 1995).

The only materials that cannot be encapsulated within liposomes are substances that are not soluble in either lipid or aqueous phases. It can also be difficult to successfully entrap molecules which have significant solubility in both phases. Because of this, there can be a problem with using liposomes for flavour encapsulation, as most flavour compounds have some solubility in both phases (Reineccius, 1995b). A technique called active loading can be used to entrap such compounds (Deamer *et al.*, 1972; Cullis *et al.*, 1989; Madden *et al.*, 1990). A non-permeating buffer ion such as glutamate is entrapped inside the liposomes at a low pH. The extra-liposomal buffer is then replaced with an iso-osmotic buffer at pH 7 containing the active compound (usually a lipophilic amine). The actives then diffuse through the membrane in the uncharged form, and are converted to a charged species inside liposome. The charge reduces the molecules affinity for the lipid phase, resulting in an accumulation of solute inside the liposome as long as a pH difference is maintained between inside and outside. Alternatively, charged lipids may be incorporated into the membrane at low pH, followed by adjustment of the suspending medium to neutrality.

### *2.7.1 Hydrophilic entrapment*

As mentioned in section 2.6.3, hydrophilic entrapment is proportional to the total internal volume of the liposome (Weiner, 1995), but is also a factor of the phospholipid concentration. The use of high concentrations of the hydrophilic material increases the active material to phospholipid ratio, making more efficient use of the phospholipid available. The types of liposome structure formed also affects hydrophilic entrapment. With MLVs, the presence of the internal bilayer membranes reduces the entrapped aqueous space for a given liposome diameter, and therefore the internal aqueous volume of a unilamellar vesicle will be greater than that of a multilamellar one.

Vemuri *et al.* (1990) used a Microfluidizer to produce liposomes, reporting that hydrophilic encapsulation reduced sharply after the first pass through the interaction chamber, and reduced slightly further upon subsequent passes. This directly correlated with the changes in liposome diameter, leading them to conclude that encapsulation efficiency is a function of vesicle size. The larger the diameter of the vesicle, the more captured volume per surface

area, and therefore the higher the encapsulation efficiency per gram of phospholipid. Martin (1990) stated that an efficiency of approximately 70% was the theoretical maximum, due to 30% of the liquid filling the gaps between the spheres.

Reported entrapment efficiencies for hydrophilic materials vary greatly depending on the details of the preparation method and specific hydrophilic compound. Mayhew *et al.* (1984) used a Microfluidizer to encapsulate cytosine arabinoside (Ara-C), and obtained entrapment efficiencies ranging from 5-75% depending on the operating conditions and concentrations of phospholipid and Ara-C. Cullis *et al.* (1987) compared the entrapment efficiencies for different vesicle types and different preparation procedures as reported by a number of authors. The standard thin-film liposome preparation method usually gives an entrapment efficiency of only 1-9%, but repeated freezing and thawing of the solution can increase the efficiency to 35-88%. SUVs produced by detergent removal had an entrapment efficiency of 12%, while LUVs produced by the same method could have efficiencies as high as 42%. Some of these reported differences might have been due to the material being entrapped, as this was not considered in the comparison. However, the nature of the entrapped material can be a quite significant factor, especially if the material has a hydrophobic component or interacts with the bilayer molecules through charge attraction or repulsion. The dehydration-rehydration method used by one group resulted in entrapment efficiencies ranging from 1 to 34% for proteases and proteins of varying hydrophobicities (Picon *et al.*, 1994).

One of the major advantages liposomes have over other methods of microencapsulation is their ability to retain hydrophilic molecules within their structure while in solution (Kirby, 1991). As most food systems are water-based rather than solvent-based, this is a very important characteristic for food applications.

### *2.7.2 Hydrophobic entrapment*

For highly hydrophobic materials, entrapment is usually close to 100% regardless of the liposome type and composition, and entrapment efficiency is thus determined by the total amount of phospholipid present (Weiner, 1995). For molecules of lower hydrophobicity, the location of the compound within the liposome and its entrapment efficiency will depend on its partitioning between the hydrophilic and hydrophobic phases. Valenti *et al.* (2001) reported that both of the multi- and uni-lamellar vesicles used in their experiments showed a

high entrapment efficiency for an essential oil (around 78-80%), and that there was no significant difference in encapsulation efficiency between the two. This is not unexpected, as hydrophobic materials will be carried in the membrane, and there should be the same area of membrane regardless of whether it is arranged in uni- or multilamellar vesicles.

Because the hydrophobic actives are carried in the bilayer, there is a limit to how much can be incorporated into the liposome without destabilising the system. Van Nieuwenhuyzen and Szuhaj (1998) suggested that maximum loading of hydrophobic materials does not exceed approximately 25 wt% of the phospholipid. Arnaud (1995a) recommended that high concentrations of highly hydrophobic substances such as oils should be incorporated into liposome products in the form of separately stabilised droplets.

Burke *et al.* (2001) reported that the hydrophobic radical cation of β-carotene (a product of β-carotene oxidation) is able to interact with water-soluble species. Studies using DSC by Shibata *et al.* (2001) found that despite being categorised as a hydrophobic molecule, β-carotene has an amphiphilic nature, which may explain its ability to interact with hydrophilic compounds.

### *2.7.3 Removal of unentrapped material*

The calculation of the percentage capture of actives is based on the removal of unincorporated material and the assumption that the remaining material is all entrapped. This requires a method to separate free and entrapped actives. New (1990a) outlined two separation methods that could be used on small scales - the minicolumn centrifuging method and potamine aggregation. The method for determining the amount of entrapped material will of course vary with the different actives used, and may require the disruption of the liposome structure to allow all entrapped material to be accounted for. This is usually done based on either size or density differences (New, 1995c; Reineccius, 1995b).

The most suitable technique will depend on the characteristics of the liposome preparation. Chromatography methods will not be appropriate for large-scale separation, and dialysis cannot concentrate the system beyond a relatively dilute point. Centrifugation may appear relatively straightforward, but New (1990c) stated that the density of a phosphatidylcholine bilayer was 1.0135 g/ml, increasing slightly with the addition of cholesterol and quite

dramatically with the inclusion of proteins. Aqueous solutions where there is a relatively small difference in density may require up to 200,000 *g* for 20 hours in order to sediment the liposomes. Martin (1990) commented that large neutral liposomes would sediment at fairly low gravitational forces, while smaller ones would remain in the supernatent. Therefore, centrifugation can be useful for separating large liposomes from small ones, but more precise separation can be difficult.

Despite this, separation via centrifugation seems to be the most common laboratory technique. Hashimoto *et al.* (1999) diluted the liposomal solution with phosphate-buffered saline (PBS), and then removed the unentrapped glucose by centrifuging at 130,000 *g* for 5 minutes at 20 ºC. The liposomes in the sediment were then resuspended in PSB. Similarly, Banville *et al.* (2000) recovered the liposome suspension by ultracentrifugation at 100,000 *g* for 1 hour at 20 ºC, and Vemuri *et al.* (1990) used 100,000 rpm for 30 min at 4 ºC. New (1990c) prepared the liposome suspension in 50 mM KCl or 100 mM NaCl, which was then centrifuged at 100 000 *g* for 20 min at 20°C to sediment large particles and large MLVs, and again at 159, 000 *g* for 3-4 hours. New (1990c) stated that a number of layers would separate out; the top clear layer is reported to be a pure suspension of SUVs with a mean radius of 10.5 nm, while the central opalescent layer contained small MLVs.

The use of centrifugation on a commercial scale is expensive. The purchasing of equipment, ensuring all safety standards for the equipment are met, and the on-going cost of electricity to run the centifuges mean that alternative methods of separation are likely to be required. These may include ultrafiltration or size exclusion chromatography techniques.

Dialysis involves the use of a semi-permeable membrane, through which the aqueous phase and small dissolved molecules can move while leaving the liposomes behind. This is a very gentle separation method and is unlikely to cause any disruption or damage to the liposomes, but it does take much longer than some of the other techniques. The time factor may be important when trying to determine the entrapment efficiencies of small molecules, which may diffuse through the membrane and be removed along with the unentrapped material. Valenti *et al.* (2001) purified liposome suspensions by exhaustive dialysis against water using a cellulose membrane. This separated loaded liposomes from the unincorporated essential oil, and turbidity was measured using a spectrophotometer at 500nm as an indication of reproducibility.

Column chromatography is a common method for the analysis of liposome size. Preparative-scale chromatography can be used to separate a liposome population into groups of similar sizes. However, these methods are often limited by throughput volume and can only be carried out in a batch fashion (Martin, 1990).

### *2.7.4 Entrapment efficiencies*

Entrapment efficiencies are usually expressed as the percentage of the initial amount of active added that is either incorporated inside the liposome membrane or entrapped in the aqueous spaces inside the liposome. Methods for this vary between actives, but the basic principle usually involves purifying the liposomes to remove any unentrapped material, disruption of liposomes, and then an assay to measure the level of the released actives. The difficulty comes in making this a quantitative result.

Valenti *et al.* (2001) encapsulated an essential oil, purified the liposomes using gel chromatography, disrupted the purified vesicles with methanol, and determined the concentration of essential oil using a spectrophotometer at 307nm. Standard solutions of oil in methanol were used to construct a calibration curve.

Rodriguez *et al.* (2000) used thin-layer chromatography (TLC) to determine the ratio of phospholipid/lipophilic compounds in liposomes. This was achieved through a comparison of the relative spot intensities of dipalmitoylphosphatidylcholine and the lipophilic compound, and is reported to be a simple and reproducible way to quantify the level of lipophilic compound incorporated in the liposome bilayer.

### *2.7.5 Release of entrapped material*

The release of hydrophilic entrapped material from liposomes is usually either by diffusion or by disruption of the membrane (Jackson and Lee, 1991). In general, hydrophobic molecules will not move into the external aqueous phase if they are insoluble in water, but may be lost if the liposome collides with or is attracted to a hydrophobic entity (e.g. a fat globule) (Reineccius, 1995b). Mechanisms that can be used for planned release include the

increase in permeability at the phase transition temperature, membrane disruption through the activity of an enzyme such as phospholipase, or changes in ionic concentration or pH.

#### 2.7.5.1 Mechanisms of release of entrapped material

- Diffusion – the rate of diffusion is determined by the solubility of a compound in the phospholipid bilayer and the permeability of the bilayer membrane with respect to the active material. These parameters are also affected by the vapour and/or osmotic pressure on each side of the membrane (Pothakamury and Barbosa-Cánovas, 1995; Reineccius, 1995a). In general, lipophilic molecules will not be lost to aqueous phase if they are insoluble in water, but may be lost if the liposome collides with or is attracted to a lipophilic site (i.e. a fat globule) (Reineccius, 1995b).

- Osmotically controlled release – the extraliposomal environment is much more dilute than the interior, creating an osmotic gradient across the membrane. Water is taken up, causing the liposome to swell and the membrane to stretch. Release occurs either through bursting of the liposome, or the creation of gaps in the membrane (Pothakamury and Barbosa-Cánovas, 1995; Reineccius, 1995a).

- Solvent-activated release – with reference to liposomes, this is a combination of diffusion and osmotically controlled release. For example, when liposomes in a concentrated solution are introduced to a more dilute environment (for example the dilution of a syrup using water to give a drink product, or the dilution of a concentrated solution with saliva in the mouth during consumption), the change in osmotic pressure will cause rapid uptake of solvent. This is described in the above reference to 'osmotically controlled release' (Reineccius, 1995a).

- Disruption through enzyme activity such as phospholipase provides another potential release mechanism (Jackson and Lee, 1991).

- pH-sensitive release – altering the pH of the extraliposomal solution may affect the permeability of the membrane by changing the charges on the entrapped molecules.

- Phase behaviour of liposome – at the phase transition temperature the membrane is at its most permeable. This can be altered through the selection of phospholipids or use

of sterols so that it corresponds with the desired release temperature (Weiner, 1995). Encapsulated actives can be released by adjusting the temperature of their immediate environment to the phase transition temperature using various sources of energy, such as infrared, microwave, or laser light. However, binding of actives to the lipid membranes or inclusion of other components (cholesterol) may shift the $T_c$ or remove any sign of a transition altogether (Lian and Ho, 2001).

- Melting-activated release - the liposome exterior may be coated with a impermeable material that melts at a certain temperature, thus making way for some other release mechanism such as diffusion (Reineccius, 1995a).

- Ionic concentration - $Ca^{2+}$ or $Mg^{2+}$ concentration may affect the charge on the liposomal structure, influencing stability or permeability (Reineccius, 1995a).

#### 2.7.5.2 Techniques for release in the laboratory

Pothakamury and Barbosa-Cánovas (1995) stated that the addition of surfactants (such as Tween 80) would cause the disruption of the membrane and the release of entrapped actives. Release can also be induced by the co-incubation with phospholipase A, which causes enzymatic degradation of the membrane lipids and thus allows leakage of entrapped materials from the bilayer (Zeisig and Cämmerer, 2001). Banville *et al.* (2000) entrapped vitamin D in liposomes and purified them via centrifugation. To release the vitamin D, the pellet was resuspended in 10% (v/v) Triton x-100 and the suspension sonicated for 30 minutes. Valenti *et al.* (2001) used methanol to disrupt purified liposomes containing an essential oil.

### *2.7.6 Percentage release*

To determine the percentage release by the liposome under the desired conditions it may be useful to use a marker rather than the actual compound of interest. The marker needs to have a low permeability through intact membranes, be highly water soluble and have a very low solubility in organic media, which does not associate with membranes in any way so as to destabilize or aggregate them, and can easily be separated from liposomes by conventional

methods. New (1990a) discussed four detection methods and listed some compounds that were suitable for use as markers for each method. A few markers for each technique are shown in Table 2-10.

**Table 2-10 Markers for determining percentage release from liposomes.**

| Detection method | Marker |
|---|---|
| Optical density | Sodium chromate<br>Ponceau red<br>Arnsenazo III<br>Haemoglobin |
| Fluorescence | Fluorescein<br>Carboxyfluorescein<br>Calcein |
| Enzymatic | Glucose<br>Isocitrate<br>Soybean trypsin inhibitor |
| Radiolabel | [$^{14}$C]glucose<br>[$^{14}$C]inulin<br>[$^{3}$H]DNA |

From New (1990a)

## *2.8 Sterilization techniques for liposome dispersions*

Regardless of the application, the presence of unwanted microorganisms in liposome dispersions can create problems both in terms of food safety and consumer acceptance. For products with a short shelf-life pasteurization may be sufficient, but the sterilization of the dispersion may be a more desirable option.

Zuidam *et al.* (1993) described a number of techniques which could be used for sterilization of liposome preparations. These included filtration through a 0.22 μm membrane, exposure of freeze-dried liposomes to chemical sterilising agents such as ethylene oxide, and autoclaving (121 °C for 15 minutes). Autoclaving of liposomes of different compositions and at different pH values did not appear to affect pH, size, and extent of phospholipid oxidation. However, it was noted that the autoclaving resulted in significant leakage of entrapped hydrophilic material, regardless of liposome composition or pH. Hydrophobic material remained in the membrane but there was evidence of degradation of sensitive

compounds. Tardi *et al.* (2001) produced a highly concentrated semi-solid liposome dispersion (vesicular phospholipid gel or VPG), and observed that it showed significant change in morphological and functional properties during autoclaving. However, the gel maintained its vesicular structure, sustained release, and ability to form a liposome suspension upon dilution.

Arnaud (1995a) reported that microbiological stability of selected liposome suspensions used in food applications may be attained either by heat sterilization or by formulating the product with a high sugar content. Neither of these techniques appeared to have any negative effect on liposome stability. Lichtenberg and Barenholz (1988) mentioned that irradiation may also be used for liposome sterilization, but it is a destructive technique and may affect the chemical stability of the liposomes.

## *2.9 Commercial uses of liposomes*

### *2.9.1 Pharmaceutical industry*

Liposomes have been widely studied as drug delivery systems, and since the 1970s there have been many hundreds of drugs successfully incorporated into both phases of liposomes of different sizes and compositions by a wide range of methods (Frezard, 1999). Their popularity is due to their ability to reduce toxicity, their capacity to entrap virtually any molecule regardless of its structure, and the ability to manipulate size, composition and bilayer fluidity to give desired characteristics. At present the majority of liposome use in the pharmaceutical industry is to reduce toxicity and increase accumulation at the target site(s). Enhanced safety and heightened efficacy have been achieved for a wide range of drug classes, including antitumor agents, antivirals, antifungals, antimicrobials, vaccines, and gene therapeutics (Lian and Ho, 2001).

Herslof (2000) reported that products based on liposome technology represent total sales of approximately US$400 million per annum. Companies active in manufacturing liposome-based products include Ciba-Geigy, Upjohn, Beckton Dickinson, Squibb, Liposome Technology, Vestar, and The Liposome Company (Watwe and Bellare, 1995). Lian and Ho (2001) presented a table of liposome and lipid-based pharmaceutical products currently for sale and on trial in the USA. Products on sale include three based on encapsulated

Amphotericin B, two of encapsulated Doxorubicin and one of Daunorubicin citrate. Most contain at least 30% cholesterol.

### 2.9.2 Food industry

There has been only limited development of liposome technology in the food industry compared with that seen in the pharmaceutical and cosmetics industries. Despite this, Arnaud (1995b) stated that it was the agrifood industry that had the largest number of potential applications. Zeisig and Cämmerer (2001), Arnaud (1995b) and Reineccius (1995b) reported that the limited development to date had not been due to a lack of potential applications, but to difficulties in finding safe, low-cost ingredients and low-cost processing methods suitable for producing large volumes of liposomes with consistent characteristics. The recent development of Microfluidization and pro-liposome techniques offers possible solutions to many of the processing problems, and current research into the use of cheaper commercial lecithin fractions may lead to suitably low-cost ingredients.

Potential applications of liposomes within the food industry are briefly discussed below. There is relatively little published information on the use of liposomes in foods; thus it is difficult at this stage to provide a critical analysis of the likely success of proposed applications. The lack of information may be due partly to a low level to date of commercial application (because of a lack of appropriate methods and ingredients for large-scale production) and partly to the commercial sensitivity within the food and nutraceutical industries, where many new developments are commonly kept in-house. Published research has been carried out only in the laboratory or pilot plant; there are no publications describing the commercial production of a product that this author is aware of. Lasic (1998) reported that Biozone Labs in the USA were producing liposome entrapped vitamins, minerals and herb extracts, and a brief internet search revealed a number of similar products from different companies. However, none of the sites concerned identified specific research publications supporting their claims of increased bioavailability and efficacy.

#### 2.9.2.1 Antioxidants

The entrapment of antioxidant systems is commonly cited as an example of the potential benefits of liposome technology. The move towards the replacement of saturated fat with unsaturated fat in the diet has increased the susceptibility of many fat-containing foods to oxidation, especially in emulsion-based food products such as spreads, margarines and mayonnaise. Fat-soluble antioxidants can be used to counteract this, the most effective being synthetic antioxidants. However, many of these synthetic alternatives are no longer permitted for food use in a number of countries (Kirby, 1991).

Ascorbic acid and α-tocopherol can act in a synergistic fashion as (natural) antioxidants. The α-tocopherol reacts with peroxy radicals in the continuous phase of the food to form α-tocopheroxyl radicals, which are less effective than peroxy radicals in oxidation chain reaction initiation (Stahl *et al.*, 1998). The α-tocopheroxyl radical can be reduced to α-tocopherol by ascorbic acid. This regeneration extends the antioxidant effect of the α-tocopherol. However, α-tocopherol is hydrophobic and therefore cannot interact with the water-soluble ascorbic acid. It is possible to use lipid-soluble derivatives of ascorbic acid, but effective dispersion requires high temperatures, increasing the likelihood of oxidation problems in the food system. An alternative may be to use a liposome system.

Arnaud (1995b) reported that liposome-entrapped α-tocopherol had been shown to be more effective at preventing oxidation in oil-in-water emulsions than when the free form was dissolved in the oil. Oxidation occurs first at the water-oil interface. If the liposome is situated at this interface, the α-tocopherol in the membrane could reduce the peroxy radicals before the radicals initiate oxidation (Figure 2-10). Ascorbic acid entrapped in the aqueous regions of the liposome could regenerate the α-tocopherol. Liposome entrapment of the ascorbic acid would minimise the degradation of the ascorbic acid by other food components and ensure maximum α-tocopherol regeneration.

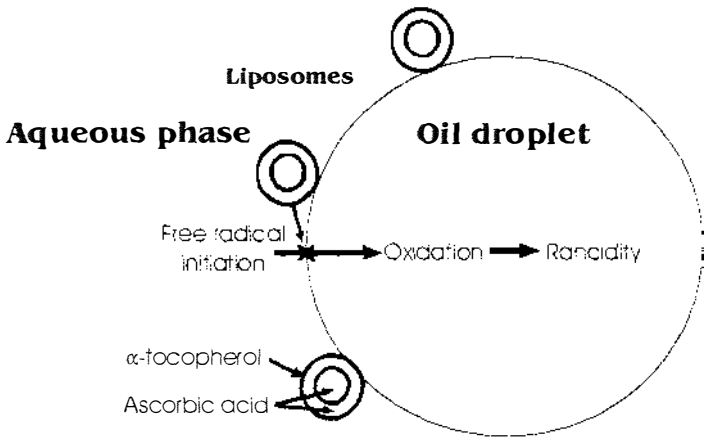



**Figure 2-10. Protection of a food emulsion by antioxidants entrapped in liposomes. Based on Kirby (1991).**

### 2.9.2.2 Isolation of components

The ability of liposomes to prevent the interaction of entrapped materials with the outside environment is the basis of numerous potential applications. Kirby (1991) gave a list of application possibilities, including the protection of hygroscopic materials from moisture and the protection of ingredients that are volatile or sensitive to heat, light or oxidation. In many food systems, a larger than functionally-necessary amount of an active compound is included in the formulation to compensate for losses during processing or storage. The reduction in normal degradation through the use of liposomes may avoid the need for this, saving money and limiting potential toxicity problems. Normally immiscible phases may be blended, and incompatible materials safely used together. Compounds with useful functional or nutritional attributes that have unpleasant odours or flavours may be confined and thus prevented from interacting with the olfactory system.

Arnaud (1995c) cited some unpublished results involving the mineral fortification of dairy products for nutritional purposes. The heat treatment of milk with added free magnesium caused protein coagulation within a few minutes, whereas the entrapment of the magnesium prior to addition resulted in no destabilisation at all. Iron sulphate causes rapid colour and flavour changes when added directly to products, but yoghurt containing iron sulphate entrapped in liposomes showed no colour change after 2 weeks.

Westhaus and Messersmith (2001) entrapped $CaCl_2$ inside liposomes with a $T_c$ of 37 °C. The liposomes were dispersed in a liquid sodium alginate solution. No change in viscosity

was noted for several days at room temperature, but the liquid gelled rapidly upon heating to 37 °C. The authors discussed the relevance of this to medical applications, but it may also be useful in providing a new method for controlling the gelation of food systems containing alginate.

Ascorbic acid has been shown to be more stable at higher concentrations. By entrapping the vitamin in liposomes, it could be held at a much more concentrated level and therefore could have a longer shelf life (Reineccius, 1995b). Using a model system, Kirby *et al.* (1991) found that 50% of liposome-entrapped ascorbic acid remained after storage for 50 days at 4 °C, whereas the free form had disappeared after 20 days. Stability in the presence of degradative substances, such as copper, ascorbic acid oxidase and lysine, was also improved.

#### 2.9.2.3 Enzymes

Enzymes are often inactivated by the conditions existing within a food system. Liposome entrapment isolates the enzyme from the surrounding food environment, enabling it to retain its activity under conditions that would otherwise impede performance or even cause denaturation. It is possible to entrap an enzyme at an optimum pH for stability or function and then change the external pH to a value that is more desirable for the food product. Enzymes are more stable in concentrated solutions than when diluted in a bulk food phase. Kirby (1991) suggested that stabilising materials could be entrapped with the enzyme to increase the protective effect, an example being thermostabilisers such as sugars that protect against high temperatures.

As well as protecting the enzymes from denaturation, liposomes can be used as a means of controlled release. This allows an enzyme to be added to a food system much earlier than when its action is required, without any of the negative effects that would be caused by the early addition of free enzyme. As long as the enzyme is within the liposome structure, it will not be able to interact with the substrate, remaining inert and inactive within the food matrix. By manipulating the composition of the liposome membrane, it is possible to control when the enzyme is released, and the rate of release.

An example of this application is the entrapment of proteolytic enzymes in liposomes in cheese production. Zeisig and Cämmerer (2001) have recently reviewed the progress in this

area. Encapsulation of the proteolytic enzymes have been shown to significantly reduce the normal maturation time (Law and Wigmore, 1983a,b). Addition of free enzymes to the milk causes premature proteolysis, resulting in poor curd structure and low yields. A large proportion of the enzyme is lost in the whey stream, increasing product cost through the requirement for a high initial enzyme concentration and limiting downstream whey processing options. Addition of the enzyme directly to curd results in poor enzyme distribution. Liposome entrapment of the enzymes can produce a cheese with good texture and flavour characteristics in half the normal time, with the overall enzyme requirement reduced by 100-fold (Kirby *et al*, 1987). Liposome-entrapped enzymes are not currently used in commercial production; Skeie (1994) stated that this was due to the high cost of the phospholipids needed and lack of appropriate preparation methods.

#### 2.9.2.4 Flavour and aroma

The entrapment of flavours is a major area of research on liposome applications in food systems. At present, this application is limited by cost, although the relatively high cost of liposomes is offset somewhat by a reduction in the quantity of active ingredients required (Best, 2000). Costs should decrease as the technology becomes more widespread and improves in efficiency. It should also be possible for the food manufacturer to demand a premium if products with entrapped flavour offer significant advantages to the consumer.

Liposome-entrapped water-soluble flavours remain entrapped in aqueous food systems prior to consumption (Kirby, 1991), whereas most other entrapment methods involve entities that dissolve in water. Liposomes also allow oil-soluble flavours to be suspended in aqueous media. Manipulation of the liposome bilayer composition to adjust the phase transition temperature allows flavours and aromas to remain entrapped and protected against degradation during storage, but to be released in the mouth. Alternatively, the consumer can instigate liposome rupture and flavour release immediately before consumption by re-heating the product (Arnaud, 1995c).

Van Nieuwenhuyzen and Szuhaj (1998) commented that volatile components are often lost when food is microwaved, and that the presence of lipid in the form of phospholipid helps produce a more desirable flavour-release profile in reduced-fat foods. The entrapment in

liposomes of volatile flavours thus has potential in low-fat and/or microwavable food products.

Studies have also looked at the use of entrapped flavours in cheese. The liposomes are added directly to milk during the cheese making process, and become trapped in the matrix during protein coagulation. Consumer testing found that lower levels of the entrapped flavouring agents were required to achieve the desired taste or aroma (Arnaud, 1995b).

In an interview in 2000 (Best, 2000), Dr Charlie Brain claimed to be able to "manipulate the lecithin structure in order to determine how a flavour or neutraceutical trapped inside the liposome will adhere to the mouth, throat, or GI tract". The liposomes could then slowly release their entrapped flavours, resulting in a lower requirement for flavour concentration within a product. He also stated that for some applications (an example being marinated meats), liposome systems could be used to help flavours integrate themselves into animal cells. No scientific data was presented to support these claims, and no further information has been published to date.

#### 2.9.2.5 Preservatives

During the last few decades, there has been a general trend towards reductions in the permitted levels of many food additives, and, where possible, the replacement of synthetic substances with alternatives that are perceived to be of natural origin (Kirby, 1991). However, many of these natural alternatives are not as effective as the additives they replace, are more expensive, and are often more restricted in their application. The use of liposomes potentially can overcome these disadvantages.

For instance, it has been shown that liposomes and micro-organisms accumulate in the same micro-compartments in the cheese matrix during cheese ripening (Kirby, 1991). This raises the possibility that liposomes could be used to deliver antimicrobial agents (preservatives) directly to the sites at which micro-organisms are present in foods. Such targeting would significantly reduce the overall concentration of antimicrobial agents required, and might permit the use of natural agents.

An example of this application is in the preservation of washed curd cheeses such as Edam, Emmental, and Gouda. These cheeses tend to be highly susceptible to spoilage by spore-

forming bacteria. Such spoilage can be controlled by adding nitrate to the milk during processing, but there are increasing health concerns about the use of nitrate in foods. Lysozyme, an enzyme derived from egg white, has been promoted as a replacement for nitrate in this situation. However, it binds to the casein in the milk, reducing its potency and rendering it ineffective at high spore counts. Liposome entrapment would prevent this binding, and would target the regions in the cheese matrix where the bacteria accumulate (Figure 2-11). Another potential preservative is nisin, an antibiotic produced by lactic acid bacteria. Addition of an antibiotic directly to the cheese curd would kill the starter culture that is required during the early stages of cheese production. Entrapment of the nisin in a liposome, with delayed release, would protect the starter culture during the initial stages, but later allow the nisin to act on the unwanted bacteria (Kirby, 1991).

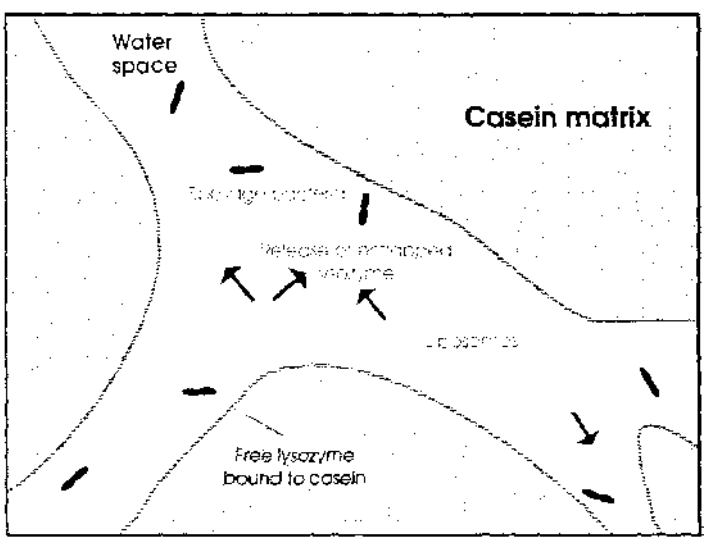



**Figure 2-11 Targeting of liposome-encapsulated lysozyme to spoilage organisms in cheese. Based on Kirby (1991).**

### 2.9.2.6 Water retention

It has been suggested by Kim and Baianu (1991) that by encapsulating an aqueous phase in liposomes in a food system it will be possible to reduce the water activity of the system, without changing the overall moisture content. If much water could be contained in liposomes, the shelf life of the product could be extended by the resulting shift of the water activity of the product matrix into a range that was less favourable for the growth of spoilage organisms. At the same time, textural characteristics could be maintained by the retention of

moisture in the product for longer periods. Unfortunately, Kim and Baianu (1991) show no data supporting their claim. Krotz (1995) mentioned the use of liposomes to entrap water, with specific reference to their ability to reduce the size of the ice crystals formed during freezing. Frozen croissants containing liposomes had significantly better aroma, flavour, impression of freshness, and bite/chew properties upon reheating when compared with standard products. However, there were no references to relevant published scientific papers to support this claim. Hawker and Ghyczy (1994) stated that liposomes are used in German bakery products to increase their shelf life, but did not give any details of this application.

While this application sounds promising, it is pointed out that PC bilayer membranes offer only minimal resistance to the movement of water (Cullis *et al.*, 1987; Gruner, 1987; New, 1990b). While the use of liposomes to minimise the damage caused by ice crystal formation during freezing would not so readily be affected by the water permeability of the membrane (as the movement of water through the bilayer will be halted by the freezing process), the reported benefits of water entrapment at ambient temperatures would be expected to be heavily dependent on the rate of water transfer through the liposome membrane.

Water transport across the bilayer membrane is directly related to the composition and properties of the membrane. The more densely packed the membrane and the higher the hydrophobicity of its interior, the lower the water permeability. Perkins *et al.* (1993) reported a permeability coefficient of ~3-6 $\times 10^{-4}$ cm/s. Cevc and Seddon (1993) stated that, in general, cholesterol-free membranes at temperatures below their phase transition temperatures would offer the most effective barrier to water transfer. Milon *et al.* (1986) measured the osmotic swelling of liposomes caused by water traveling into the vesicle and found that for their particular bilayer membrane, the water permeability of the liquid crystal-like phase was 100 times that of the gel phase. The addition of 30% cholesterol significantly decreased the permeability above the phase transition temperature, but no results were given for the effect below $T_c$. However, in the latter temperature range, water still traveled rapidly across the membrane. Other compounds have been reported to affect water permeability. Wisniewska and Subczynski (1998) looked at the effect of the incorporation of polar carotenoids on the permeability of membranes to water and reported that there was a significant reduction in permeability upon their addition to the membrane. Ho and Stubbs (1997) found that long-chain *n*-alkanols reduced water permeability through the acyl chain

region of a PC bilayer, but that the presence of short-chain *n*-alkanols increased permeability.

In summary, the effect of bilayer composition on water permeability is a complex one. Permeability can be increased through the addition of appropriate compounds, but whether this increase can sufficiently slow water loss to allow for a functional benefit in food products is not clear.

#### 2.9.2.7 Improved absorption of entrapped materials

Arnaud (1998) stated that in liposomal form, 1 g of phospholipid has an external surface area of about 200 $m^2$, and that the absorption of any molecule linked or entrapped to the liposomes would be improved due to this extremely fine dispersion. He supported this by referring to a study involving rats found that more vitamin E was absorbed in the intestinal tract of the rat in encapsulated than in the free form. Similar results with iron salts were also observed. Unfortunately no details of where these results were obtained were provided.

#### 2.9.2.8 Diet industry applications

Hawker and Ghyczy (1994) reported that the high surface area resulting from the dispersion of phospholipids in the form of liposomes and the even distribution of the fat raised the possibility of using liposomes to reduce the fat required to achieve a certain mouthfeel.

Arnaud (1998) suggested that small liposomes might be able to be used to treat obesity. At the moment this is purely theoretical, but it is based on the idea that the liposome and ingested triglycerides would be attacked by intestinal lipase to the same extent. Because of the much larger surface provided by the small liposomes compared to the relatively small one of big oil droplets, most of the triglycerides would not be hydrolysed, reducing the total fat absorption.

## 2.10 Concluding remarks

The commercial purification of MFGM phospholipids from buttermilk has provided a new highly functional and valuable ingredient from a waste stream. The production of liposomes from phospholipids extracted from buttermilk could further increase the applications and advantages of this product.

Liposomes designed for pharmaceutical and cosmetic applications have been well characterised. However, while there is a large amount of information on liposome production and behaviour, almost all of this is based on highly purified phospholipids, non-food-safe production methods and techniques which are highly resource-intensive. The heterogeneous nature of the commercial buttermilk fractions is likely to complicate analysis of the liposomes, as well as affect the overall behaviour of the dispersions. The removal of unentrapped material in a suitable manner may be particularly difficult, and the inclusion of hydrophobic material without the use of solvent may also prove a complicated task. However, there are a wide variety of potential applications for liposomes in the food industry, and the unique composition of the dairy phospholipid fractions may offer advantages over other phospholipid sources.

The production of liposomes from commercial MFGM phospholipid fractions using methods suitable for the food industry will no doubt create a number of challenges. However, if successful, it offers great potential for not only increasing the value of the fractions, but for the development of an exciting new area of research into the applications of liposomes in food systems.

# Chapter 3: Selection of phospholipid fractions and method of liposome production method

## *3.1 Introduction*

Fonterra Co-operative Ltd produces five phospholipid fractions at its Complex Lipid Plant in Edgecumbe, New Zealand. At present, no other company manufactures a commercial phospholipid fraction derived from milk, and to date there is no known literature regarding the use of commercial dairy phospholipids for the production of liposomes. In comparison, there is a huge range of different phospholipid products commercially available that are extracted from soy oil, varying widely in purity and composition.

There are a number of techniques that can be used to produce liposomes (section 2.5 in *Chapter 2: Literature Review*), but the three most commonly recommended for large-scale production are Microfluidization, Extrusion and a Proliposome system. The production method is known to be an important factor in determining the size and structure of the liposomes (New, 1990c). Each processing method is generally recognized as producing liposomes with a certain structure, and changing production variables will not result in a change in lamellarity. For example, the standard thin-film preparation procedure is known to produce multilamellar liposomes (Jackson and Lee, 1991; Picon *et al.*, 1994), which may be converted into small unilamellar liposomes through sonication (Chapman, 1984; Jackson and Lee, 1991; Kim and Baianu, 1991) or extrusion (Schnider *et al.*, 1994; Turanek, 1994). In contrast, variables such as the size of liposomes produced by a given method may often be manipulated by controlling certain production variables.

It was necessary to determine which dairy- and soy-derived phospholipids would be the most useful for comparison within the objectives of this thesis, and the most suitable liposome production method. This chapter covers the sourcing of potential phospholipid fractions, the identification of the best technique for liposome preparation, and the process for selecting the three phospholipid fractions which would be used for the remainder of the experimental work. The selected technique was then investigated further to determine the effect of changing production variables on the characteristics of resulting liposome dispersions.

## 3.2 Phospholipids

### 3.2.1 Dairy phospholipids

Samples of five dairy phospholipid fractions were provided by Fonterra Co-Operative Group, New Zealand. The commercial names for the products were Phospholac 500, Phospholac 600, Gangolac 500, Gangolac 600 and Fractolac 500. The cost for the fractions ranged from approximately NZ$50/kg for Phospholac 500 to NZ$400/kg for Phospholac 600.

The dairy phospholipid fractions were all found to be dispersible in water with the aid of a mechanical mixing device, so all five were included in stability trials. The main differences in composition between the fractions are summarised in Table 3-1.

**Table 3-1 Composition of the dairy phospholipid fractions (g/100g powder) as provided by Fonterra Co-operative Ltd (New Zealand).**

| Component | Fractolac 500 | Gangolac 500 | Gangolac 600 | Phospholac 500 | Phospholac 600 |
|---|---|---|---|---|---|
| Total lipid | 10 | 32-36 | 30-40 | ≥ 85 | 80-85 |
| - *Neutral lipid* | * | * | * | ≤ 55 | 2-8 |
| - *Polar lipid* | * | * | * | ≥ 30 | 75-83 |
| - *PC* | 4-6 | * | * | ≥ 12 | 30-40 |
| - *PE* | 1-3 | * | * | ≥ 6.5 | 7-10 |
| - *SM* | 2-5 | * | * | ≥ 7.0 | 18-20 |
| - *PS* | 4-6 | * | * | ≥ 1.5 | 3-5 |
| Moisture | ≤ 5 | ≤ 5 | ≤ 5 | ≤ 5 | ≤ 3 |
| Lactose | ≤ 5 | 55-60 | 50-60 | ≤ 10 | ≤ 10 |
| Ash | ≤ 8 | 3-5 | 8-15 | ≤ 8 | ≤ 12 |

**(* = value not given)**

### 3.2.2 Soy phospholipids

It was initially hoped to use soy lecithin products specifically designed for liposome production in the experimental work. The only such products found were the Epikuron range from Degussa Texturant System, Hamburg Germany. The price of these products ranged from NZ$750-1400/kg, with commercial-sized minimum purchase quantities. This price was outside the range of both the project budget and most food companies.

Samples of other lecithins recommended for emulsification applications in food products were considered, with only those fractions able to be dispersed in water considered for further experiments. The following phospholipid fractions were found to be dispersible in water:

- Precept 8160, a powdered enzyme-modified lecithin from Central Soya Lecithin Group, Indiana USA. Price NZ$15/kg

- Centrophase HR-2B, a medium viscosity liquid lecithin from Central Soya Lecithin Group, Indiana USA. Price NZ$9/kg

- Ultralec P, a powdered lecithin from ADM Lecithin and Monoglycerides, Illinois USA. Price NZ$7/kg

In addition to the commercial phospholipid fractions, a Sigma product P3644 (purified soy phosphatidyl choline min 30%, referred to as SigP3644 throughout this thesis) was used. This cost NZ$1200/kg, but allowed comparisons between the cheap commercial fractions and a more highly purified product.

The suppliers were unable to provide any additional composition details for any of the soy products.

## 3.3 Liposome production

### 3.3.1 Dispersion of phospholipids

The required amount of the specific phospholipid fraction to make a 1% lipid dispersion was simply added to the aqueous phase and blended thoroughly. The phospholipid fractions dispersed in MilliQ water ranged in pH from 5.6 to 6.4. Therefore, to ensure comparisons between the fractions were made at the same pH, the liposome dispersions were made in an imidazole buffer, containing 20mM imidazole, 50mM sodium chloride, and 0.02% sodium azide in Milli Q water, adjusted to pH 7, with 1M hydrochloric acid.

Initially blending was performed using a standard Kenwood Food Processor (5 minutes, medium speed). However, this incorporated a large amount of air, causing cavitation during Microfluidization (section 3.3.2) and damaging the interaction chamber. After replacement of the interaction chamber, dispersions were prepared using a JKA Ultra-Turrax® (JKA, Staufen, Germany). The phospholipids were blended for 3 minutes on medium speed, left overnight to fully hydrate, then mixed for an additional 3 minutes before Microfluidization. This resulted in a significant reduction in air entrapment.

### 3.3.2 Microfluidization

A M-110Y Microfluidizer® (Figure 3-1 and Figure 3-2) was used to provide the necessary shear to overcome the activation energy for liposome formation (Microfluidics International Corp., MA-Newton, USA). Although commercial scale models are available, this model is designed for pilot-plant scale homogenisation and has a theoretical minimum sample size of approximately 60 ml. It is capable of operating at up to ~1,600 bar (23,000 psi) but the maximum pressure provided by the laboratory compressed air supply was 1200 bar (~18,000 psi).

The phospholipid dispersion (prepared as described in section 3.3.1) was poured into the inlet reservoir, and the Microfluidizer® pump started. The dispersion was drawn into a small compartment and pressurised through the action of a large piston air pump. This hydraulic piston amplifies the preset pressure of 5 bar to $\geq$ 1200 bar hydrostatic pressure (determined by the setting of the pressure gauge). The fluid was then pumped under this high pressure

through a F12Y-type interaction chamber made of an aluminium oxide type ceramic and with a flow channel diameter of 75 μm at the narrowest point (shown in Figure 3-3). This separated the liquid into two streams and brought them back together with high force. The liposome suspension then travelled through a cooling coil and exited through the product outlet tube.

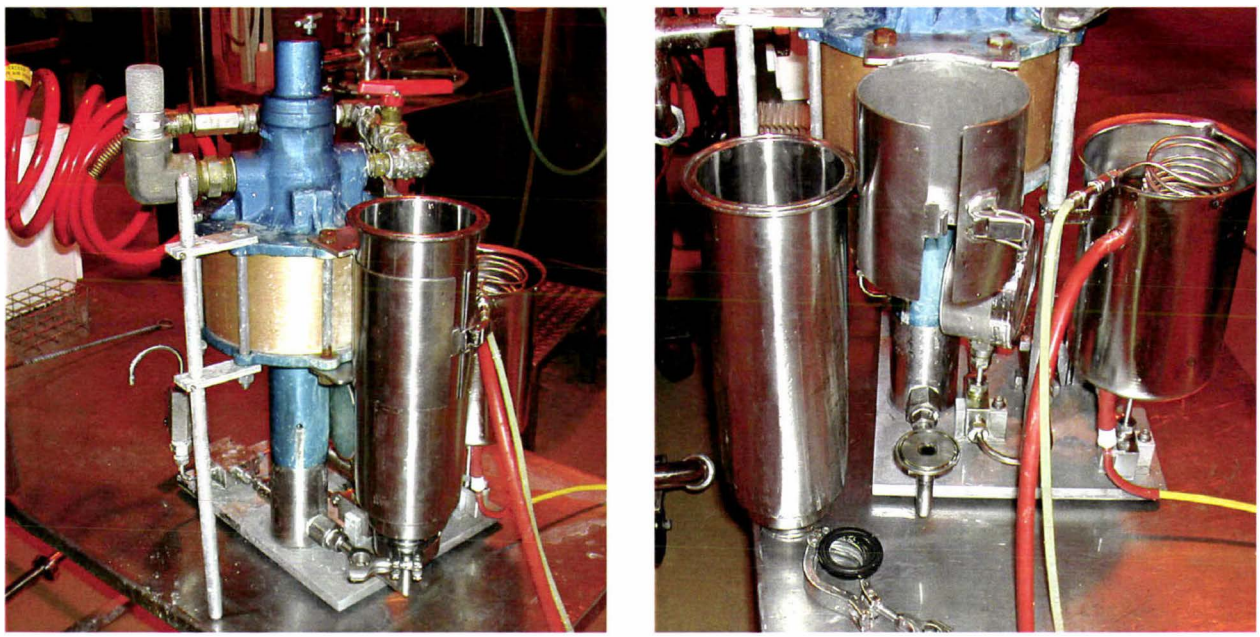


**Figure 3-1 Photographs of Microfluidizer® 110Y.**

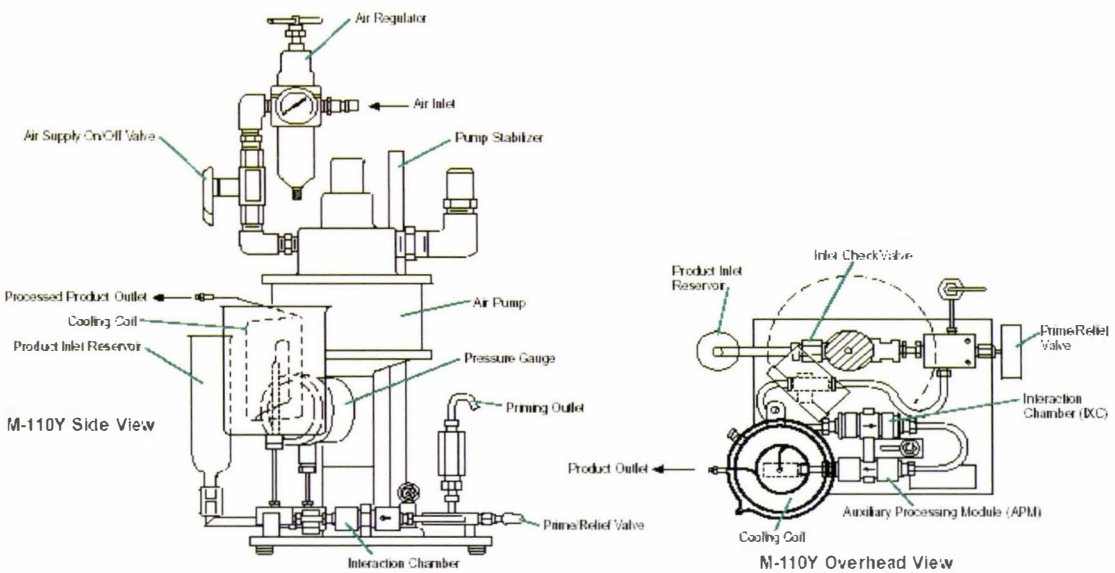



**Figure 3-2 Schematic diagram of the Microfluidizer® 110Y (provided by Microfluidics).**

**Figure 3-3 Schematic cross-section through the dispersion zone of the Microfluidizer®, the Y-interaction chamber (left) and enlarged view of the liquid jet flow (right).**

During this process, the large bilayer sheets are broken up into smaller fragments. To minimise surface energy, the ends wrap around forming bilayer vesicles known as liposomes. The suspension is usually recycled through the Microfluidizer® several times to reduce the average liposome size (Vemuri, 1990; Barnadas-Rodriguez and Sabés, 2001).

Although some literature claims that only unilamellar liposomes are produced by Microfluidizer® (Jackson and Lee, 1991), other sources state that multilamellar liposomes may also be produced (New, 1990c; Arnaud, 1995; Chen *et al.*, 2001a). This is most likely to occur when a smaller vesicle(s) become trapped inside a larger one.

Initially, as the phospholipid was dispersed the viscosity of the suspension increased significantly, particularly at phospholipid concentrations above 15% (w/w). The observed increase in viscosity is most likely due to the interaction of the large bilayer sheets formed upon hydration of phospholipids (section 2.3.1 in *Chapter 2: Literature review*). After Microfluidization, the viscosity of the liposome suspension was similar to the viscosity of water, much lower than that of the unprocessed dispersion. This is presumably because there are significantly fewer interactions between the liposomes than between the large bilayer sheets.

The ceramic Y-75µm chamber used for the preliminary work was replaced due to an increase in flowrate through the chamber and a corresponding drop in system pressure. This was caused by cavitation of air in the chamber, entering both through the holding tank inlet and through fittings and joins that vibrated loose during processing. A second ceramic chamber was purchased, with Loctite used on all fittings to help prevent air from being sucked in through joins and the holding tank was re-mounted to isolate it from vibration. As mentioned in section 3.3.1, an Ultra Turex was used to prepare subsequent phospholipid

dispersions to reduce the incorporation of air, and dispersions with obvious foaming were de-aerated under vacuum.

For more information on the use of a Microfluidizer® in the production of liposomes, see section 2.5.2 in *Chapter 2: Literature review*.

### *3.3.3 Extruder*

A small extruder (Figure 3-4) was manufactured in the Institute of Food, Nutrition, and Human Health engineering workshop, Massey University (Palmerston North, New Zealand). It was based on a simple barrel and plunger design, and used Nuclepore 0.1 μm and 0.2 μm filters purchased from Northern Lipids (Vancouver, Canada).

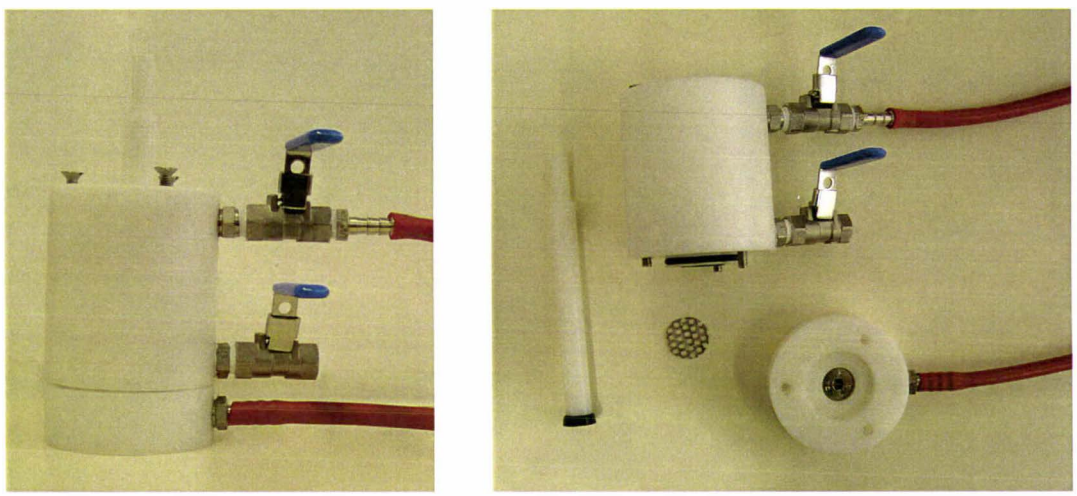


**Figure 3-4 Photographs of the small acetal plastic extruder produced in the Institute of Food, Nutrition, and Human Health engineering workshop.**

The casing of the extruder was made from food quality plastic (acetal). The detachable base held a fine mesh to support the delicate filters. Liquid that had passed through the filter was collected via an outlet in the base. The plunger was operated by an Instrom 4502 Texture Analyser so that it could be raised and lowered in a controlled and reproducible manner. The phospholipid solution (prepared as outlined in section 3.3.1) would be forced through the filters by the plunger. The turbulence and energy introduced to the system by the interaction between fluid and the walls of the pores should allow the formation of liposomes (see section 2.5.1 in *Chapter 2: Literature review* for more detail). One manufacturer of extruders specifically for liposome production (Northern Lipids) recommends that each

solution be passed through the extruder ten times to reduce the average particle size and produce a narrow size distribution.

The production of liposomes by this extruder was extremely time consuming. It was only possible to produce very small volumes of filtrate with each pass, and the filters tore easily when the Instron was set at speeds above 2ml/min. The filters became blocked after 4-6 passes and had to be replaced.

### *3.3.4 Proliposome technique*

The Proliposome technique is touted as being extremely quick, simple, and capable of achieving very high entrapment efficiencies of both hydrophilic and hydrophobic materials. The technique, which is covered in more detail in section 2.5.3 in *Chapter 2: Literature review*, is based on the simple addition of water to a carefully chosen blend of ingredients leading to the spontaneous formation of liposomes.

It is possible to buy pre-made proliposome mixes from Lucas Meyer. However, they only produce the proliposome mixes from synthetic or soy-derived phospholipids and not from any dairy fractions. In order to provide a valid comparison between the dairy and soy phospholipid fractions, it is essential that the liposome preparation method used be consistent.

EP patent No. 0158441 details a method for the production of a proliposome mix from suitable membrane lipids (natural or synthetic lecithins, purified or heterogeneous mix) and a water-miscible organic liquid which is a solvent for the lipid.

Example 1 in the patent was followed using various commercial phospholipid fractions as described in section 3.2. The method involved dissolving 500mg lecithin and 100mg SPAN in 400mg ethyl alcohol at 50 °C, then adding 100mg water and the mixture held for 15 minutes before cooling to 25 °C. Once cool, the sample was vigourously hand-shaken while 4ml of a 50mM phosphate buffer was added in a dropwise fashion, and for 1 minute thereafter. The sample was allowed to equilibriate for 30 minutes at 25 °C, with 1 minute of further shaking after 15 and 30 minutes. A further 6ml of buffer was added and the sample shaken for 1 minute every 15 minutes for 30 minutes.

Unfortunately this process did not seem to work as described. Particle size distributions obtained using PCS showed average particle diameters of close to 1µm with poly dispersity values of close to 1.0. This suggests that there was not a complete transformation into liposomes, with many large particles remaining. Different solvents and solvent:phospholipid ratios, as well as using magnetic stirring on high speed were trialled, but none of the combinations used produced suitable liposome populations.

### *3.3.5 Conclusions*

The Microfluidizer® was effective and easy to use, and produced large quantities of liposomes in a reproducible manner without requiring the addition of solvents or the ongoing purchase of components. Producing liposomes using the extruder may have been possible, but would have been extremely time-consuming and the high turnover of the filters would have made the process very expensive. Although a commercial extruder would have obviously performed much better, it would have required a large capital outlay as well as the ongoing purchase of filters.

Based on this information, it was decided to use the Microfluidizer® for all liposome preparations described in this thesis.

## *3.4 Selection of phospholipid fractions*

### *3.4.1 Methods*

The standard processing method outlined in section 3.3.2 was used to produce a 1% lipid dispersion of liposomes from each of the phospholipid fractions listed in section 3.2.

The average hydrodynamic diameter of the liposome dispersions was measured using a photon correlation spectrometer (PCS), the Zetasizer 4 (Malvern Instruments Ltd, UK). The measuring specifications used were as follows:

Sampling time: 99 seconds

Measurements per sample: 3

| | |
|---|---|
| Medium viscosity: | 1.054 cP |
| Medium RI: | 1.34 |
| Typical Liposome RI: | 1.45 (Blessing *et al.*, 1998; Ardhammer *et al.*, 2002) |
| Scattering angle: | 90° |
| Temperature: | 25 °C |

Samples of the liposome dispersions were diluted to the required turbidity (<250 KCps) using imidazole buffer. Preliminary experiments had found no effect of concentration on measured diameter for any of the three phospholipid dispersions at turbidities of up to 500 KCps.

An indication of the stability of the dispersions was obtained by storing the liposome dispersions at 4 or 20 °C, and monitoring changes in average particle size over time. The number of phospholipid fractions needed to be reduced to allow for more in-depth study of a smaller number of samples, so samples showing a lack of stability would be excluded from further experiments. The amount of phospholipid, triglycerides and other components present in each fraction was also considered.

### *3.4.2 Results and discussion*

The average hydrodynamic diameters of liposomes prepared from the different fractions are shown in Table 3-2. Liposomes produced from Phospholac 500 and Precept 8160 averaged approximately 260 nm in diameter, significantly larger than those produced from the other fractions. The average liposome diameter for Fractolac and Precept samples more than doubled after storage for 60 days at 20 °C, while the other samples appeared relatively stable. Phospholac 600, SigP3644, and Ultralec P all produced liposome populations with average diameters of less than 100 nm.

Fractolac and Precept 8160 were removed from the trial due to their lack of stability when stored at 20 °C for 60 days. Although Phospholac 500 did not demonstrate any significant increase in liposome size during storage, the large initial particle size may be an indication that the neutral fat present (approximately 55%) is forming a fine emulsion in addition to

liposomes during Microfluidization. Centrophase HR was also found to have a high level of neutral fat. The liposome dispersions made using Phospholac 500 or Centrophase HR were opaque and milky, consistent with the formation of an emulsion system. Gangolac 500 and 600 had an initial average liposome diameter similar to Centrophase HR, but the suspensions were relatively translucent and had no evidence of an emulsion system. However, their low lipid content meant that much larger quantities were required to obtain a given lipid concentration compared with Phospholac 600. It is also possible that the large percentage of non-lipid components present could interfere with or complicate analytical and characterisation techniques.

**Table 3-2 Average hydrodynamic diameter ($Z_{ave}$) of liposomes prepared from various commercial phospholipid fractions.**

| Phospholipid fraction | Source | Initial $Z_{ave}$ (nm) | $Z_{ave}$ (nm) (60 days, 4 °C) | $Z_{ave}$ (nm) (60 days, 20 °C) |
|---|---|---|---|---|
| Fractolac | Dairy | 151 | 145 | 330 |
| Gangolac 500 | Dairy | 135 | 142 | 136 |
| Gangolac 600 | Dairy | 139 | 143 | 135 |
| Phospholac 500 | Dairy | 258 | 256 | 260 |
| Phospholac 600 | Dairy | 92 | 92 | 93 |
| Centrophase HR | Soy | 136 | 144 | 145 |
| Precept 8160 | Soy | 260 | 252 | 735 |
| SigP3644 | Soy | 79 | 80 | 83 |
| Ultralec P | Soy | 75 | 78 | 79 |

Phospholac 600, SigP3644, and Ultralec P were selected as the three fractions that would be used in all subsequent experiments.

### *3.5 Effect of Microfluidization production variables on liposome characteristics*

The size of the liposomes produced by a Microfluidizer® may be influenced to some degree by the pressure used and the number of times the streams interact (Barnadas-Rodriguez and

Sabés, 2001). Cycling the dispersion through the homogeniser several times is reported to reduce liposome size and narrow the particle size distribution (Jackson and Lee, 1991). Unfortunately, the structure of liposomes produced by Microfluidization has not been studied as comprehensively as some of the more common production methods, and there are conflicting reports of unilamellar (Jackson and Lee, 1991; Chatterjee and Banerjee, 2002) and multilamellar (New, 1990c; Arnaud, 1995b; Chen *et al.*, 2001a) liposomes being produced. Zeisig and Cämmerer (2001) avoid this debate by simply stating that Microfluidized liposomes will have one or more bilayers.

This section describes the effect of pressure, phospholipid concentration, and number of passes through the Microfluidizer® on liposome size. Although it is largely determined by production method, lamellarity is not usually affected by these production variables and is therefore included in *Chapter 5: Physico-chemical characterisation of liposome dispersions.*

### *3.5.1 Methods*

Two factorial experiments were conducted looking at the effects of the number of passes through the interaction chamber and the Microfluidizer® operating pressure or the concentration of the phospholipid dispersion on the size of the liposomes produced.

Phospholipid dispersions of between 1 and 10% phospholipid (w/w) were cycled through the Microfluidizer® up to 10 times at pressures ranging from 700 to 1100 bar (10,000 to 17,000 psi). Liposome size and polydispersity were measured using a Zetasizer 4 (Malvern Instruments Ltd, UK) as outlined in section 3.4.1.

### *3.5.2 Results and discussion*

#### 3.5.2.1 Initial observations

The first pass through the Microfluidizer® had the most significant effect on liposome size, resulting in a decrease in average hydrodynamic diameter from over 500 nm to between 80-150 nm (Figure 3-5). Successive passes continued to reduce the average size, but the changes were quite small. Increasing the number of passes had no significant effect on the

particle size distribution. The type of phospholipid used had a significant influence on liposome diameter. The SigP3644 and Ultralec dispersions appeared similar in size, but liposomes produced from Phospholac had a hydrodynamic diameter that was on average more than 30 nm larger than the liposomes obtained from the other fractions.

Figure 3-6 shows the effect of the operating pressure on the average liposome diameter. To improve the resolution on the graph, the y-axis has been restricted to between 50 and 160 nm, and the measured size for zero passes has not been included.

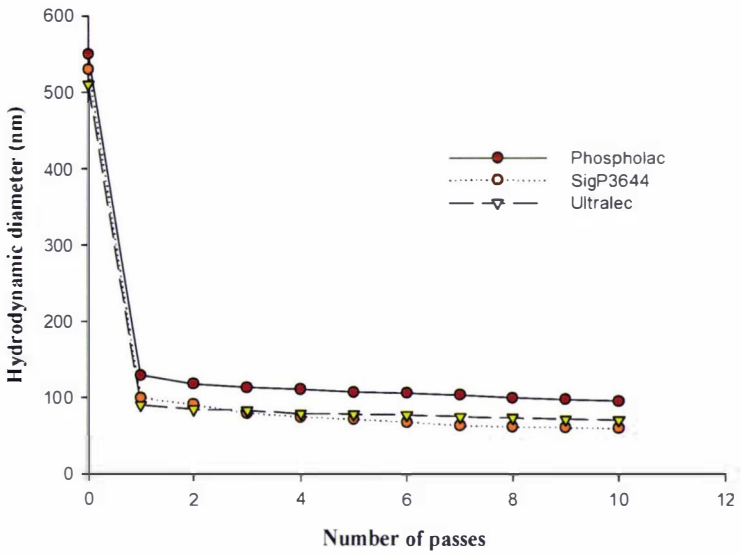



**Figure 3-5 Effect on liposome size of the number of passes through the Microfluidizer® at 1100 bar using a 10% phospholipid solution.**

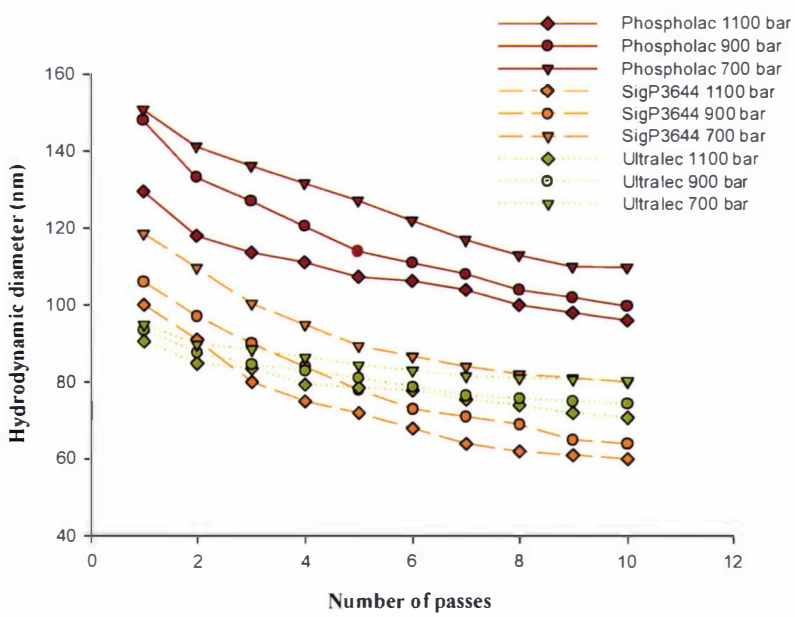



**Figure 3-6 Effect of Microfluidizer® pressure on hydrodynamic diameter for a 10% phospholipid dispersion.**

The trends in the average diameter at the 3 different operating pressures were approximately in parallel. Increasing either the operating pressure or the number of passes decreased the hydrodynamic diameter of the liposomes. It was evident that the effects of the pressure and the number of passes were additive. The Phospholac dispersion had the largest average hydrodynamic diameter for all combinations of pressure and passes. The Ultralec dispersion appeared to have the lowest average liposome size for 3 passes or less, but as the number of passes increased the Ultralec curve flattened while the liposomes produced from SigP3644 continued to decrease in size. After 10 passes there was no difference between Utralec and SigP3644 dispersions manufactured at 1100 bar, but the SigP3644 dispersion had the smallest average diameter at 700 and 900 bar.

Decreasing the phospholipid concentration from 10% to 5% appeared to decrease liposome size for both SigP3644 and Ultralec dispersions, as well as for Phospholac dispersions for 4 or more passes (Figure 3-7). There seemed to be no significant difference between 5% and 1% phospholipid. This suggests that the 10% phospholipid dispersion was less sensitive to the shear and turbulence produced by the microfluidization process. This would be expected as the higher viscosity in more concentrated dispersions could resist deformation and breakup of liposome particles.

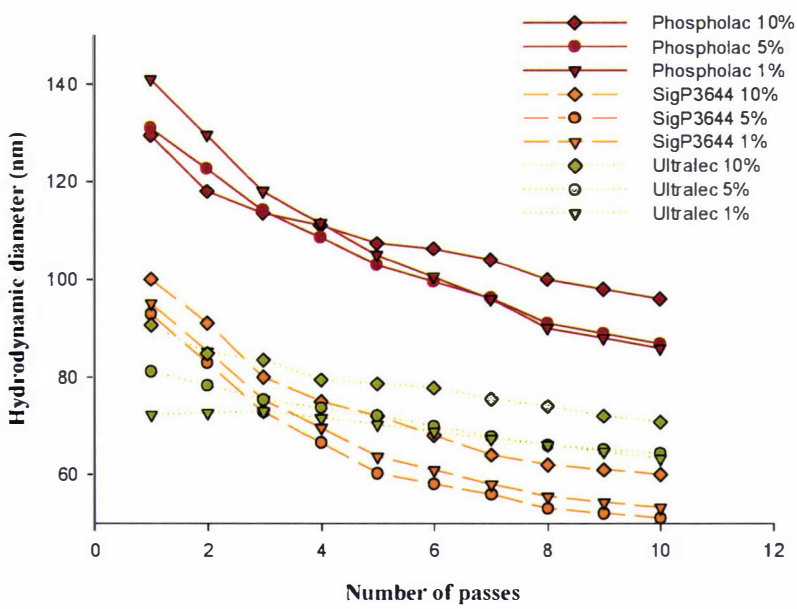



**Figure 3-7 Effect of phospholipid concentration on average hydrodynamic diameter of liposomes formed during Microfluidization at 1100 bar.**

### 3.5.2.2 Statistical modeling

To obtain quantitative information on the statistical significance of these observations, the results for this section were modelled using a general linear model. The model did not include the results for zero passes. To remove the curvature observed in Figure 3-7 and Figure 3-7, the log of the liposome diameters was used.

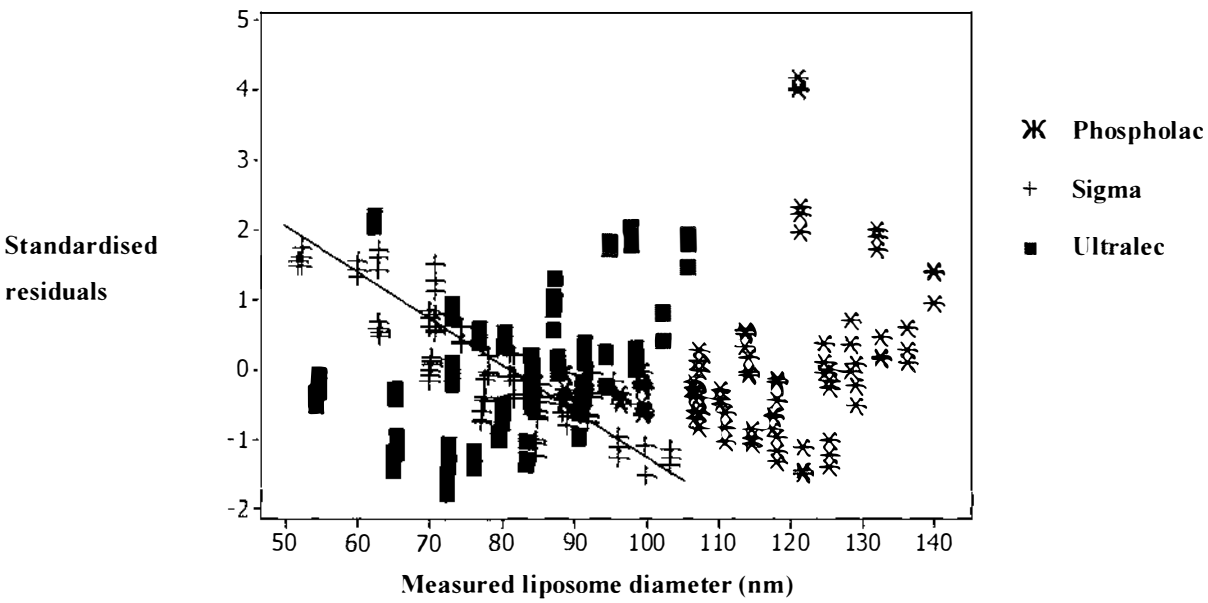



**Figure 3-8 Standardised residual plot for general linear ANOVA without phospholipid × number of passes term.**

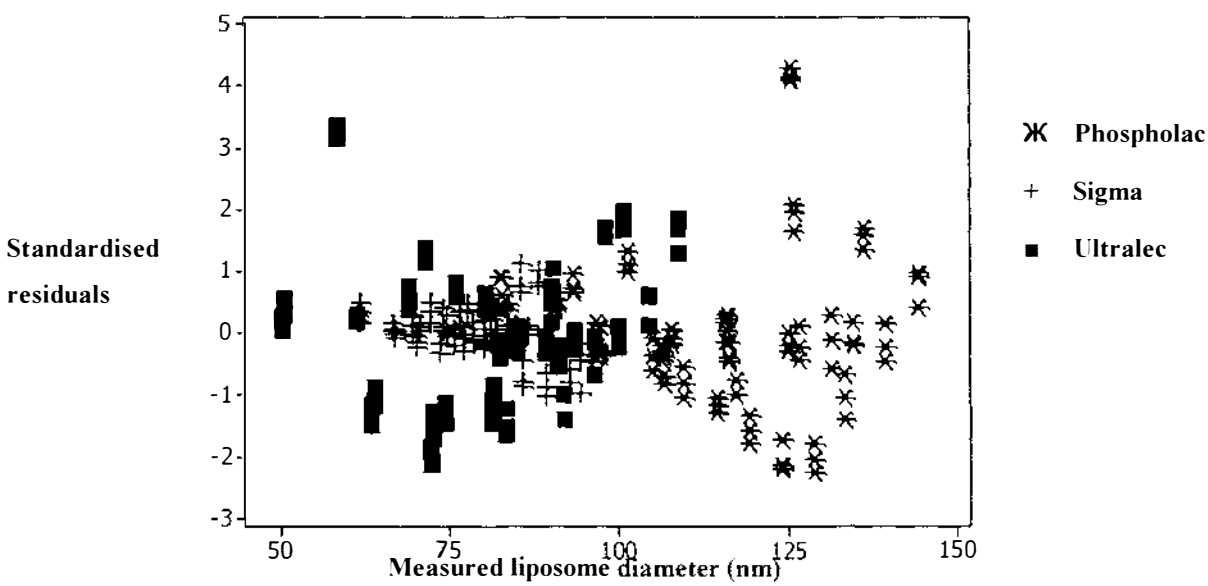



**Figure 3-9 Standardised residual plot for general linear ANOVA with phospholipid × number of passes term.**

As suggested from the previous graphs, the number of passes, pressure, concentration and the phospholipid fraction all had a highly significant effect on average liposome diameter ($p \leq 0.001$), as was the interaction between phospholipid fraction and the number of passes for the SigP3644 fraction. This interaction was not significant for either the Phospholac or Ultralec fractions, but its inclusion removed a linear correlation between residual size and average diameter for the SigP3644 data as shown in Figure 3-8 and Figure 3-9. There are a few outlying residual values, but almost all values are between -2 and 2, indicating a good fit between the model and the data. A residuals-squared value of 95.5% was obtained for this model.

The general equation for the average liposome diameter of a 10% Ultralec dispersion processed at 1100 bar was:

$$ln(\text{average liposome diameter}) = 4.67 - 0.0395 \times \text{number of passes}$$

To allow for variations to the general equation, the values given in Table 3-3 should be added to or subtracted from the constant term. In addition, for a SigP3644 dispersion an extra term of '-0.16 × number of passes' should also be included. For example, a 10% SigP3644 dispersion passed through the Microfluidiser® 6 times at 700 bar is likely to have an average liposome diameter of:

$ln(\text{average liposome diameter}) = 4.67 + 0.078 - (0.0395 \times 6) - (0.016 \times 6)$

$= 4.748 - 0.237 - 0.096$

$= 4.415$

$\text{Average liposome diameter} = \exp(4.415)$

$= 83 \text{ nm}$

This compares well with the measured average diameter under these conditions of 86 nm.

The values in the effect column of Table 3-3 provide useful information on the influence the variable has on the average liposome diameter, both in terms of the relative size of the effect and whether it will increase or decrease liposome size. Dispersions produced from Phospholac will have a significantly higher average size than those from Ultralec, while those from SigP3644 are slightly smaller than Ultralec at low numbers of passes but the

difference between the two fractions increases proportionally with the number of passes. Concentration has a relatively small effect on liposome size, but the lower phospholipid concentrations both have a lower average size than the 10% phospholipid dispersion. There is no significant difference between processing at 1100 bar and 900 bar, but production at 700 bar will result in a larger average liposome diameter.

**Table 3-3 General linear model terms for prediction of average hydrodynamic diameter as a function of phospholipid type, phospholipid concentration and Microfluidizer® pressure.**

| Variable | Value | Effect |
|---|---|---|
| Phospholipid fraction | Phospholac | 0.256 |
| | SigP3644 | -0.048 |
| | | plus additional term: |
| | | -0.016 × number of passes |
| Concentration | 1% dispersion | -0.034 |
| | 5% dispersion | -0.020 |
| Pressure | 700 bar | 0.078 |
| | 900 bar | No significant change |

### 3.5.2.3 Discussion

The observed effect of pressure, passes, and phospholipid concentration fit well with those reported in the literature, but it appears that most experiments using a Microfluidizer® have used much lower pressures than those recommended by Microfluidics and therefore used in these experiments.

Koide and Karel (1987) were among the first to use a Microfluidizer® (model M-110) to produce liposomes, reporting an average liposome diameter of 196 after 10 passes at 140 bar (2,050 psi). Barnadas-Rodríguez and Sabés (2001) used a smaller lab-scale Microfluidizer® (model 110S) to investigate the effect of a number of production variables on liposome size. They reported that mean liposome diameter decreased with increasing pressure (maximum pressure 4 bar, 58 psi) and number of cycles, but that there was no effect of phospholipid concentration. However, Bachmann *et al.* (1993) used a high-pressure homogenizer to produce liposomes and found that although higher homogenization pressures and repeated

recirculation lead to reductions in vesicle diameter and heterogeneity, size reduction was less effective at phospholipid concentrations above 10%. Škalko *et al.* (1998) reported a liposome sizes of 250-300 nm after 3 passes and 99-110 nm after 10 passes through a 110S Microfluidizer®, but do not state the pressure used. Peel (1999) used a high pressure homogenizer to process pre-formed multilamellar vesicles, and found that after 5 passes at 130 bar (1,900 psi) the average diameter stabilised at approximately 125 nm. Brandl *et al.* (1998) used a high pressure homogeniser at pressures of up to 140 MPa (1,400 bar; 20,000 psi), and obtained liposome dispersions with median diameters of < 40 nm.

### *3.6 Effect of processing on phospholipid oxidation*

The peroxide value and level of conjugated dienes present in each of the three fractions was measured before and after Microfluidization to provide an indication of the effect of the processing on phospholipid oxidation. Details of the methods used are given in section 6.2, *Chapter 6: Liposome stability*.

There was no increase in either the peroxide value or conjugated diene level in any of the phospholipid solutions immediately after Microfluidization or after a week stored at 5 °C.

This suggests that the processing conditions used to produce liposomes from the phospholipid dispersions do not promote the oxidation reactions which lead to the formation of peroxides or conjugated dienes. This observation is in agreement with the results of Barnadas-Rodríguez and Sabés (2001), who reported that Microfluidization did not cause any increase in phospholipid oxidation.

### *3.7 Conclusions*

The dairy phospholipid fraction Phospholac 600 and the two soy fractions SigP3644 and Ultralec P were chosen to be used in all further experimental work. These fractions were readily dispersed in an aqueous system and cycled through a Microfluidizer® to produce liposomes. The liposome populations showed minimal changes in particle size during storage at 4 or 20 °C for 60 days.

The particular phospholipid fraction used to produce a liposome dispersion had a significant effect on the average liposome diameter. Phospholac dispersions had a larger average liposome diameter than either SigP3644 or Ultralec dispersion under all conditions examined. As expected, pressure, passes and phospholipid concentration also had significant effects on liposome size. Increasing the pressure or number of passes resulted in a decrease in average liposome diameter, with a larger reduction in liposome size at lower phospholipid concentrations. A basic model allowing the prediction of average liposome diameter for a given set of conditions was developed. This model had an $R^2$ value of 95.5%, and offers an estimation of the likely effect on liposome size of any proposed changes to the processing method.

It was necessary to determine standard processing conditions to be used throughout this work, and to ensure small fluctuations in the variables would not result in significant changes in the average size of the liposome dispersion. However, this had to be balanced with time and equipment constraints. Unless stated otherwise, all subsequent liposome dispersions were produced from 10% phospholipid dispersions being passed through the Microfluidizer® 5 times at 1100 bar (17,000 psi).

There was no evidence of the Microfluidizing process causing any oxidative damage to the system.

# Chapter 4: Characterisation of phospholipid fractions

## *4.1 Introduction*

Liposomes are made primarily of phospholipids, and the phospholipid headgroup and specific fatty acids attached largely determine their behaviour. The analysis of a phospholipid fraction can provide information on the likely characteristics of liposomes produced from the fraction, including expected stability in different conditions (van Nieuwenhuyzen and Szuhaj, 1998).

Liposomes used for delivering drugs must be consistent and well characterised to ensure efficacy and prevent negative side effects. To this end, the phospholipids used in the pharmaceutical industry tend to be highly purified and well defined. The fractions usually only contain a single type of phospholipid, and may have only one specific fatty acid chain present. Phosphatidyl choline is the most common phospholipid used (New, 1990b), although it can be desirable for the liposomes to contain a mixture of phospholipids. Negatively charged phospholipids are often added at 5-20 mol% to increase electrostatic repulsion of the vesicles and thus improve stability (Martin, 1990; Weiner, 1995). The phospholipid blend used in such a situation will often be produced in the laboratory by mixing several purified fractions to ensure an exact and consistent composition. These pure phospholipid fractions are often very expensive, and the use of liposomes produced from such fractions in food applications may not be economically viable unless the end product commands a high price.

In the food industry, the purity (and hence price) of the phospholipid fractions commonly used tends to be much lower. Even the fractions which have been through a number of purifying steps are still relatively heterogenous (van Nieuwenhuyzen and Szuhaj, 1998) and contain several different phospholipid types and a wide range of fatty acid chains.

The different types of phospholipid most often found in commercial phospholipid fractions include phosphatidyl choline (PC), phosphatidyl serine (PS), phosphatidyl ethanolamine (PE), and phosphatidyl inositol (PI). Dairy phospholipid fractions may also contain significant amounts of sphingomyelin (SM). The phospholipid classes present will affect a

number of liposome characteristics, including surface charge, resistance to destabilising influences (i.e. high ionic concentration), and membrane permeability.

The fatty acid profile of phospholipid fractions is dependent on the origin of the fraction (i.e. animal or plant) and the processing steps (i.e. use of hydrogenation). The types of fatty acids affect the packing of the lipid bilayer, influencing phase transition temperature, membrane permeability and stability, and susceptibility to degradation reactions, such as oxidation (New, 1990a).

This chapter presents results on the characterisation of the phospholipid fractions in their original powdered state, including proximate analysis, ionic composition, total phosphorous content, phospholipid class and fatty acid composition.

## *4.2 Materials and methods*

### *4.2.1 Proximate analysis*

A proximate analysis of the phospholipid samples (Phospholac, SigP3644, and Ultralec) was undertaken to provide information on the overall composition of the fractions. Full details of these three phospholipid fractions and the reasons behind their selection are given in *Chapter 3: Selection of phospholipid fractions and method of liposome production.*

#### 4.2.1.1 Moisture content

Moisture was determined by the loss in weight of samples dried at 105 °C for 24 hours in an air oven. The analysis was performed in triplicate.

#### 4.2.1.2 Protein content

The crude protein content of foods is usually derived by multiplying the nitrogen content by an empirical conversion factor. However, the presence of nitrogen in each of the phospholipid molecules means that this approach cannot be used. Therefore, the ratio of nitrogen to phospholipid (both in g/100g phospholipid fraction) was used to provide an indication of the relative amount of protein in the samples. The nitrogen content of the samples was determined by the Kjeldahl method (AOAC official method 991.20). The

samples and blank were digested at 220 °C for 60 min in a Kjeltec 1030 System (Tecator, Sweden). The methods used for determining phospholipid concentration are given in section 4.2.2.

#### 4.2.1.3 Fat content

Fat content was determined by Soxhlet extraction (AOAC official method 963.15). Approximately 0.5g of dried phospholipid fraction was accurately weighed into an extraction thimble, and subjected to intermittent extraction using diethyl ether for 5 hours. Rotary evaporation was used to evaporate the solvent from the extracted fat, and flask containing the extracted fat dried in an air oven until constant weight.

#### 4.2.1.4 Cholesterol content

The amount of cholesterol present in the dairy phospholipid fraction was measured using gel chromatography based on the AOAC standards 933.08, 970.50 and 970.51 (AgriQuality, Auckland, New Zealand). Although there may be some level of plant sterols present in the soy phospholipid fractions, these were not measured as no organization in New Zealand provided analysis of all three of the major sterols found in soy oil (sitosterol, campesterol and stigmasterol). The soy phospholipid fractions will not contain any cholesterol.

#### 4.2.1.5 Ash

The ash content was determined by dry ashing, where the sample is heated in a muffle furnace at 550°C for 5 hours, cooled in a dessicator, and weighed.

The mineral content was determined in more detail using an acid digest and Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) (AgriQuality, Auckland, New Zealand).

### *4.2.2 Phospholipid concentration*

Neutral fat (triglycerides) will not contribute to the formation of liposomes. Therefore, it is important to base experiments on phospholipid concentration rather than total fat.

Phosphorus content was determined by Fonterra Research Centre (Palmerston North, New Zealand) using an acid digest and Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES). An acidic solution of a product was prepared using nitric acid and hydrochloric acid, and a 3-stage digestion performed using a CEM Mars5 Microwave Digestor:

Stage 1 - 300W at 60% power for 3 minutes

Stage 2 - 600W at 60% power for 12 minutes

Stage 3 - 600W at 80% power for 10 minutes

The digested sample was then analysed using Varian vista CCD Simultaneous ICP-OES.

The $^{1}$P-NMR analysis of phospholipids types performed by Spectral Service (section 4.2.3.2) also determined total phosphorus content.

### *4.2.3 Phospholipid type*

Three methods were used to determine the phospholipid types present in the phospholipid fractions - High Performance Liquid Chromatography (HPLC), $^{31}$Phosphorous Nuclear Magnetic Resonance ($^{31}$P-NMR) and Thin Layer Chromatography (TLC).

TLC has been commonly used to determine phospholipid composition for many years. It is relatively simple and avoids the possible problems with UV detection that may be experienced in HPLC. However, it only provides qualitative results unless two-dimensional TLC or Automated Multiple Development TLC with post-derivatisation and a scanner is used. HPLC provides quantitative results and can be performed using relatively common laboratory equipment. To obtain accurate results with either method, it is recommended that a separate standard be used for each source of phospholipids due to the different distribution of fatty acids. These standards are expensive, and only available for relatively common phospholipids. This is a major problem with the use of chromatography methods for identification of dairy phospholipids, as almost all standards are based on phospholipids extracted from soy, egg or animal organs.

NMR exposes samples to radio frequency signals (10-600 MHz) and measures the resonance signal of certain atomic nuclei. The resonance frequency of phosphorus bound to other atoms differs slightly depending on the structure of the particular molecule. This means that each phospholipid type gives a separate signal, even when analysed as part of a mixture. The signal intensity is directly proportional to the number of phosphorus atoms present, allowing quantitative results to be obtained. However, in many samples the formation of vesicles and micelles broadens the phospholipid signals, resulting in overlapping and higher background noise. Spectral Service (Köln, Germany) have developed a reagent which is added to the samples to prevent the formation of phospholipid structures without affecting the $^{31}$P-NMR signal. This increases accuracy and precision of the technique, and this method has been chosen by the ILPS (International Lecithin and Phospholipid Society, a subgroup of the American Oil Chemists Society) as the reference method to calibrate phospholipid standards used in HPLC identification (Diehl, 2001, 2002) . The use of an internal standard allows determination of the absolute amount of each phospholipid without requiring specific standards of the different phospholipid sources.

#### 4.2.3.1 High performance liquid chromatography (HPLC)

The phospholipid fractions were prepared by dissolving 0.1g of dry powder in chloroform:methanol (50:50 v/v), and analysed using a 4.6 mm i.d. x 250 mm PVA-sil column (YMC-Pak, Japan) and an Alltech Varex ELSD MKIII version 2 detector. The ELSD drift tube temperature was set at 80 °C with a gas flow of 2.09 SLPM. A relatively complicated flow regime using three solvent mixtures was used as shown in Table 4-1 and Figure 4-1.

**Table 4-1 Flow regime for HPLC analysis of phospholipid fractions. Solvent A: n-Hexane:TBME (98:2 v/v); B: Iso-propanol:Choloroform:Acetonitrile (84:8:8 v/v/v) with 2.5% glacial Acetic acid; C: Iso-propanol:Water (50:59 v/v) with 2% Tri-ethylamine.**

| Time (mins) | Flow (ml/min) | A (%) | B (%) | C (%) |
|---|---|---|---|---|
| **0** | 1 | 95 | 5 | 0 |
| **10** | 1 | 80 | 20 | 0 |
| **20** | 1 | 44 | 52 | 4 |
| **50** | 1.4 | 34 | 52 | 14 |
| **50.1** | 1.4 | 30 | 70 | 0 |
| **55** | 2 | 95 | 5 | 0 |
| **60** | 2 | 95 | 5 | 0 |
| **62** | 1 | 95 | 5 | 0 |

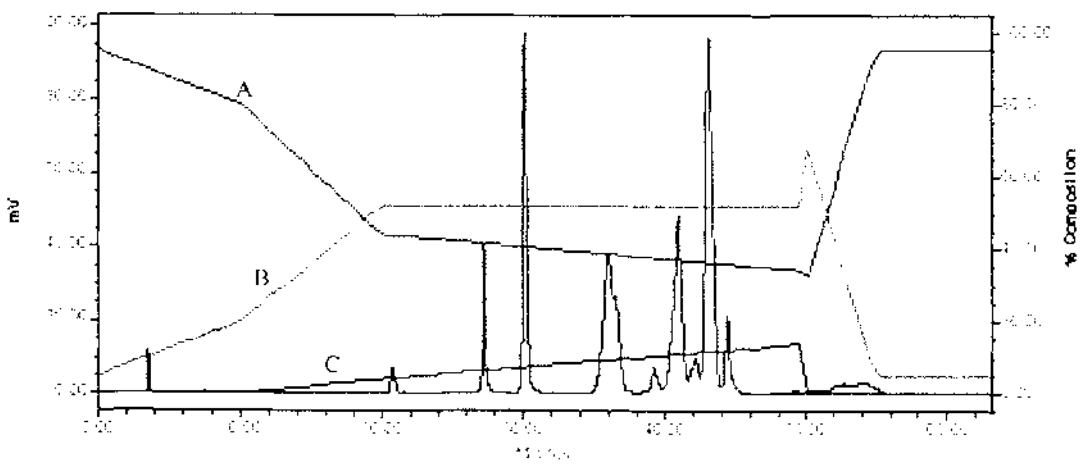



**Figure 4-1 Flow regime for HPLC as outlined in Table 4-1.**

### 4.2.3.2 Nuclear Magnetic Resonance (NMR)

The $^{31}$P-NMR analysis of the phospholipids samples was performed by Spectral Service (Köln, Germany) using a Bruker AC-P 300MHz Nuclear Magnetic Resonance Spectrometer.

#### 4.2.3.3 Thin layer chromatography (TLC)

Phospholipid fractions were dissolved in chloroform:methanol (50:50 v/v) mixture then loaded onto a foil-backed Silica 60 TLC plate. The spots were allowed to dry for 5 minutes. The plate was put in a glass TLC tank containing chloroform:methanol:water (60:35:5 v/v/v) mixture and the solvent allowed to run 70% the height of the plate. The plate was removed and air-dried for 10 minutes. The plate was then put into a TLC tank containing hexane:diethyl ether (70:30 v/v) and 1% glacial acetic acid and the solvent run in the same direction up to 95% of the plate. The plate was air-dried for a further 10 minutes before developing.

The plate was then submerged in 10% $CuSO_4$/8% $H_3PO_4$ solution for 2-3 seconds and any excess solution removed using a paper towel. The plate was placed on hotplate pre-heated to 180-200 °C, and the spots visualised as charred brown areas after 2-5 minutes.

### *4.2.4 Fatty acid profile*

The fatty acid profile of the three fractions was determined using gas chromatography (GC).

#### 4.2.4.1 Lipid Extraction

An internal standard, cholesteryl heptadecanoate (Sigma, St. Louis, Missouri) was added to samples prior to lipid extraction. Lipid from 1.0g of sample was extracted as follows. 3.75 ml of chloroform and methanol in a ratio of 1:2 (by volume) was added to 1g of the phospholipid fraction and the solution vortexed for 1 minute then centrifuged at 1500 g for 5 minutes. The supernatant was retained and the pellet re-extracted as above, adding 1 ml of water to maintain the correct ratio of solvents. The two supernatants were combined and 2.5 ml of chloroform and 0.08% NaCl (by weight) in water were successively added with 2 minutes of vortexing after each addition. This produced a biphasic solution that was centrifuged at 1500 g for 10 minutes to maximise the phase separation. The lower chloroform phase containing the plasma lipid was removed and placed in the freezer overnight. After removal of any remaining upper phase, the lipid extract was evaporated to dryness under a stream of nitrogen and reconstituted in 50 µl of chloroform. A blank sample (1 ml of water) was simultaneously extracted. All chloroform and hexane used were

pesticide residue analysis grade and methanol was analytical reagent (AnalaR) grade (BDH Chemicals, Poole, England).

#### 4.2.4.2 Fatty acid methyl ester preparation

Three millilitres of a 6% (by vol) concentrated sulphuric acid in methanol solution were added to the isolated lipid fraction as a methylating reagent. Fatty acids were methylated by acid-catalysed transesterification at 80°C for 12 hours in a sealed tube. After cooling to room temperature, 2 ml of hexane followed by 1 ml of water were added to the sample with 2 minutes of vortexing separating each addition. The upper hexane layer containing the fatty acid methyl esters (FAMEs) was removed and stored at -20°C until gas chromatographic analysis. Sulphuric acid was AnalaR grade (BDH Chemicals, Poole, England).

#### 4.2.4.3 Separation of free fatty acids

The methylated free fatty acids were separated by thin layer chromatography (TLC). TLC plates were prepared by coating glass plates with a 0.50 mm layer of silica gel G (Merck, Darmstadt, Germany); 55 grams dissolved in 100 ml of a 0.02 mol/l sodium acetate trihydrate (BDH Chemicals, Poole, England) solution. A solvent phase of toluene separated the lipid extracts into free fatty acid, and cholesterol. Plates were sprayed with 0.1% (by weight) 8-anilino-1-napthalene sulfonic acid (ANS) and free fatty acid bands visualised under UV light. The blank sample, containing only the free fatty acid standard, was used to confirm the location of the free fatty acid band.

#### 4.2.4.4 Gas chromotography

The hexane in which the FAMEs were dissolved was evaporated under a stream of nitrogen in a rotary evaporator, and the FAMEs reconstituted in 50 µl of hexane for analysis of fatty acid composition by gas chromatography (GC). FAMEs were separated using a BPX-70 capillary column, 100 m x 0.22 mm i.d., 0.25 µm film (SGE, Melbourne, Australia). The gas chromatographic system consisted of a 6890 GC equipped with an autosampler (HP7673) and Chem Station integration (all Hewlett Packard, Avondale, PA). The column oven was held at an initial temperature of 165°C for 52 minutes, then increased at a rate of 5°C/minute to a final temperature of 210°C for 59 minutes (total run time 120 minutes). Both the injector and flame ionisation detector ports were at 250°C. Carrier gas flow (helium) was maintained

at 1.0 ml/min (linear gas velocity 20 cm/sec) throughout the temperature program with an inlet split ratio of 30:1.

Fatty acid peaks were identified by retention time matching with authentic standards, including a composite standard made from commercially available methyl esters (NuCheck Prep, Elysian, Minnesota and Sigma, St.Louis, Missouri).

Results of the GC analysis were transferred by a Microsoft Excel macro program into a calculation spreadsheet which corrected for blank areas and computed the fatty acid composition on a molar percent basis (mol%) using the total integrated area. For the unidentified area an average molecular weight of the identified fatty acid methyl esters, weighted according to peak area, was used to calculate mol% composition.

## *4.3 Results and discussion*

### *4.3.1 Proximate analysis*

The results of the proximate analysis of each phospholipid fraction are shown in Table 4-2. SigP3644 had the highest total lipid content, almost 15% more than Phospholac, but contained less than half the ash content of the other two fractions. The lower lipid content of Phospholac is balanced by a higher protein content, as indicated by the higher nitrogen:phospholipid ratio. There were also differences in the carbohydrate and moisture contents of the three fractions. The cholesterol content of the Phospholac was very small, only 0.032%.

The Ultralec fraction had a high level of divalent cations, with >200 mg of both calcium and magnesium per 100g of the phospholipid powder. This greatly exceeds the <10mg/100g for $Ca^{2+}$ or $Mg^{2+}$ found in either of the Phospholac or SigP3644 fractions. Phospholac contained higher levels of the monovalent cations (potassium and sodium), while SigP3644 had relatively low levels of all four cations.

**Table 4-2 Composition of phospholipid fractions as determined by proximate analysis.**

| Component | Phospholac | SigP3644 | Ultralec |
|---|---|---|---|
| Lipid (%) | 53.0 | 67.7 | 61.1 |
| Cholesterol (%) | 0.032 | - | - |
| Nitrogen:Phospholipid ratio | 0.033 | 0.014 | 0.023 |
| Moisture (%) | 5.6 | 3.8 | 3.1 |
| Ash (%) | 14.8 | 5.7 | 13.6 |
| - calcium (mg/100g) | 5.8 | 3.6 | 200 |
| - magnesium (mg/100g) | 1.8 | 6.2 | 240 |
| - potassium (mg/100g) | 2060 | 415 | 1710 |
| - sodium (mg/100g) | 810 | <0.6 | 8.8 |

### *4.3.2 Phospholipid content*

The total phosphorus content of the phospholipid fractions, as determined by ICP-OES and $^{31}$P-NMR, is shown in Table 4-3.

**Table 4-3 Total phosphorus content of phospholipid fractions (g/kg), determined by two methods.**

| Phospholipid fraction | Total phosphorus (g/kg) | | Phospholipid (g/kg) |
|---|---|---|---|
| | ICP-OES | $^{1}$P-NMR | |
| Phospholac | 29.7 | 28.5 | 728 (73%) |
| SigP3644 | 31.5 | 30.2 | 772 (77%) |
| Ultralec | 29.4 | 27.9 | 718 (72%) |

To convert grams of phosphorous into grams of phospholipid, an average phospholipid molecular weight (Mr) of 775 (Asther *et al.*, 2001; Alvarado *et al.*, 2003) was used. It was assumed that there was no free phosphorus or other entities that contain phosphorus present in the sample, giving a molar ratio between phosphorus and phospholipids of 1:1. Using this information and the basic formula

$$number\ of\ moles = \frac{mass}{Mr}$$

the grams of phospholipid per kg of the fraction were calculated. There were no significant differences between the phosphorous contents obtained by the two different methods, and the results for all three fractions were very similar.

### *4.3.3 Phospholipid types*

The HPLC traces for the three phospholipid fractions are shown in Figure 4-2. The composition of the different phospholipid fractions as determined by the different methods is summarised in Table 4-4.

As expected, the HPLC profiles for SigP3644 and Ultralec were very similar, showing large peaks corresponding to PC and small peaks for PE and PS. The major difference between them was the size of the PI peak, which was very small for SigP3644 but for the Ultralec fraction was only slightly smaller than the PC peak. The HPLC trace for Phospholac was more complex. PC was still the largest peak, but there were small peaks corresponding to PE, PI, PS and SM.

There were also some small unidentified peaks at the beginning (24-25 minutes) of the profiles for all three fractions, and at the end (42 to 44 minutes) of the Phospholac profile. Referring to the schematic shown inFigure 4-3, it is possible that these small peaks represented phosphatidyl glyercol and lysophosphatidyl choline (LPC) respectively.

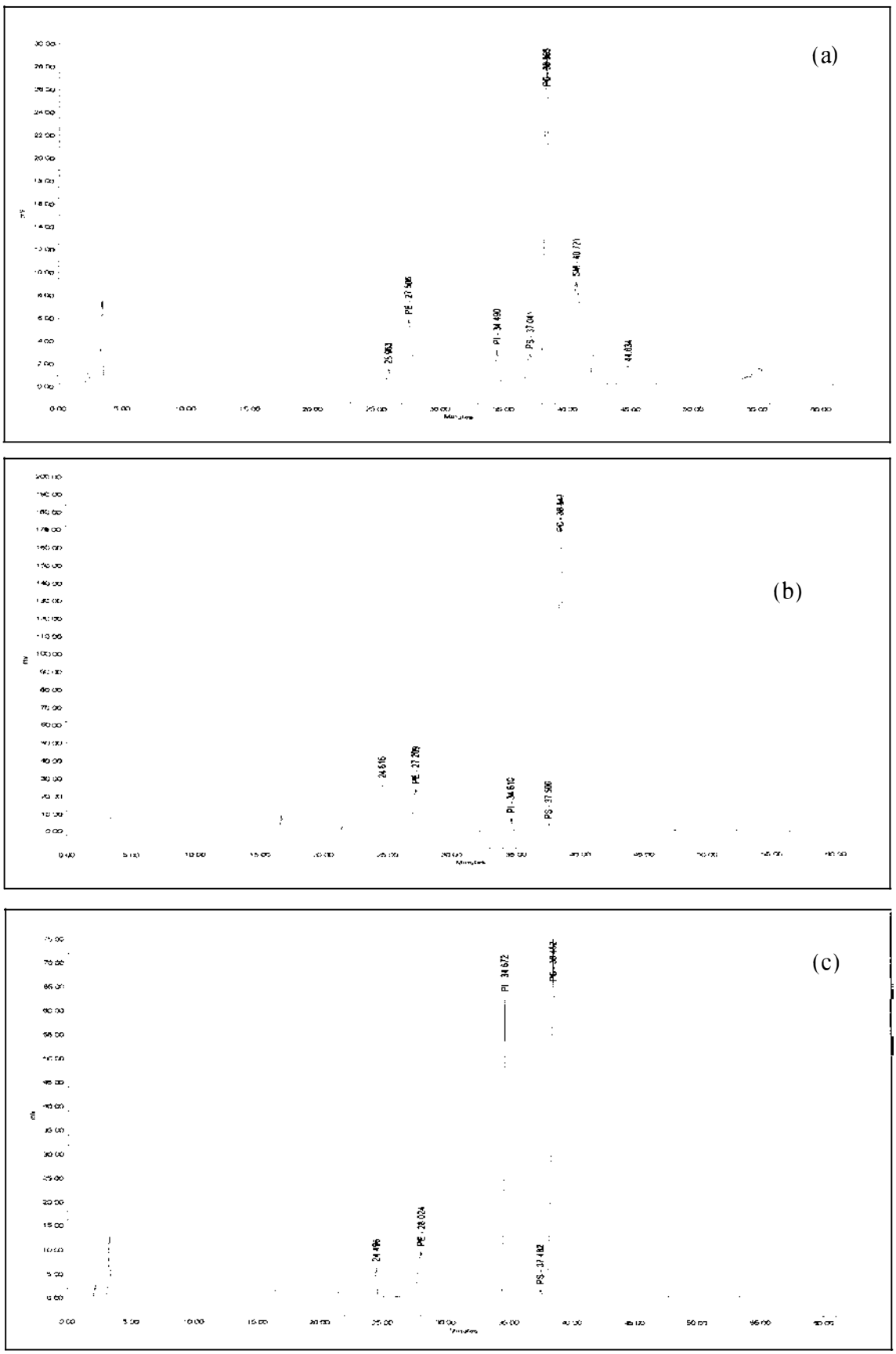



**Figure 4-2 HPLC traces for (a) Phospholac, (b) SigP3644 and (c) Ultralec as produced by the method given in section 4.2.3.1.**

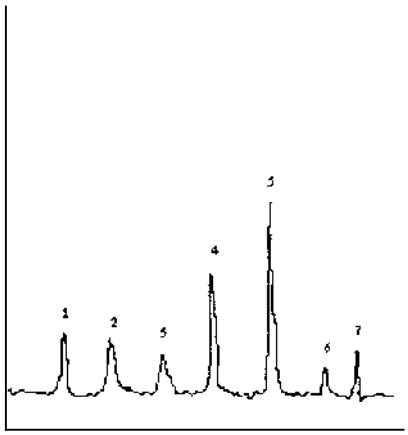



**Figure 4-3 Schematic showing expected elution positions of common phospholipids (Lutzke and Braughler, 1990; Sas *et al.*, 1999). 1: phosphatidylglycerol 2: phosphatidylethanolamine, 3: phosphatidylinositol, 4: phosphatidylserine, 5: phosphatidylcholine, 6: sphingomyelin, 7: lysophosphatidylcholine.**

The $^{31}$P-NMR results are also shown in Table 4-4. The NMR results show a higher proportion of polar lipids in the product than the total amount of lipids as measured by during the proximate analysis, but it is possible that there had not been a complete extraction of the lipids in the latter technique leading to under-estimation of the lipid content. The three fractions had similar polar lipid contents, but were quite different in terms of the relative amounts of the specific phospholipid classes. Phospholac was composed primarily of approximately equal quantities of PC, PE and sphingomyelin, with a small amount of PI and PS. This is in agreement with the literature reports on the phospholipid composition of MFGM (Boyd *et al.*, 1999; Astaire *et al.*, 2003). The SigP3644 fraction was mainly composed of PC, with about one third PE and a small amount of PI. Ultralec was also predominantly composed of PC, PE and PI, but it had less PC and more PI than the SigP3644 fraction. Approximately 5% of both SigP3644 and Ultralec belong to less common phospholipid classes grouped into the 'other phospholipids' category.

Comparing the HPLC and $^{31}$P-NMR results identifies a number of discrepancies (Table 4-4). In general, the HPLC results for PC were approximately 15 % higher than the $^{31}$P-NMR, and those for PE approximately 10% lower. The HPLC results also showed 5 mol% more PI in the Ultralec fraction than that shown by $^{31}$P-NMR.

Although it is possible that the extraction and purification of the Phospholac fraction will alter the phospholipid class distribution compared with the raw milk, it seems reasonable that

the distributions will be similar. The phospholipid content of milk as published by a variety of sources is shown in Table 4-5. The relative amounts of the different phospholipids appear more in line with the values obtained using $^{31}$P-NMR than those from HPLC.

**Table 4-4 Phospholipid class of different fractions (% of total fraction). Results obtained using methods detailed in section 4.2.2.**

| Lipid | Phospholac (%) | | SigP3644 (%) | | Ultralec (%) | |
|---|---|---|---|---|---|---|
| | $^{31}$P-NMR | HPLC | $^{31}$P-NMR | HPLC | $^{31}$P-NMR | HPLC |
| Polar Lipids | 72.0 | | 73.9 | | 68.2 | |
| Phosphatidyl choline | 23.6 | 38.4 | 40.9 | 57.3 | 24.6 | 38.9 |
| Phosphatidyl ethanolamine | 20.2 | 10.6 | 25.4 | 11.3 | 22.2 | 9.7 |
| Sphingomyelin | 22.8 | 20.4 | Not detected | Not detected | Not detected | Not detected |
| Phosphatidyl serine | 2.5 | 5.2 | Not detected | 1.1 | 0.5 | 0.9 |
| Phosphatidyl inositol | 2.6 | 3.1 | 3.6 | 1.2 | 13.3 | 17.6 |
| Other phospholipids | 0.2 | 4.8 | 4.1 | 2.9 | 6.9 | 1.2 |

**Table 4-5 Phospholipid classes present in bovine milk.**

| Phospholipid class | Amount | |
|---|---|---|
| | Morrison (1968) mol% of total lipid phosphorous | Bitman and Wood (1990) % of total phospholipid[1] |
| Phosphatidyl choline | 34.5 | 25.1-35.1 |
| Phosphatidyl ethanolamine | 31.8 | 19.8-31.1 |
| Sphingomyelin | 25.2 | 28.7-34.1 |
| Phosphatidyl serine | 3.1 | 1.9-8.5 |
| Phosphatidyl inositol | 4.7 | 4.1-11.8 |
| Lysophospholipids | 0.8 | Not given |

[1]Variation over lactation (days 3–180). Data obtained with TLC/densitometry.

The TLC plate for the identification of the phospholipids present in the three fractions is shown in Figure 4-4a. As the TLC method only uses a standard containing sphingomyelin, PC and PE, it is useful to refer to the schematic TLC plate shown in Figure 4-4b. By comparing the expected position of PS and PI relative to the phospholipids present in the standard it is possible to identify these phospholipids in the commercial fractions.

The TLC plate shows that the Phospholac fraction is primarily composed of sphingomyelin, PC and PE in roughly equal quantities. There is a small amount of PS, with no obvious PI. The Ultralec and Sigma fractions have a higher concentration of PC and PE, no sphingomyelin and noticeable amounts of PI.

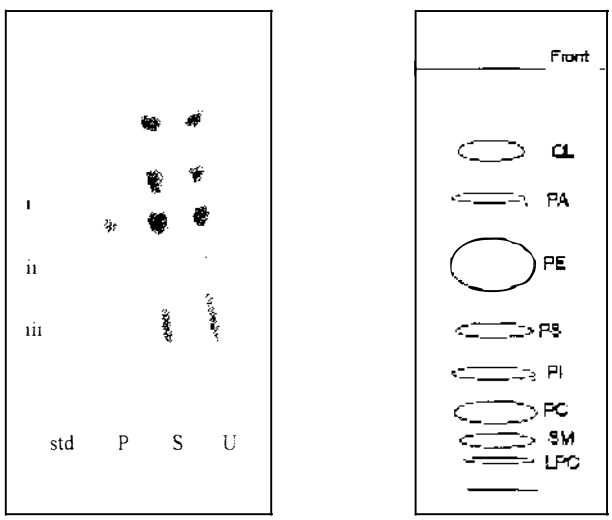



**Figure 4-4 (a) Scanned TLC plate prepared using method outlined in section 4.2.3.3. std = standard containing (i) PE, (ii) PC and (iii) sphingomyelin; P = Phospholac; S = SigP3644; U = Ultralec. (b) Schematic of a TLC plate showing order of separation of different phospholipid classes. Based on Skipski *et al.* (1962), Siek and Newburgh (1965), and Fine and Sprecher (1982). CL: cardiolipin, LPC: lysophosphatidylcholine, PA: phosphatidic acid, PC: phosphatidylcholine, PE: phosphatidylethanolamine, PI: phosphatidylinositol, PS: phosphatidylserine, SM: sphingomyelin.**

The TLC plate shows that the Phospholac fraction is primarily composed of sphingomyelin, PC and PE in roughly equal quantities. There is a small amount of PS, with no obvious PI. The Ultralec and Sigma fractions have a higher concentration of PC and PE, no sphingomyelin and noticeable amounts of PI.

There are several unidentified bands for each fraction. The dark band immediately above PE in the soy fractions is likely to be PA, as the $^{31}$P-NMR reports 2.5% and 5% present in Sigma and Ultralec respectively. Phospholac has a faint band slightly above PE, which may be cardiolipin (CL). Unfortunately the $^{31}$P-NMR does not measure CL content, but Patton *et al.* (1969) reports that up to 1% of the phosphorous present in bovine milk may correspond to CL. The Sigma and Ultralec fractions also have small spots lower than the expected location for sphingomyelin, which (referring to Figure 4-4b) seems likely to be lysophosphatidyl choline (LPC). This is supported by the $^{31}$P-NMR results which show small amounts of LPC present in both fractions (included in 'other phospholipids' in Table 4-4). Qualitative results are summarized in Table 4-6, and appear to support those obtained by $^{31}$P-NMR.

**Table 4-6 Qualitative assessment of the phospholipid classes present in different fractions by TLC.**

| Phospholipid class | Phospholac | SigP3644 | Ultralec |
|---|---|---|---|
| Phosphatidyl choline | Considerable | Considerable | Considerable |
| Phosphatidyl ethanolamine | Considerable | Considerable | Considerable |
| Sphingomyelin | Considerable | Not detected | Not detected |
| Phosphatidyl serine | Trace | Trace | Trace |
| Phosphatidyl inositol | Not detected | Moderate | Moderate |
| Other phospholipids | Not detected | Possibly | Possibly |

### *4.3.4 Fatty acid profile*

There was a significant difference between the types of fatty acid found in the two soy fractions compared to the dairy fraction (Table 4-7 and Figure 4-6). The animal-origin of the dairy fraction is reflected in the higher percentage of saturated and mono-unsaturated fatty acids, while the soy phospholipids contain more poly-unsaturated fatty acids. In general, the measured amounts of the different fatty acids agreed with the values reported for the raw materials.

**Table 4-7 Fatty acid content of phospholipid fractions.**

| Fatty acid | Phospholipid fraction (%) | | | Literature values (%) | | |
|---|---|---|---|---|---|---|
| | Phospholac | SigP3644 | Ultralec | Whole milk | MFGM | Soybean oil |
| C14:0 | 3.1 | 0.1 | 0.1 | 12 | 1.9-2.2 | T |
| C16:0 | 16.2 | 15.6 | 20.0 | 26 | 16.5-19.4 | 11 |
| C16:1 | 1.4 | 0.2 | 0.0 | 3 | 1.2-1.6 | T |
| C18:0 | 10.0 | 3.7 | 4.0 | 11 | 16.3-17.9 | 4 |
| C18:1 | 30.3 | 8.6 | 9.7 | 29 | 27.7-30.7 | 22 |
| C18:2n-6 | 4.8 | 57.5 | 51.3 | 2 | 6.1-6.9 | 53 |
| C18:3n-3 | 1.8 | 7.5 | 5.9 | T | 0.4-0.6 | 8 |
| C20:2n-6 | 0.5 | 0.3 | 0.3 | T | 0.5-1.5 | T |
| C20:3n-6 | 0.7 | 0.0 | 0.0 | T | 1.0-1.3 | T |
| C20:4n-6 | 0.7 | 0.1 | 0.2 | T | 0.9-2.2 | T |
| C20:5n-3 | 0.6 | 0.0 | 0.4 | T | 0.2-0.6 | T |
| C22:0 | 4.2 | 0.2 | 0.6 | T | 3.5-4.8 | T |
| C22:5n-3 | 1.0 | 0.0 | 0.0 | T | 0.5-0.6 | T |
| C22:5n-6 | 6.5 | 0.2 | 0.3 | T | 0.3-0.5 | T |
| C24:0 | 4.0 | 0.2 | 0.5 | T | 3.1-3.9 | T |

Literature values from Gurr *et al.* (2002), except for MFGM values which were taken from Fauquant *et al.* (2005). T: trace.

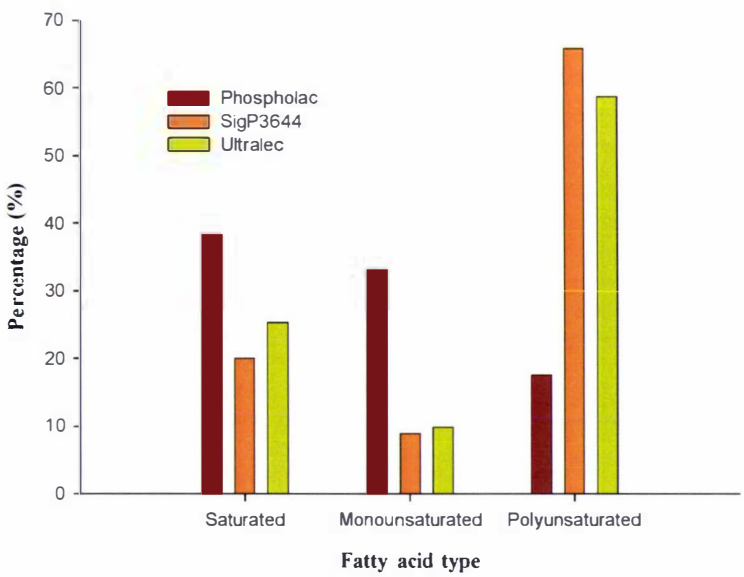



**Figure 4-6 Fatty acid types present in the different phospholipid fractions.**

## 4.4 Conclusions

There are obvious differences between the fractions in terms of basic composition, phospholipid class distribution and fatty acid profile in agreement with those expected based on the relevant literature. The soy-derived SigP3644 and Ultralec had similar phospholipid profiles, reflecting their common raw material. The primary difference between these two fractions was the degree of purification. SigP3644 had more PC and less PI than Ultralec, as well as a significantly lower ash content. The dairy-origin of Phospholac was reflected in its more highly saturated fatty acid profile, and the presence of 25% sphingomyelin. The Phospholac fraction had a significantly higher nitrogen:phospholipid ratio, with the SigP3644 nitrogen:phospholipid ratio less than half that of the dairy-derived fraction. The Phospholac fraction had a similar ash content to Ultralec, although with fewer divalent cations.

The literature suggests that these differences may mean liposomes formed from the Phospholac fraction have a higher phase transition temperature, are generally more stable, have a lower membrane permeability and have a reduced susceptibility to oxidation than liposomes produced from either the SigP3644 or Ultralec fraction. The accuracy of these statements will be investigated in the following chapters.

# Chapter 5: Physico-chemical characterisation of liposome dispersions

## *5.1 Introduction*

Knowledge of the physical and chemical characteristics of the liposome dispersion produced by a particular phospholipid fraction or processing method is very important. It allows an understanding of how liposomes are likely to behave under different conditions, and helps identify the types of applications where the specific population may be advantageous.

At a basic level, measuring the average size and size distribution of the liposome population is essential, but a more thorough characterisation involves a number of properties which may provide an indication of likely entrapment and release kinetics and the stability of the system (Jousma *et al.*, 1987).

The phase transition temperature of a liposome population directly affects membrane permeability at different temperatures and hence the rate of release of entrapped material (Zeisig and Cämmerer, 2001). The thickness of the bilayer membrane is likely to affect diffusion rates, as is lamellarity, with multilamellar liposomes thought to provide a more gradual and sustained release compared with unilamellar liposomes (New, 1990b). However, the additional bilayers present in multilamellar vesicles occupy internal space within the liposome, thus reducing the entrapped volume and resulting in lower entrapment efficiencies for hydrophilic material (Arnaud, 1995b). The surface charge is often related to stability due to electrostatic forces between the liposomes, which are important as destabilization of the system often leads to loss of entrapped material (Keller, 2001).

This chapter describes the physical and chemical characteristics of liposome systems formed from the phospholipid fractions discussed in *Chapter 4: Characterisation of phospholipid fractions.*

## 5.2 Materials and methods

### 5.2.1 Phospholipid concentration of liposome dispersions

Characterisation of liposomes requires determination of the phospholipid concentration of the sample. Therefore it is of great advantage to have a quick, reliable and relatively inexpensive method for determining phospholipid concentration.

Directly measuring the phosphate concentration requires an acid-digestion step followed by a colourmetric assay, as outlined in section 4.2.2 (*Chapter 4: Characterisation of Phospholipid Fractions*). This is very time consuming and expensive. TLC plates provide qualitative identification of phospholipids and may be converted into a quantitative measurement through weighing of the spots. However, the precision and accuracy of this technique is unlikely to be very high.

The following method was developed as a quick and cost-effective way to quantify the phospholipid content of the liposome dispersions.

Phospholipids were hydrolysed by phospholipase C (Sigma, Catalogue #P7147, 20 µl in 250 µl of 0.1 M phosphate buffer, pH 7.0) to produce a diacylglycerol moiety and phosphatidyl choline or inositol or serine or ethanolamine. Microbial lipase hydrolyses the diacylglycerol to produce two fatty acids and glycerol, and the amount of glycerol liberated is measured using glycerol kinase, glycerol phosphate oxide and peroxidase. The liposome suspension was diluted 1:1 in 10% Triton X100, with further dilutions using 5% Triton, if required. The assay was performed using a Roche Cobas Fara II analyser (Hoffmann La Roche, Basel, Switzerland). A mixture containing 4 µl of sample, 5 µl of phospholipase C and 91 µl of water was incubated for 5 minutes at 37 °C. The absorbance at 520 nm was then measured and 300 µl of the triglyceride reagent was added. The mixture was then incubated for 5 minutes and absorbances read at 10-second intervals. The reagent blank consisted of water, phospholipase C and the triglyceride reagent incubated as above.

This technique cannot determine sphingomyelin concentration, as it does not have the glyceride backbone found in the other phospholipid classes (section 2.3.1 in *Chapter 2: Literature Review*). Therefore it was necessary to multiply any results for the dairy phospholipid fraction Phospholac by 1.25 to allow for the 20% sphingomyelin present.

It was also necessary to allow for any triglyceride present in the raw material, as the assay was unable to distinguish between the glyceride backbones from phospholipid or triglyceride sources. The triglyceride content of the Phospholac, SigP3644, and Ultralec samples was measured using a triglyceride diagnostic kit (Roche Diagnostics Ltd, NZ; catalogue #2016648) and found to be negligible, so no adjustment was made for these fractions.

The assay was initially calibrated using a Roche calibrator for which the concentrations of phospholipid and triglyceride are known, then checked using two Roche controls which also have known amounts of phospholipid and triglyceride. The calibrator and controls were both part of a Triglyceride diagnostic kit produced by Roche Diagnostics Ltd, NZ (catalogue #2016648). As a final check, samples produced by the technique described in section 3.3.2 (*Chapter 3: Selection of phospholipid fractions and processing method*) were compared with those obtained for the same samples from the conventional acid-digestion method.

### *5.2.2 Hydrodynamic diameter*

#### 5.2.2.1 Photon correlation spectrometry (PCS)

The average hydrodynamic diameter of the liposomes was measured using a photon correlation spectrometer (PCS), the Zetasizer 4 (Malvern Instruments Ltd, UK) as outlined in section 3.4.1.

#### 5.2.2.2 Asymmetrical Flow Field-Flow Fractionation (AFFF)

Asymmetrical Flow Field-Flow Fractionation (AFFF) was used to provide more detailed information regarding the size distribution of the liposome dispersions. This was performed using a Postnova Avalanche AF4 AFFF (Postnova, Munich, Germany) equipped with a PostNova RI Detector (PN 3140) and a Precision Detectors PD Expert Multi-angle Dynamic and Static Light-scattering System (www.precisiondetectors.com). A channel spacer of 0.25 mm and a Field Programming Method using Power Field Decay was used. The field was initially held constant at 70% for 4.8 minutes, then decayed at the rate of -p*4.8 minutes, where p=2 (to obtain a constant fractionation power). This method was designed using the software provided with the system to give the optimum separation (in terms of maximum resolution) in 60 minutes for particles between 7.5 and 750 nm diameter. The crossflow and

expected particle size of eluted material as a function of time is shown in Figure 5-1. Channel outlet flow was held constant at 0.3 ml/min, and the total run time was 60 minutes.

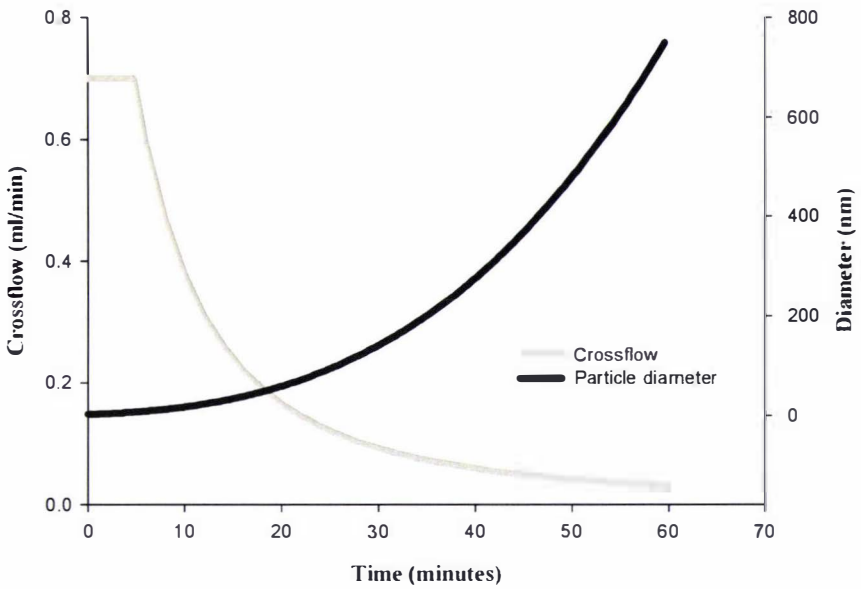



**Figure 5-1 Change in crossflow rate and eluted particle diameter over time resulting from programming conditions used for Asymmetrical Flow Field-Flow Fractionation (AFFF).**

5.2.3 *Microstructure*

ı lamellarity and particle size of liposomes.
; Transmission Electron Microscopy, Thin
d Cryo Field Emission Scanning Electron



EM)

imately 1:10 with distilled water. One drop of
p of 2% ammonium molybdate and left for 3
copper mesh for 5 minutes before the excess

liquid was drawn off with filter paper. The mesh was examined using a Philips 201C Transmission Electron Microscope (Eindhoven, Netherlands).

#### 5.2.3.1.2 Thin section TEM

The liposome dispersion was mixed with low-temperature gelling agarose. The agarose embedded samples were cut into ~1mm$^3$ cubes, put into a bijoux bottle containing 3% glutaraldehyde in 0.2M sodium cacodylate buffer and kept at 5°C for 24h. The glutaraldehyde was removed by rinsing twice with 0.2M sodium cacodylate buffer for 2 hours, then the samples were left in 1% osmium tetroxide overnight at room temperature. They were washed twice with distilled water, placed in 1% uranyl acetate for 30 minutes, then washed twice more with distilled water.

The embedded samples were dehydrated at 5°C using 25 % acetone for 15 minutes, then 50%, 70% and 90% acetone for 30 minutes each, followed by 100% acetone. The acetone was replaced with Procure 812 embedding resin, and put on rollers for 24 hours. A cube of the sample was placed into an embedding capsule and this was cured at 60 °C for 48 hours.

The embedded samples were then sectioned to a thickness of 90 nm using the Reichert Ultracut microtome. These sections were mounted on 3 mm copper grids and stained with lead citrate before examination in a Philips 201C Transmission Electron Microscope (Eindhoven, Netherlands) at an accelerating voltage of 60 kV.

### 5.2.3.2 Cryo-Field Emission Scanning Electron Microscopy (Cryo-FESEM)

Samples were plunge-frozen using liquid propane, before being frozen with slushy liquid nitrogen at -140 °C. They were then fractured with a knife on the Alto 2500 Cryo Stage (Gatan, UK), and freeze-etched by raising the temperature from -140 °C to -90 °C before returning to -140 °C. The exposed surface was then coated with Au and Pd for 120 seconds, before being examined under the JSM-6700F Field Emission Scanning EM (JEOL, Japan). An acceleration voltage of 10 kV was used.

### 5.2.4 Zeta potential

The electrical potential at the plane of shear as liposomes move in an electrical field is called the zeta potential (Jones, 1995). The zeta potential of a dispersion provides an indication of the surface charge of the particles. This is important as a surface charge determines inter-particle interactions and hence contributes towards resistance to aggregation (Lasic, 1998; Keller, 2001).

Liposome dispersions were prepared by the standard technique using 0.1 M NaCl as the aqueous phase. The pH of the dispersions was adjusted using I M HCl and 1 M NaOH, and diluted as required using 0.1 M NaCl solution that had also been adjusted to the specific pH.

Zeta potential was measured using a Zetasizer 4 (Malvern Instruments, UK) with an AZ104 cell. Five measurements of 25 seconds duration at 100 mV were used to measure the zeta potential at the stationary layer 14.63% of the capillary diameter in from the wall.

### 5.2.5 Phase transition temperature

Liposome dispersions prepared by the standard technique were concentrated using Sartorious Centrisart I 13239-E centrifuge filters. The technique involved centrifugation at 4000 *g* for 4 hours in a CentraMP4R centrifuge (International Equipment Company, MA).

The concentrated dispersions were filled into $1cm^3$ ampoules, and a CSC 4100 (Calorimetry Sciences Corporation, Spanish Fork, UT) DSC used to scan from 0-60 °C at a rate of 1 °C per minute.

### 5.2.6 Bilayer thickness

Small angle x-ray diffraction allows the determination of the lamellar bilayer thickness for the phospholipid as well as providing an indication of the lamellarity of a liposome population (Bouwstra *et al.*, 1993).

Liposome dispersions were prepared using the standard method. Aliquots were concentrated to approximately 20% phospholipid using Centrisart 1 19239E centrifugal filters at 4000 *g* for 2 hours in a CentraMP4R centrifuge (International Equipment Company, MA).

For the small angle x-ray diffraction measurements, the samples were transferred into glass capillary tubes (1.5 mm diameter, Charles Supper Company, MA, USA) and then the scattering pattern was measured during exposure to low divergence CuKα radiation that had a wavelength of 1.54 Å (Rigaku MicroMax007, microfocus rotating anode generator with Osmic multilayer confocal optics; Japan, USA). The exposure time varied from 10 to 15 min. The diffraction images were recorded on a RAxisIV++ image-plate detector (Rigaku, Fuji; Japan) placed 100-300 mm from the sample. Diffraction patterns were visualised and analysed using CrystalClear software (version 1.3.6SP0, Rigaku-MSC; Japan, USA).

The repeat distance between the lattice planes for each of the liposome dispersions was calculated within the CrystalClear software using Equation 5-1:

$$d = \frac{\lambda}{2\sin\left(\frac{1}{2}\tan^{-1}\frac{x}{X}\right)} \qquad \text{Eq 5-1}$$

where d is the repeat distance between the lattice planes, λ is the wavelength of the X-ray beam, x is the radius of the Debye ring and X is the distance between the sample and the detector. The distance X is accurate to +/- 0.2 mm and was determined from diffraction patterns taken at different rotational settings of three-dimensional crystals.

### *5.2.7 Permeability*

The rate at which the entrapped molecules pass through the bilayer will directly affect the useful shelf-life and the possible applications for that particular liposome dispersion.

Permeability can be difficult to measure, with many authors merely measuring the release of an entrapped compound (often a fluorescent marker) over time to provide an indication of the permeability of the membranes (Sagrista *et al.*, 2000; Ma *et al.*, 2002; Bayazit, 2003; Cocera *et al.*, 2003; Jin *et al.*, 2005; Ishida *et al.*, *In press*). The use of pulsed-field-gradient NMR to measure the actual rate of diffusion of molecules through liposome membranes is relatively new, so is covered in detail below. The specific technique used here combines ideas from a number of authors, and provides a more comprehensive approach than can be found in any single published article at this date (J. Hindmarsh, 2005 unpublished work).

5.2.7.1 PFG NMR diffusion measurements

PFG NMR has been widely used for the direct determination of the self-diffusion coefficients ($D$) of molecules. NMR PFG can quantify the net displacement $(r'-r)$ of group of molecules, where an individual molecule moves from $r$ to $r'$ over a given period of time. Provided the net displacements for the molecules follow a Gaussian distribution, Einstein's relationship $(r'-r)^2 = 6Dt_D$ is valid (the mean squared displacement of molecules scales linearly for the diffusion time $t_D$), and the self-diffusion coefficient can be determined.

There are a range of PFG experiments of varying complexity. In the simplest version, a 90°-180° spin-echo experiment is used together with two field gradient pulses. The pulse sequence is shown in Figure 5-2. The relative echo intensity ($E$) is a function of the experimental parameters $\delta$ (duration of the magnetic field gradient pulse), $\Delta$ (equivalent to the diffusion time $t_D$, time internal between gradient pulses) and $g$ (the magnitude of the field gradient). For the short-gradient-pulse limit (SGP limit) or narrow-pulse approximation, where motion during the duration of the gradient pulse is ignored ($\delta \rightarrow 0$ and $\delta << \Delta$), the relative echo attenuation is shown by Equation 5-2. $\gamma$ is the gyromagnetic ratio for proton NMR ($^1H$), $2.675 \times 10\ s^{-1}T^{-1}$.

$$E(g,\Delta,\delta) = \exp\left(-\lambda^2\delta^2 g^2 \Delta \cdot D\right) \qquad \textbf{Eq 5-2}$$

It is often not possible to obtain sufficiently short gradient pulse duration ($\delta$) to fulfill the SGP condition, so for finite gradient durations the diffusion time ($t_D$) may be adjusted (Equation 5-3). This means that Equation 5-2 may now be expressed as shown in Equation 5-4 (Callaghan, 1993).

$$t_D = (\Delta - \delta/3) \qquad \textbf{Eq 5-3}$$

$$E(g,\Delta,\delta) = \exp\left(-\lambda^2\delta^2 g^2 (\Delta - \delta/3) \cdot D\right) \qquad \textbf{Eq 5-4}$$

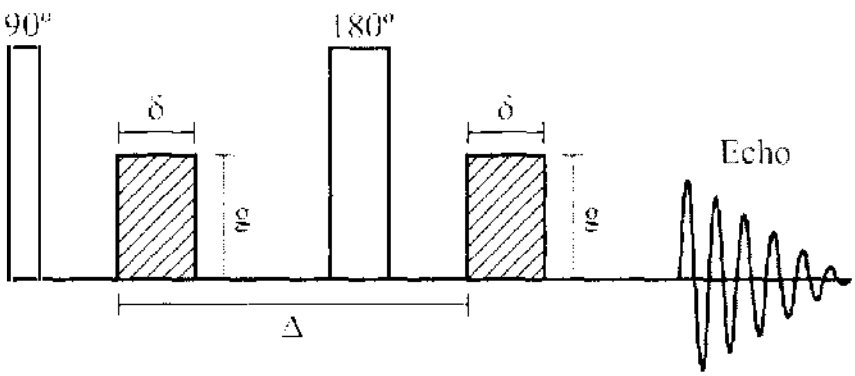



**Figure 5-2 Pulse sequence for simple PFG experiment.**

5.2.7.2 Two compartments with free and restricted diffusion

The simplest model of a liposome consists of two separate compartments (one intracellular and one extracellular) with a boundary permeability of zero, i.e. there is no exchange of molecules between the two compartments. The free diffusion in the exracellular compartment can be followed using Equation 5-4. The movement of the intracellar molecules is restricted to the volume of the liposome cell, resulting in a non-Gaussian distribution of the molecule displacements. This means that the echo attenuation can no longer be described by Equation 5-4. If the geometry of the liposome cell is approximated to a sphere, the echo attenuation of the intracellular compartment can be described by Equation 5-5 (Packer and Rees, 1971).

$$E(g,\Delta,\delta,R)=\exp\left(-2\gamma^2 g^2\sum_{m=1}^{\infty}\frac{1}{\lambda_m^2\left(\lambda_m^2R^2-2\right)}\times\left\{\frac{2\delta}{\lambda_m^2D}-\frac{2+e^{-\lambda_m^2D(\Delta-\delta)}-2e^{-\lambda_m^2D\Delta}}{\left(\lambda_m^2D\right)^2}-\frac{2e^{-\lambda_m^2D\delta}-e^{-\lambda_m^2D(\Delta-\delta)}}{\left(\lambda_m^2D\right)^2}\right\}\right)$$

**Eq 5-5**

In this equation, $R$ is the inner liposome radius, $D$ is the unrestricted self-diffusivity of the liquid and $\lambda_m$ is the $m$th positive root of Equation 5-6, where $J_n$ is a $n^{th}$ order Bessel function. Usually the 5$^{th}$ positive root is sufficient.

$$J_{3/2}(\lambda R)=\lambda R J_{5/2}(\lambda R) \qquad \textbf{Eq 5-6}$$

The total echo signal attenuation ($E_{tot}$) in a system composed of two compartments is a superposition of the weighted signal from the intracellular ($S_{intra,restricted}$) and extracellular

($S_{extra,free}$) components (Equation 5-7) (Pfeuffer *et al.*, 1998). In this equation, $p_1$ and $p_2$ are the relative volume fractions of the molecules in the intracellular and extracellular compartments respectively, and therefore $p_1+p_2=1$.

$$E_{tot} = S_{intra,restricted} + S_{extra,free} = p_1 \cdot E(g,\Delta,\delta,R) + p_2 \cdot \exp\left(-\gamma^2 g^2 \delta^2 (\Delta - \delta/3) \cdot D_2\right)$$

**Eq 5-7**

Virtually all liposome dispersions will have some sort of distribution of liposome sizes, and so it is necessary to modify Equation 5-5 to include a probability distribution of radii, *P(R)* (Packer and Rees, 1971). Since the magnitude of the NMR signal is proportional to the volume of material, a volume average of the attenuation is used to obtain *Ep*, the total signal attenuation for a liposome population (Equation 5-8).

$$Ep = \frac{\int_0^\infty R^3 P(R) E_{tot} dR}{\int_0^\infty R^3 P(R) dR}$$

**Eq 5-8**

The size distribution of systems formed through shearing (i.e. emulsions) has been found to fit a log-normal distribution (Equation 5-9), where $\sigma$ is the variance and $R_0$ is the volume weighted geometric median radius of the distribution (Hollingsworth and Johns, 2003).

$$P(a) = \frac{1}{R\sigma(2\pi)^{1/2}} \exp\left[-\frac{(\ln R - \ln R_0)^2}{2\sigma^2}\right]$$

**Eq 5-9**

### 5.2.7.3 Exchange between two compartments

A more complex (but usually more accurate) model of a liposome dispersion assumes that there is some molecular transport and exchange between the two compartments (Kärger and Heink, 1983). In this model, it is assumed that the exchanging species experiences free diffusion in each of the compartments. The rates of exchange for the molecules are combined with the diffusion equations to give the mean residence time in a pool (i.e. the time after which the population is reduced to $^1/_e$). For a system consisting of two compartments with diffusion constants $D_{1,2}$, exchange times $\tau_{1,2}$ and with relative volume fractions $p_{1,2}$ the echo attenuation is described by Equation 5-10 (Price *et al.*, 1998).

Supplementary equations explaining some of these terms are shown in Equation 5-11 through to Equation 5-15.

$$E_{tot} = p_1 \exp\left(-\lambda^2 g^2 \delta^2 (\Delta - \delta/3) D_1\right) + p_2 \exp\left(-\lambda^2 g^2 \delta^2 (\Delta - \delta/3) D_2\right)$$

**Eq 5-10**

$$D_{1,2} = \frac{1}{2}\left[D_1 + D_2 - \frac{1}{\lambda^2 g^2 \delta^2}\left(\frac{1}{\tau_1} + \frac{1}{\tau_2}\right) \mp \sqrt{\left(D_2 - D_1 - \frac{1}{\lambda^2 g^2 \delta^2}\left(\frac{1}{\tau_2} - \frac{1}{\tau_1}\right)\right)^2 + \frac{4}{\lambda^4 g^4 \delta^4 \tau_1 \tau_2}}\right]$$

**Eq 5-11**

$$p_1 = 1 - p_2 \qquad \textbf{Eq 5-12}$$

$$p_2 = \frac{1}{D_2 - D_1}\left(p_1 D_1 + p_2 D_2 - D_1\right) \qquad \textbf{Eq 5-13}$$

$$p_1 + p_2 = 1 \qquad \textbf{Eq 5-14}$$

$$p_{1,2} = \frac{\tau_{1,2}}{\tau_1 + \tau_2} \qquad \textbf{Eq 5-15}$$

To allow for the spherical geometry of the intracellular compartment, the model may be extended to include partial restriction of diffusion at the cell wall. This is achieved by replacing the intracellular diffusion coefficient $D_1$ in Equation 5-11 and Equation 5-13 with an apparent diffusion coefficient ($D_{app,intra}$) as shown in Equation 5-16 (Pfeuffer *et al.*, 1998). This is dependent on the diffusion time, and is the partial derivative of Equation 5-5, where $D_{app,intra}$ is defined for $\lambda g \delta \cdot R < 1$ only. By exchanging $D_1$ for $D_{app,intra}$ in Equation 5-11 and using Equation 5-8 and Equation 5-9, the equation is adapted to allow for a distribution of liposome sizes.

$$D_{app,intra}(t_D) = -\frac{\partial \ln E(g,\Delta,\delta,R)}{\delta\left(\lambda^2 g^2 \delta^2\right)} \cdot \frac{1}{t_D} \qquad \textbf{Eq 5-16}$$

### 5.2.7.4 Liposome Diffusion

At low concentrations, liposomes dispersed in a low viscosity liquid such as water will freely diffuse. This can be described by Stokes-Einstein equation (Equation 5-17) where $k$ is the Boltzmann constant, $T$ is the absolute temperature, $\eta$ is the viscosity of the solution and $R_{out}$ is the outer radius of the liposome cell.

$$D_{cell} = \frac{kT}{6\pi\eta R_{out}} \qquad \textbf{Eq 5-17}$$

The individual molecules forming the liposome will have a displacement due in part to their own self-diffusion within the membrane, and also due to the movement of the vesicle. The apparent diffusion coefficient $D_{app,intra}/D_1$ of the intracellular liquid may be combined with the liposome diffusion coefficient $D_{cell}$ to produce an effective diffusion coefficient for molecules inside the cell (Equation 5-18) (Goudappel *et al.*, 2001).

$$D_{eff} = D_{app,intra} + D_{cell} \qquad \textbf{Eq 5-18}$$

The liposome diffusion coefficient $D_{cell}$ can be determined from the PFG NMR diffusion measurements by tracing the attenuation of the signal from the lipid peaks in the NMR spectrum. This assumes that only a minimal amount of lipid in the sample is not contained in the liposome membrane, and therefore any movement of lipids detected by the PFG NMR experiment may be associated with the diffusion of the liposome. The value of $D_{cell}$ is obtained by fitting the attenuation data to Equation 5-4.

### 5.2.7.5 PFG NMR diffusion measurements

PFG NMR diffusion measurements were performed using a Bruker Avance 500 Mhz spectrometer with a 50 G cm$^{-1}$ z-gradient. The gradient strength ($g$) ranged from 5 to 50 G cm$^{-1}$ in 32 steps, with a gradient pulse width ($\delta$) of 5 ms. Several measurements were taken for each sample, with $\Delta$ values of 60, 80, 100 and 150 ms. The liposome dispersions used in this experiment had been prepared in distilled water to minimise signal interference from the buffer salts and were concentrated using ultracentrifugation. Initial results were compromised by the peak corresponding to the diffusion of the liposomes within the sample being significantly larger than the peak from the diffusion of the water molecules between

the intra- and extraliposomal spaces. Small buds of cotton wool were placed in the bottom of the NMR tube and sufficient of the concentrated dispersions added to moisten the wool. This reduced the diffusion of the liposomes within the sample to close to zero.

Models were fitted to the PGG NMR experimental data using the non-linear least-squares regression analyses in Matlab (Version 6.5 The Mathworks Inc). For the two compartment model with no exchange (Equation 5-7), the fitted parameters were $p_1$, $D_2$, $R_0$ and $\sigma$. For the two compartment model with exchange (Equation 5-10 through to Equation 5-16), the fitted parameters were $p_1$, $D_2$, $\tau_1$, $R_0$ and $\sigma$.

### *5.2.8 Lamellarity*

The lamellarity of a liposome is the number of concentric bilayers that are present in the vesicle. Unilamellar liposomes have only one membrane, while multi-lamellar liposomes will have two or more.

If internal membranes are occupying space inside the aqueous core of the liposome, the entrapped volume will be reduced (section 5.2.9) as will its ability to efficiently entrap hydrophilic material. However, multi-lamellar liposomes may have advantages in allowing the sustained release of entrapped material over a longer period of time. For further discussion on the lamellarity of liposomes, refer to section 2.6.4 in *Chapter 2: Literature Review*.

#### 5.2.8.1 Methods

##### 5.2.8.1.1 Manganese chloride

The technique described by Hope *et al.* (1985) was used to provide an indication of the lamellarity of the liposome dispersions. Briefly, the liposome dispersion was centrifuged at 85,000 *g* for 24 hours and the pellet resuspended in deuterium oxide to give a final phospholipid concentration of approximately 100 g/L. This was then diluted 1:1 with either water or an aqueous $MnCl_2$ solution (final concentrations 1-20mM $Mn^{2+}$). The presence of the $Mn^{2+}$ ions broadens the $^{31}$P-NMR signal from the phospholipids in the outer layer of the

external bilayer, and at a relatively high concentration only the signal from the internal phospholipids should be detected (Fresta *et al.*, 1995). The ratio of the $^{31}$P-NMR signals was calculated, using the sample free of $Mn^{2+}$ as the reference. The ratio indicates what proportion of the phospholipids present are in the interior of the liposome, for example a value of 0.5 would suggest that 50% of the phospholipids were inside the liposomes, indicating that the liposomes were likely to be primarily unilamellar. Increasing the number of membranes inside the liposome would increase the proportion of phosphorus that was unquenched, as a smaller fraction of the total phospholipid present would be on the exterior of the liposome and therefore exposed to the $Mn^{2+}$ ions.

Spectra were obtained using a 400.1 MHz NMR spectrometer operating at 162 MHz. Accumulated free induction decays corresponding to 256 transients were collected using a 9.66 μs 90° radiofrequency pulse, gated proton decoupling and a 65 kHz sweep width. An exponential multiplication corresponding to a 15 Hz line broadening was applied prior to Fourier transformation. The presence of the deuterium oxide provided a lock signal for the NMR.

The accuracy of the dilution with water or $MnCl_2$ solution was checked using the method outlined in section 5.2.1 to ensure the phospholipid concentration was the same for each sample. These were consistent to within ± 5%.

#### 5.2.8.1.2 Lanthanide shift reagent

Difficulties with the above method (as outlined in section 5.3.7.1) led to it being repeated using the lanthanides europium ($Eu^{3+}$) and ytterbium ($Yb^{3+}$) as a shift reagent. Instead of simply broadening the signal from the external phospholipids, the lanthanide shifts the peak from these phospholipids sideways from the original position (Perrett *et al.*, 1991; and Hauser, 1993a). The higher the concentration of lanthanide used, the further the signal is shifted from its original position, but the broader the new peak (Prosser *et al.*, 1998). Therefore, the optimum concentration is a trade-off between ease of differentiation from the internal phospholipids and the need to obtaining a clearly defined peak.

#### 5.2.8.1.3 Ultracentrifugation

Literature suggests that ultracentrifugation may be used to separate uni- and multilamellar liposomes. Perkins *et al.* (1993) states that 5,000-10,000 *g* is usually sufficient to sediment

MLVs, while Hauser (1993a) reports that a supernatent containing only SUVs may be obtained by centrifuging the dispersion at 100,000 *g* for 30 minutes or more. The liposome dispersions were centrifuged at 100,000 *g* for 2 hours. The layers were separated, and particle size measured (section 3.4.1). Negative staining electron microscopy was used to check the lamellarity of the liposomes.

### *5.2.9 Entrapped volume*

A simple method using $^1$H-NMR for estimating the entrapped volume per mass of phospholipid in a liposome population was used. Liposome dispersions were made using the standard production technique. No hydrophilic or hydrophobic materials were added to the vesicles. The liposome dispersion was centrifuged at 85,000 *g* for 2 hours to form a pellet. The centrifuge tube was inverted and left upside down for 30 minutes to drain all excess liquid, then the pellet surface and walls of the tube rinsed twice with deuterium oxide. The pellet was resuspended in deuterium oxide ($D_2O$) to give a final phospholipid concentration of approximately 100 g/L. The liposome membrane is highly permeable to both $H_2O$ and $D_2O$, so these rapidly equilibrate through the system. A calibration curve of $^1$H-NMR signals for known amounts of $H_2O$ in $D_2O$ is used in combination with the $^1$H-NMR signal for the sample to identify the amount of water present. Once the phospholipid concentration is known accurately, the entrapped volume can be reported as mg $H_2O$/g phospholipid.

Unfortunately there was a proportion of the liposome population which did not sediment even when centrifuged at 100,000 *g* for up to 8 hours. This has been identified as a potential problem when using centrifugation to collect liposomes in populations containing SUVs, LUVs, or where their density is close to that of the solution (Perkins *et al.*, 1993). If the majority of the liposomes remaining in the supernatant are SUVs the entrapped volume is likely to have been over-estimated as it has not taken in account the small lipid-to-volume ratio of this class of liposome. However, if they are primarily LUVs, entrapped volume may have been under-estimated as this class has a higher entrapment volume than either SUVs or MLVs.

An alternative method was used based on the membrane impermeable dye 5-6-carboxyfluorescein (CF) (Jousma *et al.*, 1987). Buffer containing 3.5 mM CF was used to produce liposome dispersions by the standard method, and unentrapped CF separated from

the liposomes and entrapped CF by passing aliquots of the dispersion through a 100×10mm column containing Bio Gel P-6 DG desalting gel (BioRad, catalogue 150-0738). The liposomes were then disrupted using Triton X-100 and the amount of CF present measured using a luminescence spectrophotometer LS50B (Perkin Elmer, Wellesley, Massachusetts, USA) with λex = 492 nm and λem = 514 nm. This was combined with the CF concentration of the initial dispersion and the phospholipid concentration of both samples to obtain the millilitres entrapped per gram of phospholipid.

### *5.2.10 Raman spectroscopy*

All spectra presented were recorded on a Bruker IFS66 FTIR spectrometer with a FRA106 Raman module attachment. The instrument was connected via optical fibres to a Nikon microscope. OPUS software was used. The detector was a liquid nitrogen-cooled Ge diode, and the excitation source was an Nd:YAG laser at 1064 nm. No white light background correction was performed.

The samples were placed directly under the microscope and the objective magnification was set to 20x. The spectral running conditions used 2000 scans from each sample, which takes 30 min to collect for a good signal-to-noise ratio, averaging at an 8 cm spectral resolution and a maximum laser power of 20 mW. This low power was chosen in order to ensure that no sample degradation occurred.

## ***5.3 Results and discussion***

### *5.3.1 Hydrodynamic size*

#### 5.3.1.1 Photon correlation spectroscopy (PCS)

Table 5-1 shows the average hydrodynamic diameter of liposome dispersions, containing no entrapped compounds, produced by 5 passes through the Microfluidizer® at 1100 bar. All samples had a high polydispersity value of approximately 0.5, indicating a very wide particle size distribution.

**Table 5-1 Average hydrodynamic diameter ($Z_{ave}$) of liposome dispersions made from a 10% phospholipid solution using a Microfluidizer® at 1100 bar for 5 passes, as measured by photon correlation spectroscopy (PCS).**

| Sample | $Z_{ave}$ (nm) |
|---|---|
| Phospholac | 95 ± 5 |
| SigP3644 | 81 ± 5 |
| Ultralec | 79 ± 5 |

Liposomes produced from the Phospholac fraction had the highest average diameter (approximately 95nm), about 15 nm larger than those made from either the SigP3644 or Ultralec fractions. Liposomes produced from SigP3644 and Ultralec had similar average diameters. These results are in general agreement with Zeisig and Cämmerer (2001), who reported that liposomes produced by Microfluidisation had an average diameter of 100-200 nm with a polydispersity of between 0.2 and 0.6. For further discussion regarding average size and polydispersity of liposome dispersions produced using a Microfluidizer® refer to *Chapter 3: Selection of phospholipid fractions and processing method.*

5.3.1.2 Asymmetric Field Flow Fractionation (AFFF)

Figure 5-3 shows the normalized response of the 90° detector for various liposome diameters (as determined by light scattering). This provided an indication of the relative proportion of liposomes present for each diameter. However, care must be taken when interpreting results as increasing the particle size results in increased scattering for the same number of particles.

Liposomes made from all three of the phospholipid fractions had a primary peak between 50 and 100 nm, with a long tail stretching past 1000 nm. The length of this tail is in agreement with the very high polydispersity values of 0.4-0.5 obtained by the Zetasizer. However, as scattering the intensity is proportional to diameter to the sixth power, it is unlikely that there were a significant number of the liposomes with an average diameter over 300 nm.

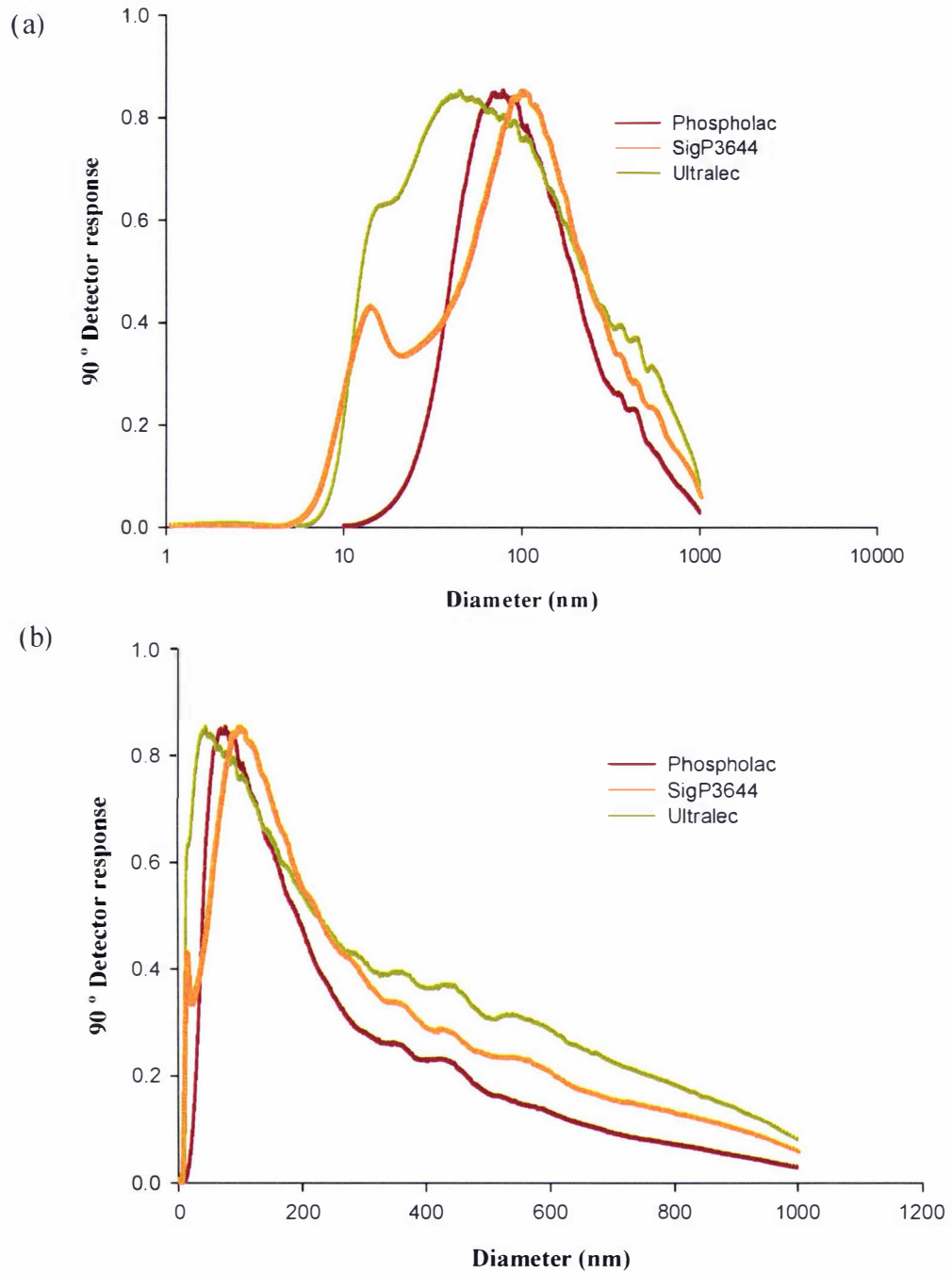



**Figure 5-3 Normalised 90° detector response for specified liposome diameters on (a) log and (b) linear scales as determined by Asymmetric Field Flow Fractionation (AFFF).**

The SigP3644 dispersion also appeared to have a small peak at 15 nm. This is smaller than the 20 -25 nm theoretical minimum liposome diameter (Reineccius, 1995b). This minimum is based on assumptions regarding thickness of the bilayer (Watwe and Bellare, 1995) and overcrowding of phospholipid headgroups causeded by surface curvature (New, 1990b). It has been suggested that Microfluidization may produce micelle-like structures (Lasic *et al.*,

1993) but other workers have refuted this (Škalko *et al.*, 1998). Assuming that the micelle is composed of a monolayer of phospholipid molecules with the hydrophobic fatty acid chains touching in the middle, its diameter would be approximately the same as the thickness of the bilayer. Watwe and Bellare (1995) report membrane thickness in liposomes as being approximately 4 nm, while the results from X-ray diffraction presented in section 5.3.6 range from 4.7-6.8 nm, too small for micelles to be responsible for the peak at 15 nm. Goormaghtigh and Scarborough (1993) used a glycerol density gradient to separate bands of SUVs, and found that 76% of the liposomes present in one of their preparations were smaller than 22 nm in diameter, and 63% had diameters of between 11 and 19 nm. Therefore, it seems probable that the small particles observed using AFFF were indeed very small liposomes.

### *5.3.2 Microstructure*

A variety of electron microscopy techniques are available, including negative staining or thin section Transmission Electron Microscopy (TEM) and Cryo Field Emission Scanning Electron Microscopy (Cryo-FESEM). In addition to simply confirming the presence of liposomes, all three methods provide information on liposome size and shape, and the TEM techniques may also give an indication of lamellarity.

An attempt was made to use image analysis computer software to obtain quantitative information regarding particle size and size distribution. Unfortunately, this was not successful due to inadequacies in the software which did not allow it to differentiate between clustered liposomes. Therefore, all size assessment of micrographs was performed manually and size ranges are only intended as an indication of typical liposome diameters.

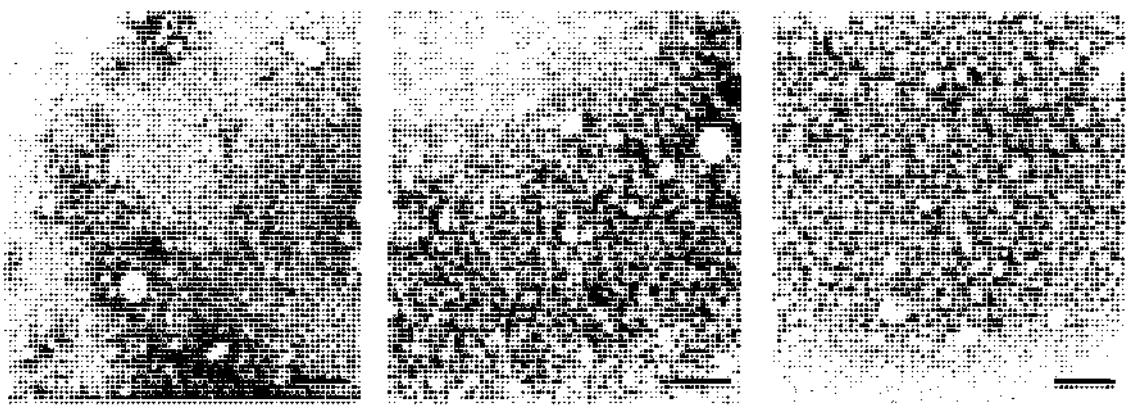
#### 5.3.2.1 Transmission Electron Microscopy (TEM)

##### 5.3.2.1.1 Negative staining TEM

The negative staining technique was very quick and easy compared with the other two methods. Typical micrographs of the different liposome dispersions are shown in Figure 5-4. The liposomes could easily be identified as discrete particles that were predominantly

spherical or rod-like in shape. The outer membrane surrounding the internal aqueous space could be clearly seen in many of the liposomes, and internal membranous structures could also be identified.

The liposomes made using the Ultralec fraction seemed to have the narrowest size distribution, mostly between 50-80 nm. The Phospholac and SigP3644 images contained a large number of very small particles (≤ 40 nm) interspersed with much larger ones (100-200 nm).



**Phospholac** **SigP3644** **Ultralec**

**Figure 5-4 Negatively stained micrographs of liposome dispersions produced as described in section 5.2.3.1. Bar = 0.3 μm.**

Another interesting observation is that many of the liposomes were not spherical, and that a variety of shapes were apparent. Small rods and oval shapes were commonly found, but even shapes as irregular as teat-like protrusions from otherwise spherical vesicles could also be seen (Figure 5-5). It is possible that the rod and sphere shapes are different views of liposome discs, but it seems unlikely that there would be such a high proportion of the vesicles in either the side plane (long rods) or front plane (spheres) without very many in the oblique plane. Škalko *et al.* (1998) showed (using freeze-fracture TEM) that rod- and peanut-shaped liposomes were produced via Microfluidization. Jin *et al.* (1999) have suggested that the assumption that liposomes are spherical particles may be incorrect, and that significant elongation of lipid vesicles occurs naturally. Clerc and Thompson (1994) outlined a possible mechanism for vesicle formation via extrusion, and stated that it was clear that vesicles produced in this manner would initially be non-spherical, but could become spherical through osmotic expansion. Perkins *et al.* (1993) reported that liposomes often adopt non-spherical and irregular shapes, and that to some degree shape can be

manipulated by osmotic gradients. Epand and Polozov (1996) also commented that liposomes produced by classic techniques are often non-spherical due to osmotic stresses caused when the membranes seal before the phospholipids are completely hydrated. Water continues to freely pass through the membrane into the liposome core, largely with the exclusion of the solutes, creating an osmotic imbalance. Deuling and Helfrich (1976) discussed the theoretical shapes of lipid bilayer vesicles and concluded that a large variety of non-spherical but rotationally symmetric shapes are possible, many of which have been observed experimentally.

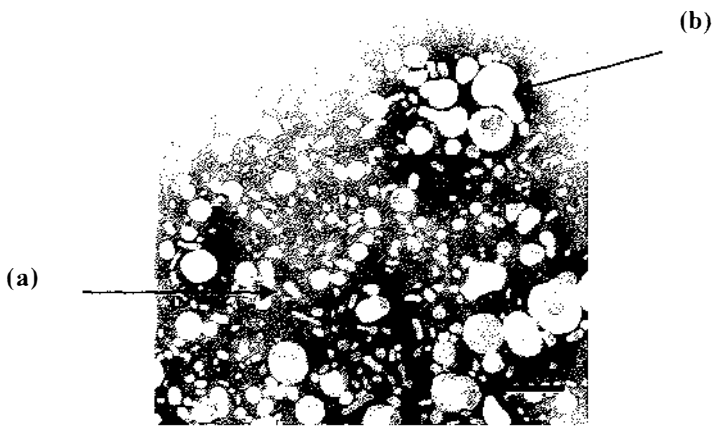
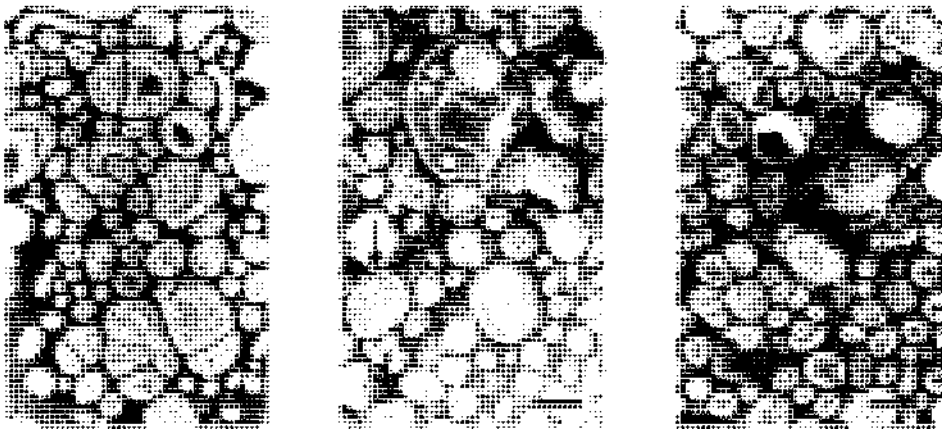



**Figure 5-5 Negatively stained micrograph of SigP3644 liposome dispersion showing irregular shape and elongation of some liposomes. a - rod shaped liposomes; b - teat-like protrusions from liposomes. Bar = 0.3 μm.**

Some of the liposome dispersions appear to have irregular membranous structures trapped inside the outer bilayer (Figure 5-6). These structures only seemed to be present in some of dispersions photographed. Although multilamellar liposomes are usually thought of as being composed of neatly stacked lamellae at regular spacing, according to Perkins *et al.* (1993), most large heterogeneous liposome systems typically contain liposomes with irregular spaced bilayers or 'liposome within liposome' structures. These are perhaps more correctly referred to as multivesicular liposomes (Talsma *et al.*, 1987; Brandl *et al.*, 1998). Freeze-fracture TEM micrographs in Škalko *et al.* (1998) of liposomes produced via Microfluidization also showed irregular shaped vesicles trapped inside other vesicles, as do those formed from phospholipid pastes produced by high-pressure homogenization (Brandl *et al.*, 1998).



**Phospholac** **SigP3644** **Ultralec**

**Figure 5-6 Irregular membrane structures shown by negative-staining TEM as described in section 5.2.3.1. Bar = 0.1 μm.**

This negative staining technique worked very well, but it did tend to produce varying quantities of large crystal-like artefacts as shown in Figure 5-7. There does not seem to be any published reports of such artefacts being caused by the staining process, but it is thought that these are caused by a reaction between the stain and some component in the liposome preparations. The possible relationship between the presence of the artefacts and the buffer salts was eliminated when it was found that the crystal structures appeared even when the liposomes were produced using phospholipids dispersed in deionised water. The commercial phospholipid fractions contain a wide variety of non-phospholipid compounds that could be responsible for the appearance of artefacts. Despite the formation of the artefacts, there were usually a large number of grid squares free from these artifacts, providing excellent micrographs of the liposomes as shown above.

**Figure 5-7 Crystal-like artifact produced during negative staining TEM as described in section 5.2.3.1. Bar = 1μm.**

5.3.2.1.2 Thin section TEM

Typical micrographs of Phospholac and Ultralec liposome dispersions are shown in Figure 5-8. The liposomes appeared to have clumped together, with groups of densely-packed liposomes surrounded by empty space. Many of the vesicles in the clumps seemed non-spherical, although the liposomes in the less crowded areas had retained their spherical shape. This suggests that some of the non-spherical nature of the liposomes may be due to membrane deformation caused by the crowding within the liposome clumps. However, some liposomes appeared shrunken and collapsed, which does not seem to be as a result of tight packing. It seems likely that dehydration steps used during preparation for TEM were responsible for the clumping of the liposomes, and may have contributed to the non-spherical nature of the vesicles.

The apparent multi-lamellar nature of many of the liposomes was obvious, with others appearing to only have a single membrane. It was difficult to know whether these were indeed uni-lamellar liposomes, or whether the sample has been sectioned between the inner and outer membranes of those liposomes without cutting through the inner bilayers.

Visual analysis of the images indicated that the Phospholac liposomes were primarily between 80-100 nm in diameter, slightly smaller than the 80-150 nm seen in the micrographs of Ultralec liposomes.

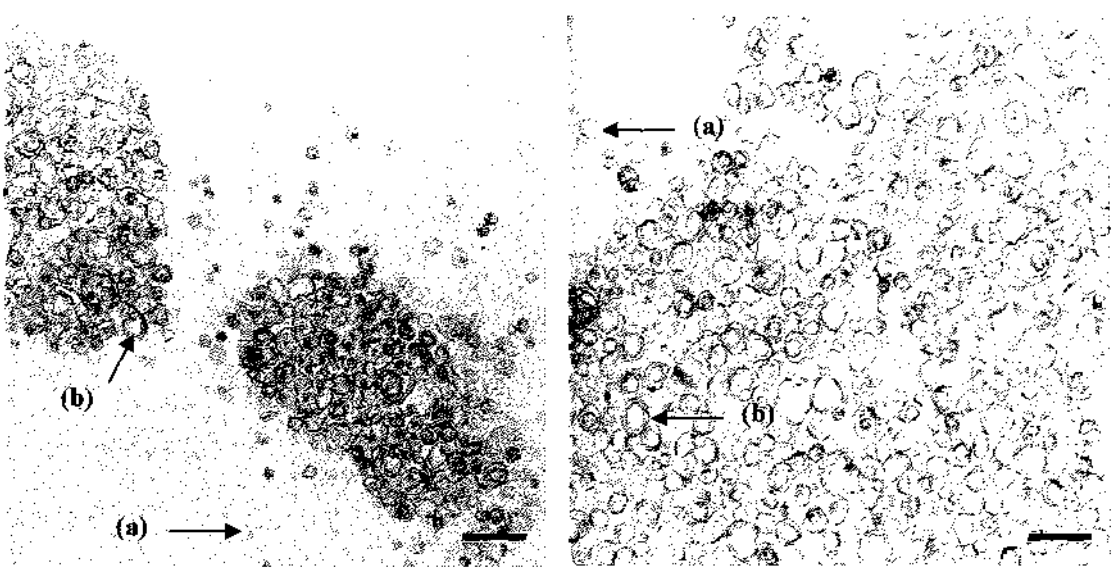
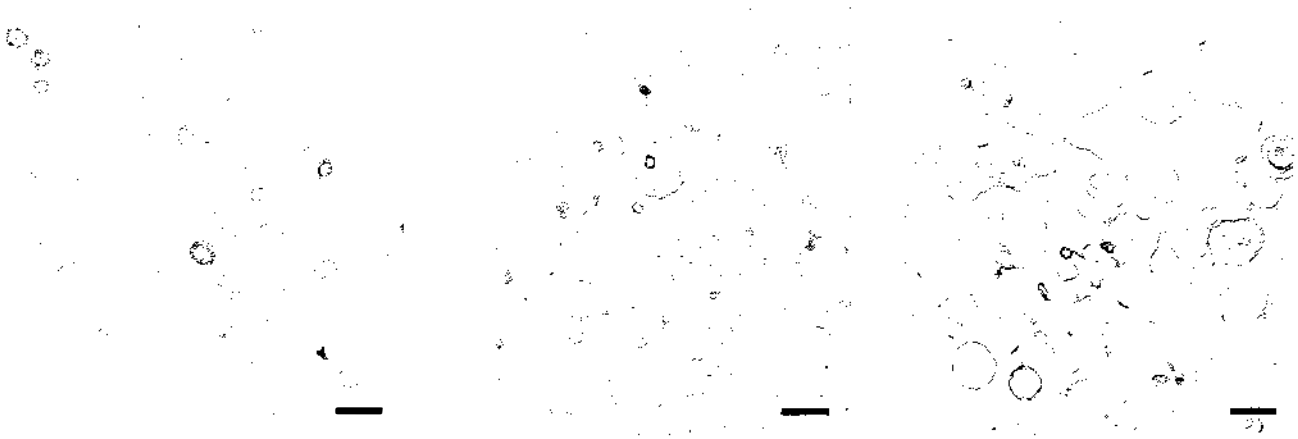



**Figure 5-8 Thin section TEM images of liposome dispersions produced as described in section 5.2.3.1. (a) unilamellar and (b) multi-lamellar liposomes. Bar = 0.3 μm.**

An attempt was made to reduce the severity of the dehydration step by substituting acetone with ethanol at the same concentrations. The micrographs obtained are shown in Figure 5-9. There was little or no clumping in any of the micrographs. However, many of the liposomes still appeared dehydrated and collapsed, although there were a number of intact spherical vesicles present in each micrograph. The Phospholac liposome dispersion seemed to be less severely damaged than the SigP3644 and Ultralec liposomes, with the latter the most affected. Sizes ranged from 50-150 nm for the Phospholac fraction, 80-250 nm for the SigP3644, and 100-500nm for the Ultralec. These size ranges are generally much larger than those obtained using photon correlation spectrometry (section 5.3.1) or other electron microscopy techniques, and may be a reflection of damage caused by the thin section processing. Thin section TEM is therefore not recommended for use on liposome dispersions



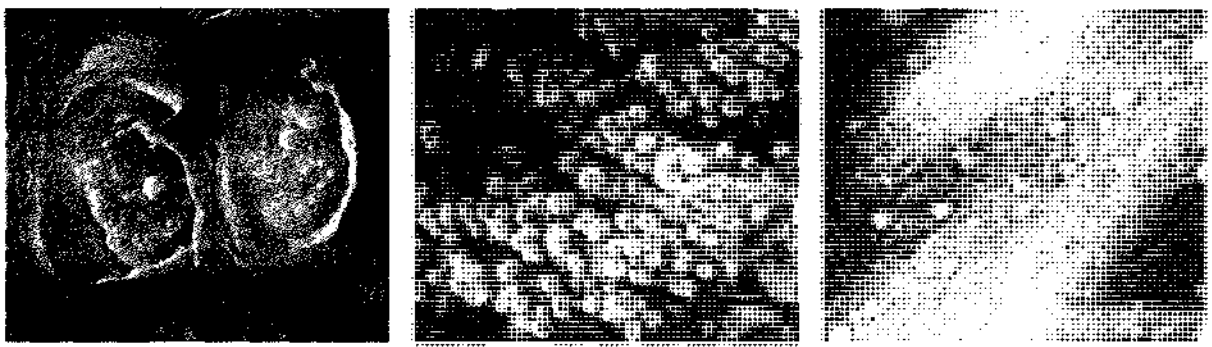
**Phospholac** **SigP3644** **Ultralec**

**Figure 5-9 Thin section TEM images of liposome dispersions produced as described in section 5.2.3.1,but using ethanol instead of acetone during the dehydration step. Bar = 0.3 μm.**

### 5.3.2.2 Cryo-FESEM

Micrographs produced by the Cryo-FESEM technique provide three-dimensional confirmation of structures consistent with that of liposomes - smooth spheres with a membrane surrounding an internal compartment. Typical micrographs for each of the liposome dispersions are shown in Figure 5-10. As expected, the approximately spherical vesicles had a wide particle size distribution, with both large and small liposomes evident in all three dispersions. In the micrograph shown in Figure 5-11, a number of liposomes can be

seen that have been broken during the Cryo SEM preparation process. This has revealed an internal space which had been fully enclosed by the outer shell of the liposome structure.



**Phospholac** **SigP3644** **Ultralec**

**Figure 5-10 Cryo-FESEM micrographs produced by method outlined in section5.2.3.2. Bar = 0.5μm.**

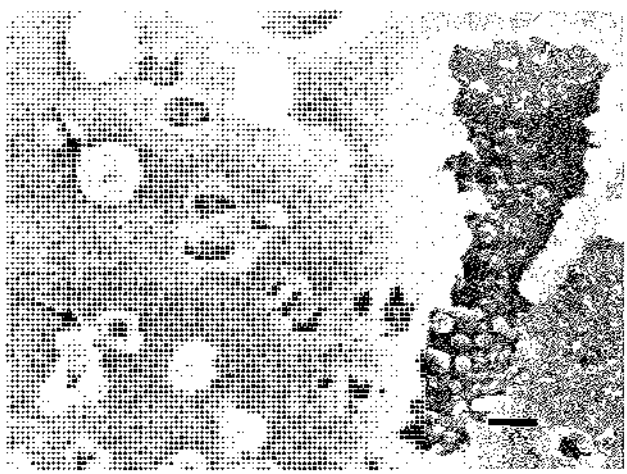


**Figure 5-11 Cryo-FESEM micrograph produced from SigP3644 liposome dispersion showing fractured liposomes revealing membrane shell surrounding internal cavity. Bar = 0.1μm.**

The liposomes in the SigP3644 dispersions seemed to be mainly between 150-300 nm in diameter, although a number of much smaller liposomes of approximately 80 nm in diameter were also present. These could be seen more clearly in Figure 5-12. Similarly, the Ultralec dispersion appeared to contain liposomes across a wide size range - from less than 100 nm up to 300 nm in diameter. Although the quality of the Phospholac images was not as good as that of the SigP3644 dispersions, it was still possible to see a number of large liposomes with diameters around 200 nm as well as some very small ones of approximately 40 nm in diameter.

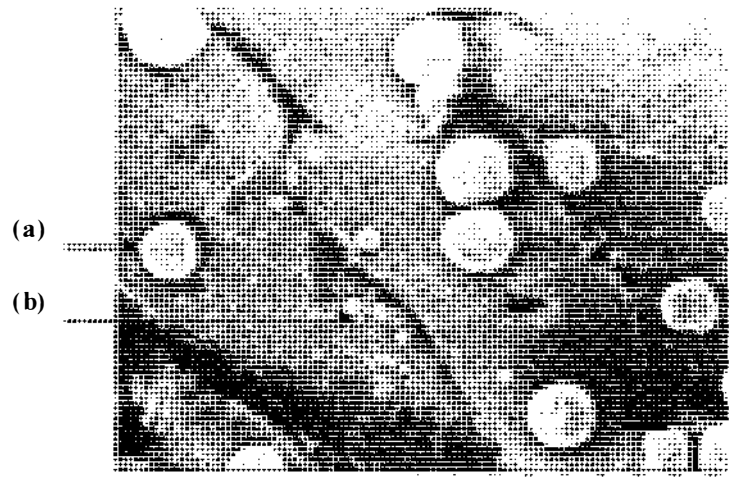



**Figure 5-12 SigP3644 Cryo-FESEM micrograph produced using method outlined in section 5.2.3.2 showing both large (a) and small (b) liposomes. Bar = 0.3µm.**

The ability to visualise the liposomes using a variety of electron microscopy techniques has provided valuable information regarding the characteristics of the different liposome populations. The TEM electron micrographs of all three liposome dispersions showed multivesicular vesicles where there were liposomes inside liposomes or membraneous material inside the outer bilayer (Figure 5-13), but there were relatively few images of true multilamellar liposomes with concentric, evenly spaced bilayers.

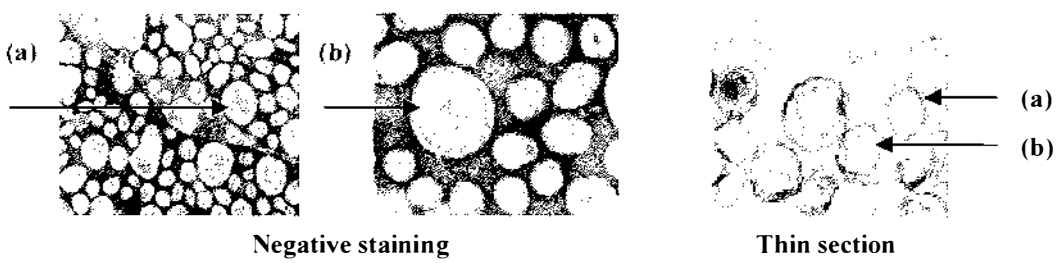



**Figure 5-13 TEM of liposome dispersions showing (a) unilamellar and (b) multilamellar liposomes.**

Based on negative staining TEM, there may be a slightly higher proportion of multivesicular or multilamellar vesicles in the dispersions produced from SigP3644 and Ultralec fractions, but it is difficult to determine whether this is an accurate reflection of the lamellarity of the populations. There is also the possibility that the multilamellar structures may be artifacts caused by the staining or embedding process. The problems encountered with the thin-section method are outlined above. Hauser (1993a) states that negative staining is troubled by its inherent probability of generating artificial lipid structures, presumably due to the interaction of the stain with other compounds present in the system. Cryofixation techniques are accepted as being more reliable in their preservation of the native structure, but are time-

consuming and unable to provide information on lamellarity. It seems that use of all available techniques with careful interpretation of results taking into account conflicting or supporting information from other sources is the best way to fully characterise liposome dispersions. Lamellarity is investigated further in section 5.3.7.

Table 5-2 shows the liposome diameters as determined using a variety of techniques. There are obvious differences between the values obtained by the different techniques, which is to be expected based on the principles behind each method. Negative staining and thin section TEM show a cross-section through the liposome, which will not always be through the centre of the liposome and will therefore not always reflect the true liposome diameter. The $Z_{ave}$ obtained by PCS gives an indication of the average hydrodynamic diameter of the liposomes within a population, but does not provide information on the size distribution. The polydispersity is very high (0.4-0.5), suggesting a very wide distribution, as was shown by the AFFF results.

**Table 5-2 Comparison of liposome sizes measured using different techniques.**

| Phospholipid fraction | PCS* ($Z_{ave}$, nm) | AFFF Peak range (nm) | Cryo-FESEM (est. range, nm) | Negative staining TEM (est. range, nm) | Thin-section TEM (est. range, |
|---|---|---|---|---|---|
| Phospholac | 95 ± 5 | Peak centre: 80<br>Narrowest distribution | 40-200 | ≤40-200 | 50-150 |
| SigP3644 | 81 ± 5 | Peak centre: 100<br>Small peak at 14nm | 80-150 | ≤40-200 | 80-250 |
| Ultralec | 79 ± 5 | Peak centre: 50<br>Widest distribution | 100-300 | 50-100 | 100-500 |

*Polydispersity of 0.4-0.5 for all three fractions, indicating a very broad particle size distribution

Overall, there seems to be a general agreement regarding all three liposome dispersions having a very broad size distribution, with an average liposome diameter between 50-200 nm. While it is useful to have an understanding of the average liposome size and the width of the size distribution, the main use of liposome size throughout this thesis is as an indicator of liposome stability. The robustness of the $Z_{ave}$ value means that although the absolute value may differ from the average liposome diameter as measured by other techniques, it can still be confidently used to identify changes and follow trends within the system.

### 5.3.3 Zeta potential

The zeta potential values for liposomes at various pH values are shown in Figure 5-14. The three liposome dispersions demonstrated similar shaped curves, with negative potentials at pH values above 3 and a flat region between pH 5 and 10. The SigP3644 liposome dispersion had the highest zeta potential value, ~ -80 mV between pH 5 and 10 compared with ~ -65 mV for both Phospholac and Ultralec liposome dispersions. Below pH 5, the zeta potentials for all dispersions became rapidly less negative and approaching zero. The liposome dispersion made from Phospholac appeared to have a potential of zero at pH 2.6, rising to a positive potential of 15 mV at pH 2.0. The Ultralec dispersion had a potential of zero at pH 2.0, but the potential for SigP3644 liposomes had not yet reached zero at this pH. There are very few zeta potential values for liposomes reported in the literature, and the significant effect of the composition of buffer salts used on the potential renders any comparison between different systems difficult. However, a zeta potential of approximately -60 to -80 mV is much larger than those of between 0 and -43 mV reported for other liposome dispersions (Talsma *et al.*, 1989; Ruel-Gariepy *et al.*, 2002).

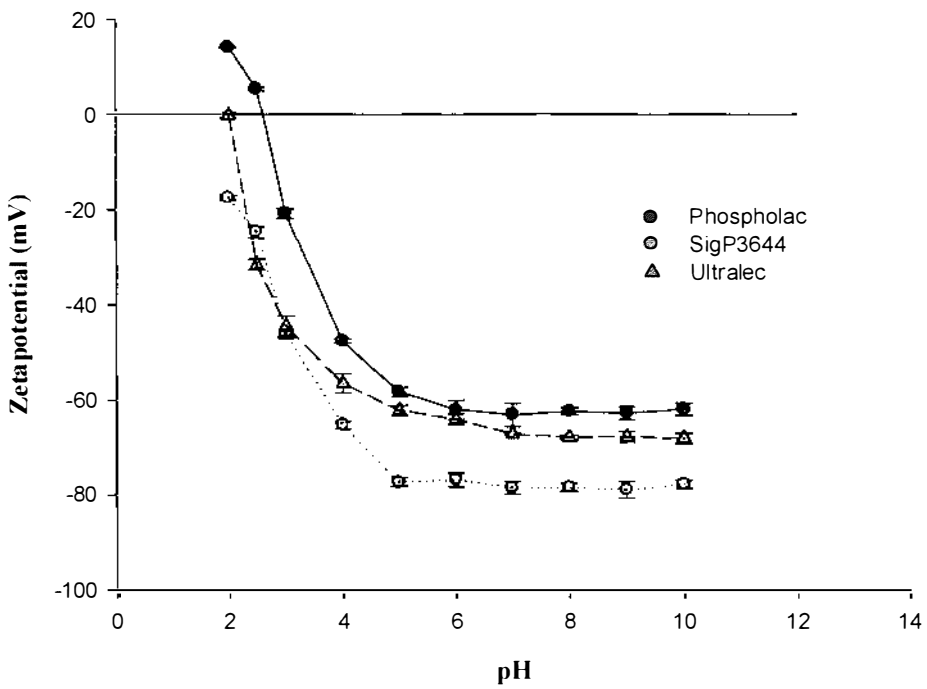



**Figure 5-14 Zeta potential versus pH curve for liposomes produced from Phospholac, SigP3644 and Ultralec**

Based on these results, it would be expected that liposomes produced from SigP3644 are likely to have a higher degree of charge repulsions ≥ pH 5 than liposomes produced from

either Ultralec or Phospholac. These repulsions may provide the SigP3644 liposome dispersions with enhanced resistance to aggregation or coalescence within this pH range. From pH 2.5 to 4 there is no significant difference between SigP3644 and Ultralec, and any difference in stability between the fractions is unlikely to be due to charge repulsions.

The relationship between the zeta potential and the phospholipid composition of the three fractions is discussed in *Chapter 8: General discussion.*

### *5.3.4 Phase transition temperature*

The DSC thermogram is shown in Figure 5-15. There was a broad endothermic peak between 20 and 35 °C for the Phospholac liposome dispersion, with the peak at approximately 28 °C. This probably corresponds to the bilayer membranes transforming from gel to liquid-crystal state. There were no significant features present between 0 and 60 °C in the liposome dispersions made from SigP3644 or Ultralec.

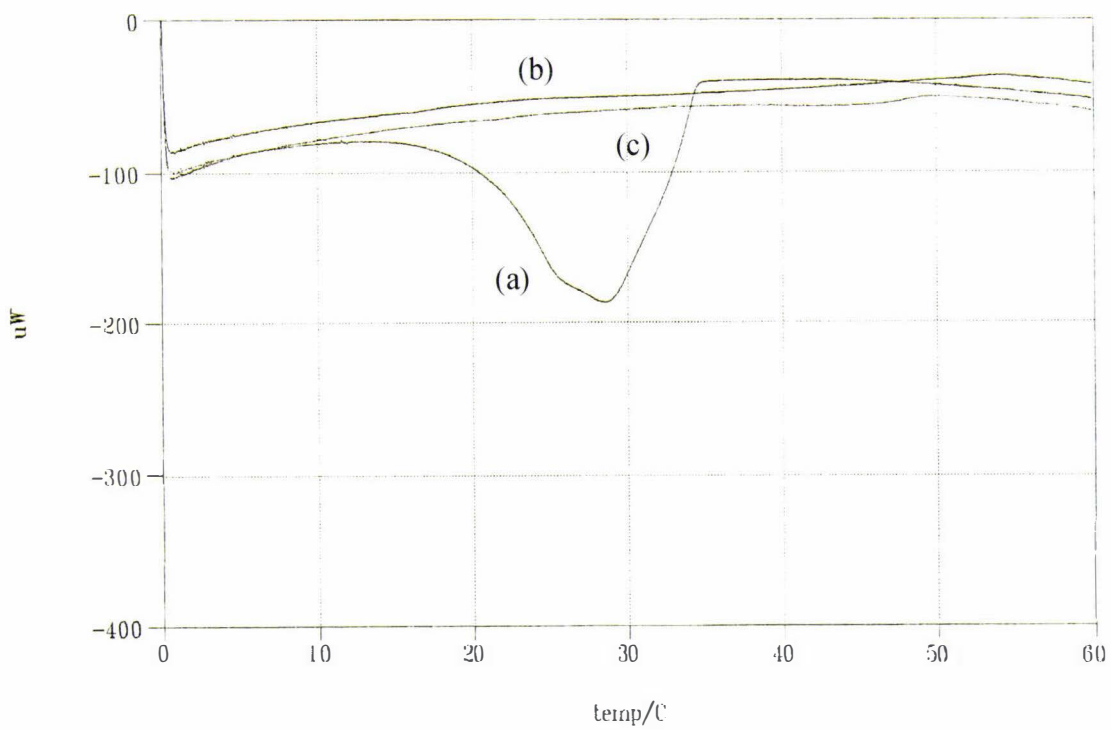



**Figure 5-15 DSC thermogram for liposome dispersions made using different phospholipid fractions. (a) = Phospholac; (b) = SigP3644; (c) = Ultralec.**

These results were as expected based on the origin of the phospholipid fractions. As discussed in section 2.3.2 (*Chapter 2: Literature Review*), non-hydrogenated soy phospholipids usually undergo phase transition between -15 to -5 °C, while phospholipids from animal sources have phase transition temperatures above 0 °C. Milk spingomeylin has been reported as having a $T_c$ of 35 °C at concentrations of <70 wt% (Malmsten *et al.*, 1994).

It is extremely difficult to separate any thermal effects caused by changes in the structure of the membrane around 0 °C from the extremely large peak resulting from the freezing/thawing of the water in aqueous phase. This can be avoided by working with concentrated glycerol solutions to depress the freezing point of the water. However, simply knowing that the phase transition temperature for the SigP3644 and Ultralec fractions was below 0 °C was specific enough for the purposes of this work.

The width of the observed endothermic peak (Figure 5-15a) is a reflection of the complicated mixture of phospholipid classes and types of fatty acids present in the fraction (Lee, 1977a,b), and is similar to endotherms obtained for biological membranes (McElhaney, 1996). Each different combination of head-group and fatty acid will have a slightly different phase transition temperature, resulting in the co-existence of gel and fluid phases and a total phase transition over a wide range of temperatures (Marsh, 1996). Biltonen and Lichtenberg (1993) reported that SUVs with diameters below 35nm produced a much broader transition at a temperature approximately 4 °C lower than that of LUVs or MLVs with the same composition. Lichtenberg *et al.* (1988) found that liposomes with diameters less than about 35 nm had a lower phase transition temperature than liposomes with diameters between 40-70 nm. They suggested that this is due to the tight curvature of SUVs reducing the packing density of the phospholipid molecules, resulting in fewer van der Waals forces than would be present in larger molecules. The AFFF results (section 5.3.1.2) show the Phospholac liposome dispersion does contain some vesicles with diameters less than 35 nm, which may be contributing to the breadth of the endothermic peak shown in Figure 5-15a.

The position of the endothermic peak for liposomes made from Phospholac may offer both advantages and disadvantages in terms of the possible use of liposomes in food systems. Liposomes in products stored at refrigerator or low-ambient temperatures will have their membranes in the gel phase. The literature states that membranes in the gel phase are likely to be more stable and retain entrapped solutes for longer periods than similar liposomes membrane in the fluid phase. This suggests that the Phospholac dispersions may have

stability advantages at refrigeration temperatures over other dispersions with phase transitions below 4 °C. However, at temperatures corresponding to the endothermic peak, the Phospholac liposome membranes are expected to increase in permeability, and solute retention is likely to decrease. This may be useful if the release of entrapped material is desirable upon heating or consumption of the food product, but could also be of concern if storage or transport at these temperatures is required.

The stability and permeability of liposomes produced from the soy fractions may still be affected by changes in temperature, but these changes will be predominantly caused by mechanisms other than the phase transition of the membranes.

### *5.3.5 Bilayer thickness*

The results from the small angle x-ray diffraction of the concentrated liposome suspensions at 20 °C are shown in Figure 5-16. This temperature was above the phase transition temperature for the SigP3644 and Ultralec liposomes, but below the transition temperature for the Phospholac liposomes. A sample of buffer was used as a control.

A very broad and intense ring for each of the liposome suspensions was observed. There was also a faint outer ring visible as a blue halo on the images, but this was too weak to show up as a significant peak on the intensity traces. The primary ring in the Ultralec sample was particularly wide and diffuse, and the intensity trace showed a relatively flat peak. Upon closer inspection, it appeared that there may be a slight reduction in intensity in the middle of this ring, suggesting that it could be two broad overlapping peaks (Figure 5-17).

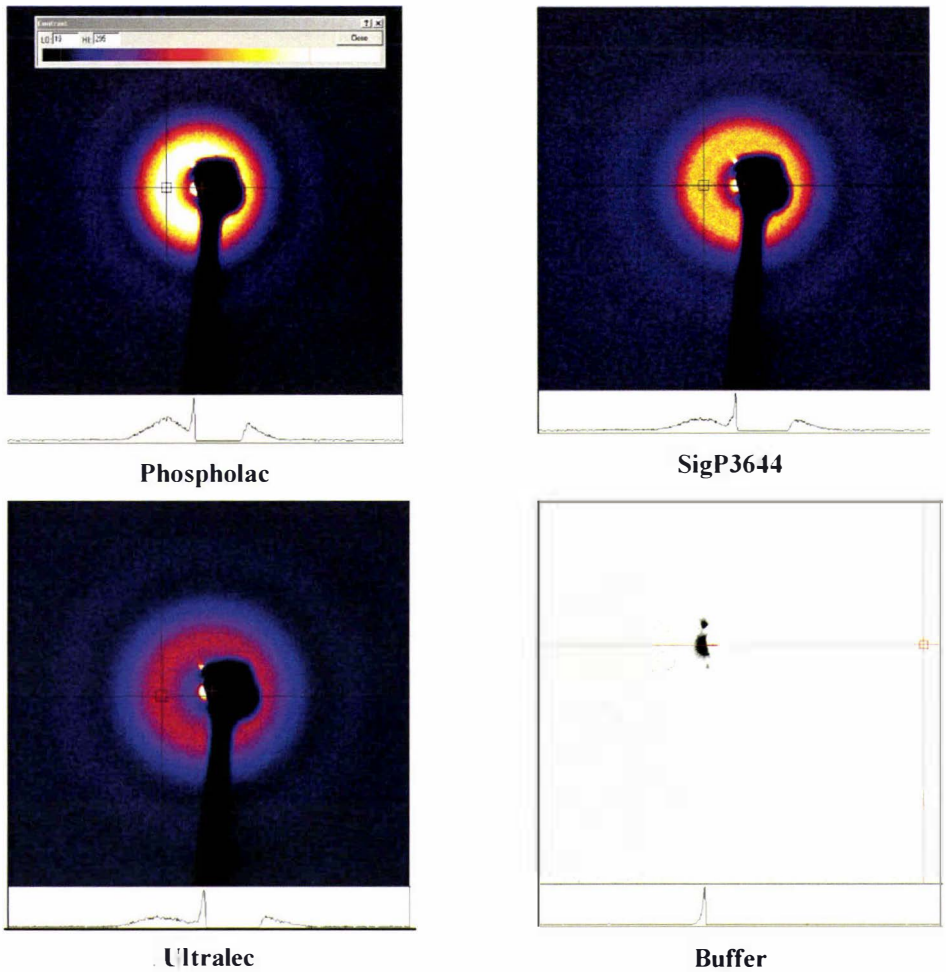



**Figure 5-16 X-ray scattering patterns for liposome dispersions and buffer (control) at 20 °C.**

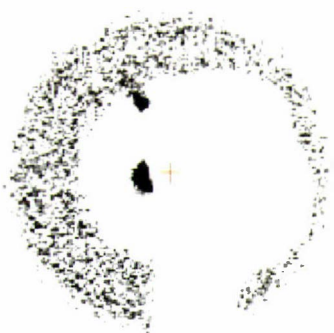


**Figure 5-17 Close-up of Ultralec ring at 20 °C.**

X-ray scattering curves from dispersions containing only unilamellar liposomes are typically very broad and flat, and usually have only a single symmetric peak. Those from multilamellar liposomes exhibit first- and second-order diffraction peaks at regular intervals,

i.e. 1/d, 2/d etc. (Hauser, 1993a; Moody, 1993; Kinnunen *et al.*, 2003). A primarily unilamellar population with a small number of bi- or tri-lamellar vesicles is likely to result in a broad, asymmetric peak (Bouwstra *et al.*, 1993).

The more concentric bilayers present at equal distances within the liposomes, the lower the concentration required to produce sharp diffraction peaks. If the majority of the multi-lamellar liposomes present are only bi- or tri-lamellar, they need to constitute a significant proportion (>20%) of the total liposome population before they will have a clear effect on the x-ray scattering curve (Bouwstra *et al.*, 1993). If different multilamellar liposomes have different lamellar spacings either within the same vesicle or between different vesicles, the diffraction patterns for the system may average out to a broad smoother peak (Moody, 1993). In addition, multivesicular liposomes (such as those shown in Figure 5-13) will be recognised as unilamellar structures by the diffuse small angle X-ray diffraction (Talsma *et al.*, 1987).

**Table 5-3 Positions of diffraction maxima obtained by small X-ray diffraction of liposome dispersions at 20 °C.**

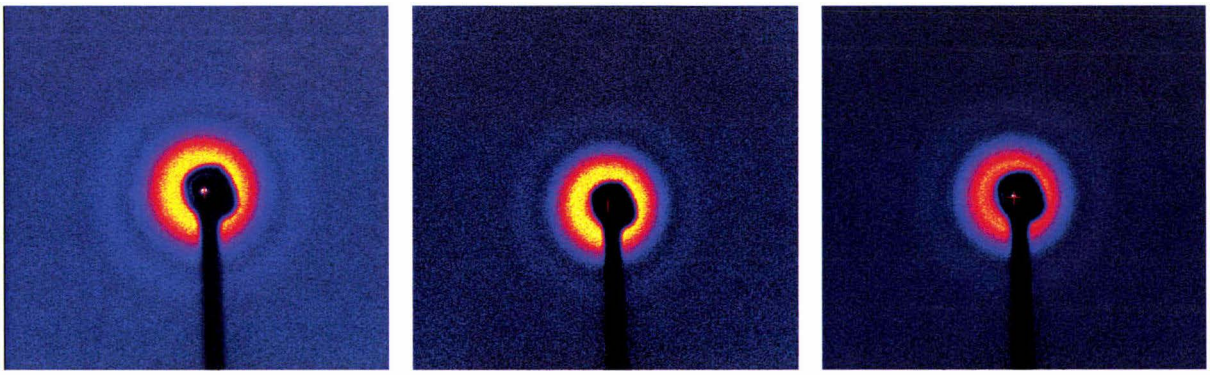
| Phospholipid fraction | Inner ring (Å) | Outer ring (Å) | Peak width at $^1/_2$ peak height (Å) |
|---|---|---|---|
| **Phospholac** | 68 ± 1.5 | 22 ± 0.5 | 50 |
| **SigP3644** | 53 ± 0.5 | 18 ± 1.0 | 42 |
| **Ultralec** | 66 ± 1.0 / 47 ± 1.0 | 20 ± 1.0 | 42 |

Table 5-3 shows the positions (in Å) of the diffraction maxima for each of the three liposome suspensions. The inner ring distances provide an indication of the bilayer thickness for the liposomes in each of the dispersions. Phospholac liposomes had the largest bilayer or membrane thickness, over 15 Å or 1.5 nm wider than the SigP3644 liposomes. The Ultralec fraction may have two separate liposome populations, one with a membrane thickness of 66 Å and the other with thickness of approximately 47 Å. This suggests possible segregation of different phospholipids present in the fraction into different liposomes. The X-ray scattering band was quite wide for both Phospholac and SigP3644, which may be masking an overlap of more than one band but could also be due to the low degree of order present in the system.

The symmetric broad scattering curves obtained for the three phospholipid fractions suggest that virtually all the liposomes are either unilamellar or multivesicular. The faint outer ring observed was unlikely to be due to the presence of multilamellar vesicles as it was not a fraction of the primary peak i.e. 22 Å is not $^1/_2$ of 68 Å. It does however indicate that there is a degree of order at between 18 and 20 Å, which was too small to be the length of a single phospholipid molecule. The interior of the liposome membrane is an ordered arrangement of the fatty acid chains, and it is possible that this ring could reflect the average length of the fatty acid portion of the phospholipid. The carbon-carbon bond is approximately 1.54 Å, with a bond angle of about 109.5 degrees. This means that the vertical length of a $CH_2$ group along the chain is approximately 1.26 Å. Therefore, a C18 chain would be approximately 22.7 Å in length. This hypothesis is supported by Coster (1999), who states that a similar diffuse ring at the same relative position to the bilayer membranes is caused by acyl chains. SigP3644 and Ultralec both contained a higher proportion of long chain fatty acids than Phospholac (section 4.3.4 in *Chapter 4 Characterisation of phospholipid fractions*), but they also had a higher proportion of double bonds which would shorten the overall chain length. The lack of definition for this faint outer ring may be a reflection of the wide variety of fatty acids present in each of the phospholipids fractions.

Figure 5-18 shows the x-ray scattering patterns for liposome dispersions held at 40 °C, with the position of the diffraction maxima presented in Table 5-4. At this temperature, the liposomes in all three dispersions will be in the fluid state.

The x-ray scattering patterns are similar to those observed at 20 °C, with a bright ring close to the beam-stop at approximately 50-70 Å, and a second diffuse ring at 18-20 Å. The main ring is much broader at 40 °C than at 20 °C, and it is difficult to tell exactly where the intensity maxima peak is. There may also be a third very faint ring on the Phospholac image at approximately 13 Å. The increase in width and diffusivity of the main ring may reflect a more heterogeneous liposome population caused by the higher temperatures. As the temperature increases, the fluidity of the bilayer increases, allowing more movement within the bilayer and possibly a greater range of bilayer widths.



**Phospholac** **SigP3644** **Ultralec**

**Figure 5-18 X-ray scattering patterns for liposome dispersions at 40 °C.**

**Table 5-4 Positions of diffraction maxima obtained by small X-ray diffraction of liposome dispersions at 40 °C.**

| Phospholipid fraction | Inner ring (Å) | Outer ring (Å) | Peak width at $^1/_2$ peak height (Å) |
|---|---|---|---|
| **Phospholac** | 65 ± 3 | 20 ± 1 / 13 ± 2 | 62 |
| **SigP3644** | 52 ± 3 | 18 ± 1 | 53 |
| **Ultralec** | 59 ± 3 | 20 ± 1 | 50 |

Marsh (1996) states that the lipid bilayer is 'considerably' thinner in the fluid state than the gel state, but no examples giving actual numerical changes in bilayer thickness were provided. Malmsten *et al.* (1994) commented that the repeat distance of the lamellar phase for milk SM changed by <5 Å between the gel and fluid phases. There did not appear to be a significant reduction in bilayer thickness for the Phospholac dispersion as the temperature was increased from 20°C to 40 °C (Table 5-5). The similar thicknesses of the Phospholac and Ultralec membranes mean that any differences in permeability between these dispersions is unlikely to be simply due to bilayer thickness, however this may contribute to more permeable membrane in SigP3644 dispersions.

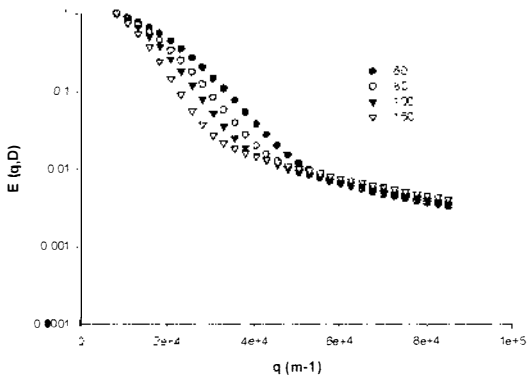
**Table 5-5 Comparison of phospholipid bilayer thickness as determined by small angle X-ray diffraction at 20 and 40 °C.**

| Phospholipid fraction | Bilayer thickness (Å) | |
|---|---|---|
| | Liposome dispersion at 20 °C | Liposome dispersion at 40 °C |
| **Phospholac** | 68 | 65 |
| **SigP3644** | 53 | 52 |
| **Ultralec** | 66 | 59 |

### *5.3.6 Permeability*

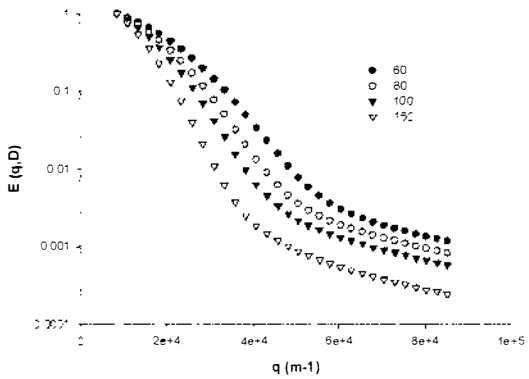
The membrane permeability of the various liposome dispersions will depend on the specific molecule diffusing through the bilayer. However, measuring the rate at which a particular species permeates the membrane should provide an indication of the relative permeabilities of the different liposome dispersions. Water was chosen as an appropriate species as it was known to be present in all of the liposome systems and had been used for similar techniques in the published literature.

The first step in determining the permeability of water through the bilayer was to confirm that there was indeed exchange between the water trapped inside the liposome and the bulk aqueous phase. Figure 5-19 shows plots of the relative signal attenuation for the water peak for values of $\Delta$ between 60 and 150 using both the model that is based on no exchange between compartments (Equation 5.7) and the model that assumes some exchange will be taking place (Equation 5-10). When these plots are compared with the experimental data obtained from the liposome dispersions, it is obvious that the model that includes exchange provides a much closer fit to the actual results.



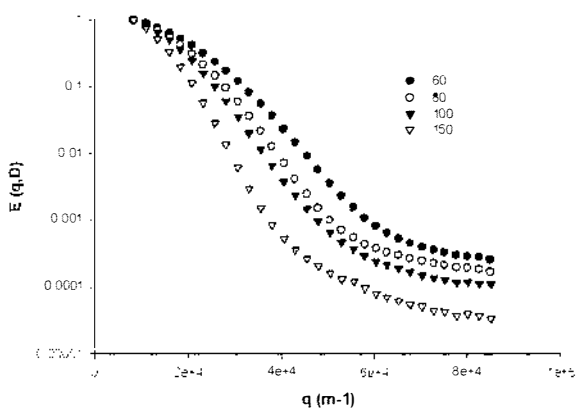

Model with no exchange (Equation 5-7)

a=5e$^{7}$, sigma=0.8, Diff=1.0e$^{-9}$, p1=0.05, Diff=1e$^{-9}$ m$^{2}$/s




Model with exchange (Equation 5-10)

a=5e$^{7}$, sigma=0.8, p1=0.05, t1=0.05sec, Diff =1e$^{-9}$ m$^{2}$/s

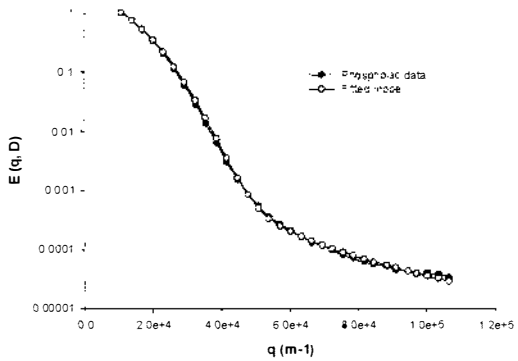



Typical response for liposome dispersions

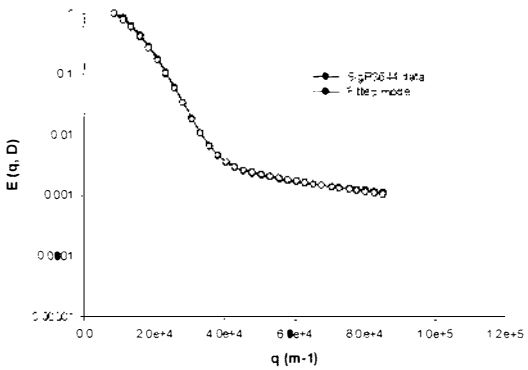
(Actual data from a Phospholac dispersion)

**Figure 5-19 Plots of the relative signal attenuation for the water peak measured using the PFG-NMR technique outlined in section 5.2.7.**

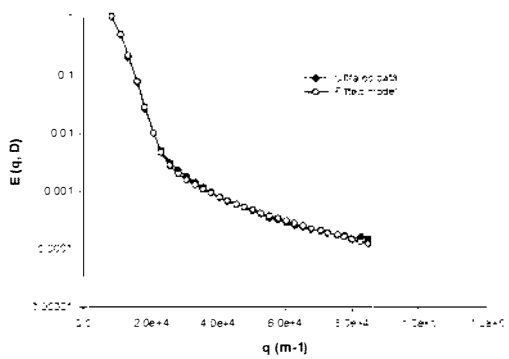
The experimental data overlaid with the fitted model for each of the three liposome dispersions is shown in Figure 5-20. The plots shown used different q and D values so cannot be directly compared, but it can be seen that the fitted model closely matches the experimental data.




Phospholac liposome dispersion




SigP3644 liposome dispersion




Ultralec liposome dispersion

**Figure 5-20 Experimental data and fitted models for the signal attenuation of the water peak for the different liposome dispersions using the PFG-NMR technique outlined in section 5.2.7.**

Using the equations presented in section 5.2.7, coefficients for the diffusion of the water molecules in the bulk aqueous phase were calculated (Table 5-6). Assuming a log-normal distribution of liposome sizes for each of the dispersions, the mean liposome radius could be estimated. These figures suggest that the liposomes produced from the Phospholac fraction had the smallest mean diameter, approximately half that of the Ultralec liposome dispersion. Liposomes made from the SigP3644 fraction had the largest overall size.

The liposome diameters estimates using the PFG NMR are within the same approximate order of magnitude as those obtained from the particle sizing techniques discussed earlier (section 5.3.1 and 5.3.2). However, the estimated mean diameter acquired through this method is at the higher end of the range indicated by the electron micrographs. This is not unexpected, as Figure 5-1 shows that the size distribution of the liposome dispersions is not strictly a log-normal distribution. The PFG NMR mean sizes are also based on a volume-weighted average, which will favour the larger liposomes in spite of the small vesicles being

more numerous. Like many models, the one used for this experiment is not derived from a true physical representation of the system, but it still provides a useful indication of the system behaviour.

**Table 5-6 Summary of water permeability through the liposome membrane at 20 °C**

| Sample | Bulk water diffusion (D2 & D1) ($m^2/s$) | Fitted to volume-weighted log-normal distribution | | | | |
|---|---|---|---|---|---|---|
| | | Mean liposome radius (r) (nm) | Standard deviation | Fraction of water that is inside liposomes | Mean exchange time (seconds) | Mean permeability (m/s) |
| **Phospholac** | 3.41E-10 | 470 | 0.85 | 0.043 | 0.098 | $1.60E^{-06}$ |
| **SigP3644** | 3.3E-10 | 685 | 0.76 | 0.041 | 0.049 | $4.66E^{-06}$ |
| **Ultralec** | 3.26E-10 | 172 | 0.9 | 0.053 | 0.044 | $1.30E^{-06}$ |

The mean permeability of the three dispersions is also shown in Table 5-6. These results indicate that water diffuses through a liposome membrane made from the SigP3644 fraction at approximately $4.7E^{-06}$ m/s, three times faster than it is able to permeate membranes composed of the Phospholac or Ultralec fractions.

These values fit well with the 3-6 × $10^{-6}$ m/s reported by Perkins *et al* (1993) for the average permeation coefficient for water diffusing through liposome membranes, and are close to the $10^{-5}$ m/s mentioned by Epand and Polozov (1996). They also agree with the results from the small-angle x-ray diffraction (section 5.3.5), which found that the average bilayer thickness of the Phospholac and Ultralec dispersions was approximately the same, but it was smaller for liposomes produced from the SigP3644 fraction.

The effect of temperature on the water permeability of the liposome membranes is shown in Table 5-7. Further details of these measurements are discussed in section in *Chapter 7: Entrapment of hydrophobic and hydrophilic compounds in liposome dispersions.* Increasing the temperature from 20 to 40 °C does not appear to significantly affect the permeability for either the SigP3644 or Ultralec membranes, although it is possible that there is a slight decrease in permeability across this temperature range for the Phospholac dispersion.

**Table 5-7 Effect of temperature on the membrane permeability of water.**

| Sample | Mean permeability of water through the liposome membrane (m/s) | | | |
|---|---|---|---|---|
| | **20 °C** | **28 °C** | **30 °C** | **40 °C** |
| **Phospholac** | $1.6E^{-06}$ | $1.5E^{-06}$ | $1.4E^{-06}$ | $1.2E^{-06}$ |
| **SigP3644** | $4.7E^{-06}$ | | | $4.8E^{-06}$ |
| **Ultralec** | $1.3E^{-06}$ | | | $1.1E^{-06}$ |

The results seem to indicate that there is no effect of membrane phase or of phase transition on the permeability of the liposome membranes to small molecules such as water. Literature states that the permeability of the membrane should peak at the phase transition temperature, but there is no indication of any increase in Phospholac membrane permeability at 28 or 30 °C. The presence of high concentrations of cholesterol can reduce or remove evidence of this transition, but *Chapter 4: Characterisation of the phospholipid fractions* found that the Phospholac fraction contained less than 0.05% cholesterol, too low to have any significant affect on the phase transition. The transformation of the Phospholac membrane from the gel to the liquid crystal phase should result in an increase in membrane permeability as the packing density of the membrane decreases, but the experimental data show a small decrease in measured permeability.

The increase in temperature did not change the phase of the SigP3644 and Ultralec membranes and so the lack of a significant affect of the temperature on permeability seems reasonable. In addition, it would be expected that the denser packing of the Phospholac membrane in its gel phase at 20 °C would result in a lower membrane permeability compared with the liquid crystal arrangement of the SigP3644 and Ultralec dispersions. However, while the Phospholac dispersion has a much lower permeability than the SigP3644 sample, there is very little difference between the Phospholac and Ultralec samples.

### 5.3.7 Lamellarity

#### 5.3.7.1 Manganese chloride

Figure 5-21 shows that addition of increasing amounts of $Mn^{2+}$ ions resulted in successively smaller proportions of the phosphorus contributing to the $^{31}$P-NMR peak. The Phospholac and SigP3644 dispersions seemed to plateau with approximately 40% and 15% unquenched phosphorus respectively, but the Ultralec dispersion had no remaining unquenched phosphorus after the addition of 10g $MnCl_2$/l. These values are obviously well below the minimum of 0.5 expected, based on the simplified theory behind this technique.

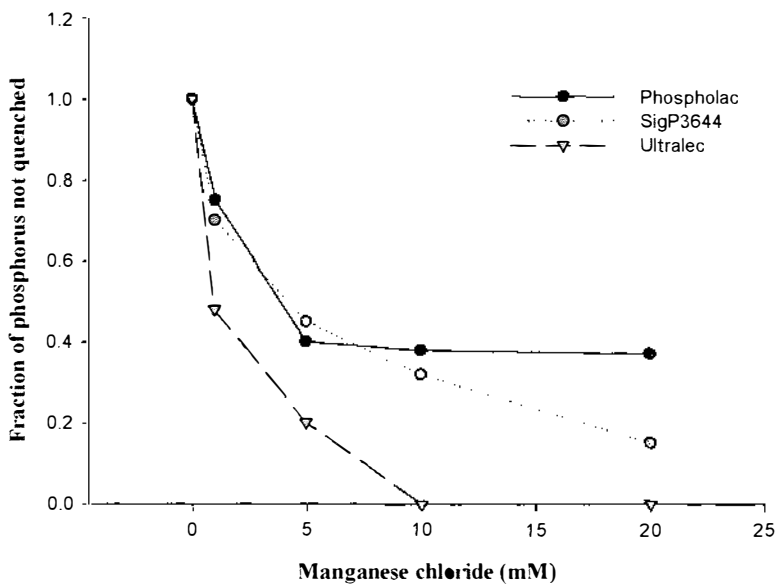



**Figure 5-21 Proportion of original phosphorus not quenched at varying $Mn^{2+}$ concentrations.**

The external and internal surface areas of liposomes of different diameters were calculated based on the equation for the surface area of a sphere and using the bilayer thickness as calculated in section 5.3.5. The approximate proportion of the total surface area that would be in the interior of the vesicle was determined, and is shown for a variety of liposome diameters in Table 5-8

**Table 5-8 Proportion of phospholipid expected in the interior of unilamellar liposome based on various diameters using Phospholac bilayer thickness determined in section 5.3.5.**

| Liposome diameter (nm) | Fraction of phosphorus on the liposome interior |
|---|---|
| 150 | 0.45 |
| 120 | 0.43 |
| 100 | 0.42 |
| 90 | 0.41 |
| 80 | 0.40 |
| 70 | 0.38 |
| 60 | 0.36 |

The unquenched phosphorous fraction of 0.4 observed for the Phospholac liposomes fits with a predominantly unilamellar population with an average diameter of approximately 80 nm. This is in agreement with the estimated average liposome diameter for Phospholac liposomes as determined by a variety of techniques of between 80-100 nm (Table 5-2). However, the value of 0.15 obtained for the SigP3644 liposomes would imply an average diameter well below that reported by PCS, AFFF, or any of the electron microscopy techniques.

Initially it was thought that the $Mn^{2+}$ ions were diffusing through the liposome membrane and were therefore quenching a portion of the phosphorus in the interior of the vesicle. Perkins *et al.* (1993) states that although this method has been used successfully, underestimation of lamellarity may occur if the agent permeates the bilayers. They suggested that this can be determined by monitoring the signal as a function of time. According to Hope *et al.* (1985), PC membranes are relatively impermeable to $Mn^{2+}$, with the signal intensity not changing over a period of days. In the present study, there were only 30 minutes between the addition of the $MnCl_2$ solutions and the $^{31}P$-NMR measurements for the experiments using the commercial phospholipid fractions, and repeating the measurement several hours later showed no change in the original result. Hauser (1993a) comments that packing defects present in the liposomes may make internal phospholipids accessible to otherwise impermeable reagents, and it is possible that some of the liposomes used in the current work may have had defects due to severe membrane curvature. However,

the AFFF particle size distribution (Figure 5-3) showed that while both SigP3644 and Ultralec dispersions contained a number of very small liposomes, so too did the Phospholac dispersion.

The most probable explanation was signaled by a significant increase in turbidity for many of the SigP3644 and Ultralec liposome samples upon the addition of the $MnCl_2$. Increases in the turbidity are usually an indication of significant increase in particle size, often caused by liposome aggregation or fusion. The changes in particle size caused by increasing the $MnCl_2$ concentration of the liposome dispersion and corresponding electron micrographs are shown in Figure 5-21 and Figure 5-22 respectively. The addition of $Mn^{2+}$did not result in any significant change in the average diameter of the Phospholac liposome dispersion, but there were rapid increases in liposome size for the SigP3644 and Ultralec dispersions upon addition of >5 mM $Mn^{2+}$. This suggests aggregation or fusion of the liposomes.

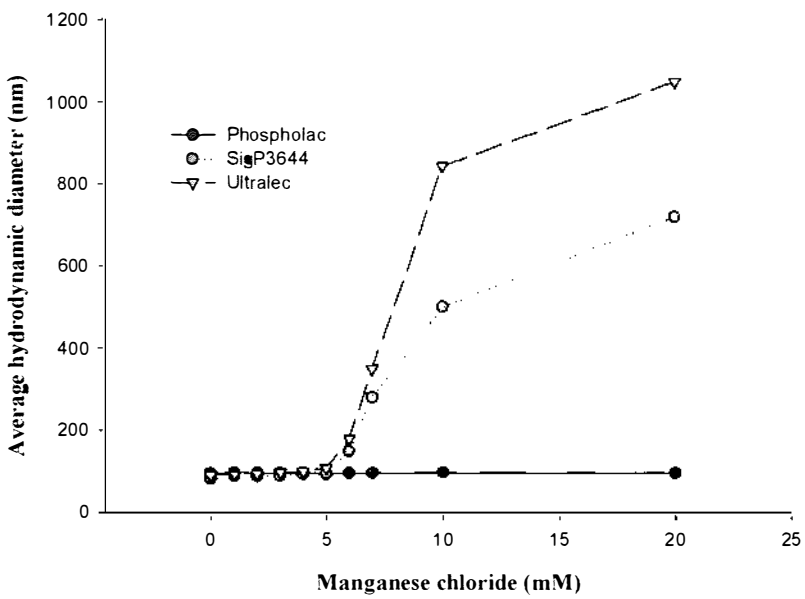



**Figure 5-21 Effect of $Mn^{2+}$ ions on the hydrodynamic size of liposome dispersions.**

The negative staining TEM micrographs of dispersions containing 20 mM $Mn^{2+}$ showed little of the original liposome structure remained in either the SigP3644 or Ultralec sample, but the Phospholac dispersion appears to contain a significant number of intact liposomes.

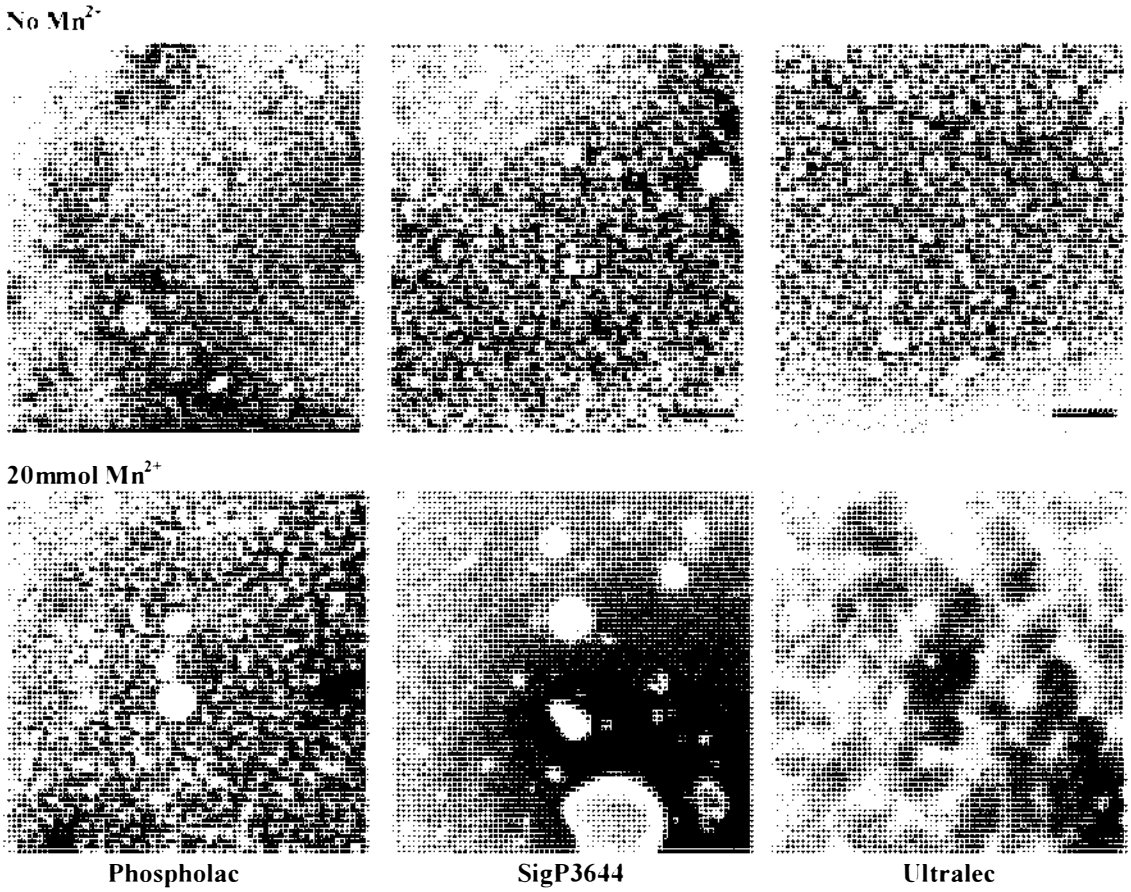



**Figure 5-22 Negatively stained TEM micrographs showing the effect of $Mn^{2+}$ ions on liposomes.**

5.3.7.2 Lanthanide ions

A second method was trialed using lanthanide ions (europium and ytterbium) to shift the $^{31}P$-NMR peak corresponding to the external phosphorus upfield from the peak corresponding to the internal phosphorus.

Addition of 5mM europium seemed to spread out the initial single signal from the phosphorus and allow different peaks to be distinguished, possibly corresponding to the different phospholipid classes. There were 3 overlapping peaks for the Phospholac dispersion, which is composed of roughly equal quantities of PC, PE, and sphingomyelin. The spectra for the SigP3644 and Ultralec dispersions showed two primary peaks with an additional smaller peak for the Ultralec sample. Both these fractions are composed primarily of PC and PE, but Ultralec also contains a significant amount of PI. However, this level of europium was not adequate for identifying internal and external phosphorus, and addition of higher concentrations caused an increase in turbidity for the SigP3644 and Ultralec

dispersions. Ytterbium (III) was selected as an alternative, as Prosser *et al.* (1998) found ytterbium ions produced the best resolution of the commonly-used lanthanide ions for $^{31}P$ and $^{13}C$ spectra. Unfortunately, there was significant increase in turbidity observed for the SigP3644 and Ultralec dispersions and results indicate that there was also some disruption to the Phospholac dispersion.

Figure 5-23 shows the spectra for Phospholac dispersions with and without added $Yb^{3+}$ ions. Intact liposomes with no added $Yb^{3+}$ ions produced a single slightly asymmetric peak with a noisy baseline. The addition of ytterbium resulted in the shifting of some of this peak towards the right, but this was overlapped by a very broad peak of about $^1/_4$ the height of the main peak. The spectra obtained from liposomes disrupted through the addition of Triton X-100 seem to have a similar broad peak, as well as a small narrow peak possibly indicating that not all the liposomes have been disrupted. The presence of the broad peak in both samples suggests that the $Yb^{3+}$ may be destabilising and disrupting the liposome dispersions even though there were no significant visual changes in turbidity.

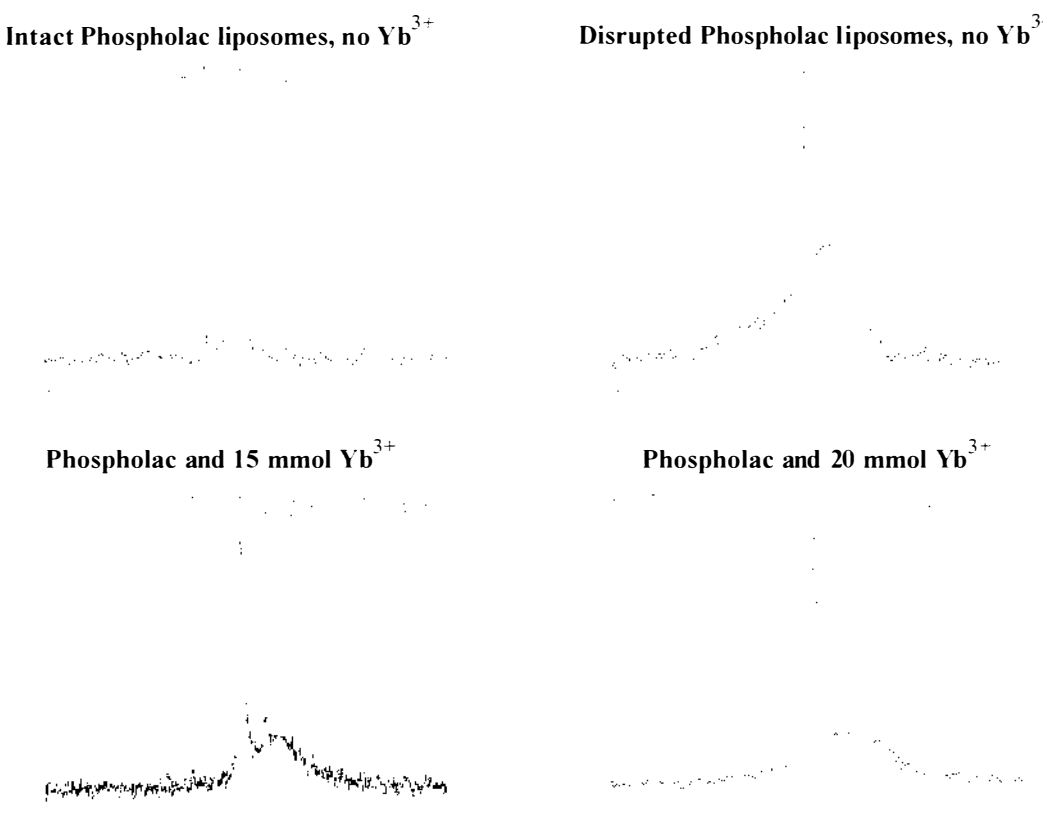



**Figure 5-23 $^{31}P$-NMR spectra for Phospholac dispersions with and without the addition of Ytterbium (III) ions.**

The lack of success of the $^{31}$P-NMR techniques was surprising given the reported use of the methods for determining liposome lamellarity (Fresta *et al.*, 1995; New, 1995b; Jousma *et al.*, 1987). However, the reported phospholipid mixtures are all based on highly purified phospholipids in a buffer solution, and it is possible that there has been some reaction between the $Mn^{2+}$ and other components of the commercial phospholipid fractions used in the present study. The literature also appears to have focused on liposomes produced from PC, PS and/or cholesterol, and the high proportion of PE present in the three commercial phospholipid fractions may have contributed to the liposome instability in the presence of the shift reagents.

#### 5.3.7.3 Ultracentrifugation

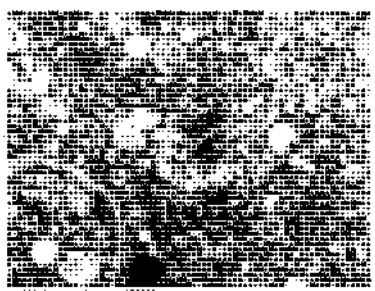
Ultracentrifugation has been reported to separate unilamellar and multilamellar liposomes without requiring the addition of potentially destabilising compounds. Samples of the liposome dispersions were centrifuged at 100,000 *g* for 2 hours, and separated into 3 layers - a pellet, a clear supernatant, and a more viscous liquid phase which settled to the bottom of the centrifuge tube but did not sediment. Ultralec also had a thick white cream layer at the top of the centrifuge tube.

The turbidity of the supernatant was too low for PCS, suggesting that this layer did not contain a significant number of liposomes. Table 5-9 shows the particle size for the other layers. The size of the liposomes forming the pellet was significantly larger than the overall average size for all liposome dispersions, and the middle layer had a corresponding decrease in size. There was no reduction in polydispersity for the middle layer compared with the initial liposome dispersion, indicating that the sedimentation of the more dense and larger liposomes had not significantly altered the width of the particle size distribution. This may suggest the presence of large particles with a density similar to water. Such particles are most probably unilamellar, as multilamellar liposomes with a higher proportion of membraneous material are more likely to have a greater density difference compared with the aqueous phase. The cream-like layer that rose to the top of the centrifuge tube was only produced by the Ultralec dispersion, and had the largest overall particle size and the highest polydispersity value.

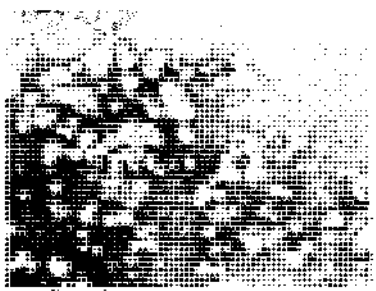
**Table 5-9 Particle size of layers formed during ultracentrifugation of liposome dispersion.**

| Phase | Phospholac | | SigP3644 | | Ultralec | |
|---|---|---|---|---|---|---|
| | $Z_{ave}$ (nm) | Polydispersity | $Z_{ave}$ (nm) | Polydispersity | $Z_{ave}$ (nm) | Polydispersity |
| **Overall** | 95 | 0.43 | 85 | 0.40 | 80 | 0.33 |
| **Pellet** | 175 | 0.16 | 145 | 0.34 | 145 | 0.26 |
| **Middle** | 72 | 0.43 | 72 | 0.43 | 75 | 0.40 |
| **Cream** | Not present | | Not present | | 150 | 0.55 |

**Phospholac**



**Pellet**



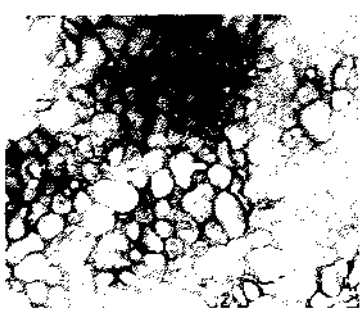
**Middle phase**

**SigP3644**

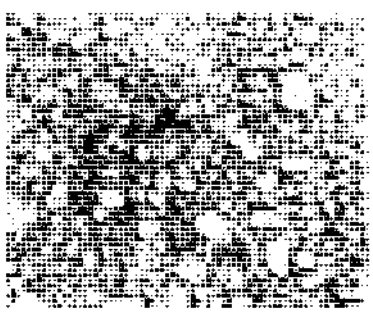
**Pellet**

**Middle phase**

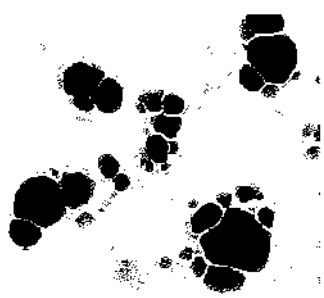
**Ultralec**



**Pellet**



**Middle phase**



**Cream**

**Figure 5-23 Negatively stained TEM micrographs of different phases formed by ultracentrifugation of the liposome dispersions.**

Negative staining micrographs (produced using the technique described in section 5.2.3.1) for each layer can be seen in Figure 5-23. There does not appear to be any clear relationship between the layers and liposomes lamellarity, with multivesicular liposomes present in the middle layer of the SigP3644 dispersion and unilamellar liposomes present in the pellets of all three dispersions. The dark bubbles shown in the Ultralec cream layer have a similar appearance to fat globules in an emulsion. This suggests that they may be triglycerides which have aggregated due to the centrifugal force. However, the triglyceride content was measured using a Roche diagnostic kit (5.2.1) and found to be negligible.

### *5.3.8 Entrapped volume*

The milliliters of water entrapped per gram of phospholipid in liposome populations as determined by the deuterium oxide and carboxyfluorescein methods are shown in Table 5-10.

**Table 5-10 Entrapped volume of empty liposomes.**

| Sample | Entrapped volume (ml $H_2O$/g Phospholipid) | |
|---|---|---|
| | $D_2O$ method | Carboxyfluorescein method |
| Phospholac | 2.0 | 0.8 |
| SigP3644 | 4.3 | 1.0 |
| Ultralec | 4.6 | 1.5 |

The entrapped volumes determined by the two methods were significantly different, although the Phospholac dispersion had the smallest entrapped volume and the Ultralec dispersion the largest for both methods. The values obtained from the entrapment of carboxyfluorescein are at the lower end of the range of 1-8 ml/g reported for SUVs and MLVs (Hope *et al.*, 1985; Hauser, 1993a; Arnaud, 1995a), while those from the deuterium oxide method are in the middle of this range. Possible explanations for the higher value for the $D_2O$ technique include the failure of all the small liposomes to pack into the pellet or that unentrapped water was still present in the pellet, either of which would artificially increase the apparent amount of water trapped per gram of phospholipid. The CF results do appear to be lower than expected based on the average liposome diameters, but are still in line with

those reported in the literature. Hope *et al.* (1985) reported entrapped volumes of between 1.1-2.5 µl $H_2O$/µmol phospholipid (1.5-3.5 ml $H_2O$/g phospholipid) for liposomes with a mean diameter of 70-95 nm produced by a variety of techniques from egg and soy phospholipids.

It is puzzling to note that Phospholac has the largest average liposome diameter as determined by PCS (Table 5-2) but the smallest entrapped volume. Although the other fractions would produce a greater number of liposomes for a given mass of phospholipid, the volume of a sphere is proportional to $r^3$ and so it would be expected that the larger particles would entrap more water per gram of phospholipid.

One possible explanation for this is linked to the Phospholac sample appearing to contain a large number of very small liposomes along with a few large liposomes (EM results in Table 5-2 and discussion in section 5.2.3). The much smaller liposomes would naturally have a smaller than expected entrapped volume, and while the larger liposomes may increase the average PCS diameter due to their significant effect on light scattering (intensity is proportional to $d^6$), their entrapped volume may not fully compensate for the high proportion of small vesicles. However, Figure 5-3 (particle size distribution for the dispersions as measured by AFFF) shows that SigP3644 and Ultralec dispersions both have a higher proportion of very small vesicles than the Phospholac dispersion.

Another possible explanation is that the Phospholac fraction produces a higher proportion of multilamellar or multivesicular liposomes, the internal bilayers taking up space inside the vesicles and reducing the amount of water entrapped. The Phospholac liposome dispersion may also contain more non-spherical liposomes, the elongation reducing the volume entrapped for a given surface area. There is evidence of both multivesicular and non-spherical liposomes in the present study, but the results are unable to confirm any difference between the dispersions produced by the different phospholipid fractions.

Whatever the explanation, if the entrapped volume results are correct it would appear that liposomes produced from the Phospholac fraction will not be as efficient at entrapping hydrophilic material as those produced from the SigP3644 or Ultralec fractions. This is investigated experimentally in *Chapter 7: Liposome entrapment of hydrophobic and hydrophilic compounds in liposome dispersions.*

### 5.3.9 Raman spectroscopy

The Raman spectra of the three liposome dispersions were quite similar (Figure 5-24), indicating that the molecular structure of the samples are also similar. However, the observed differences indicate that the Phospholac contains structural groups that do not appear to be present (or present but to a lesser extent) in the other two samples. This may be due to the presence of sphingomyelin, PE, PS, or even the different fatty acid composition of the Phospholac.

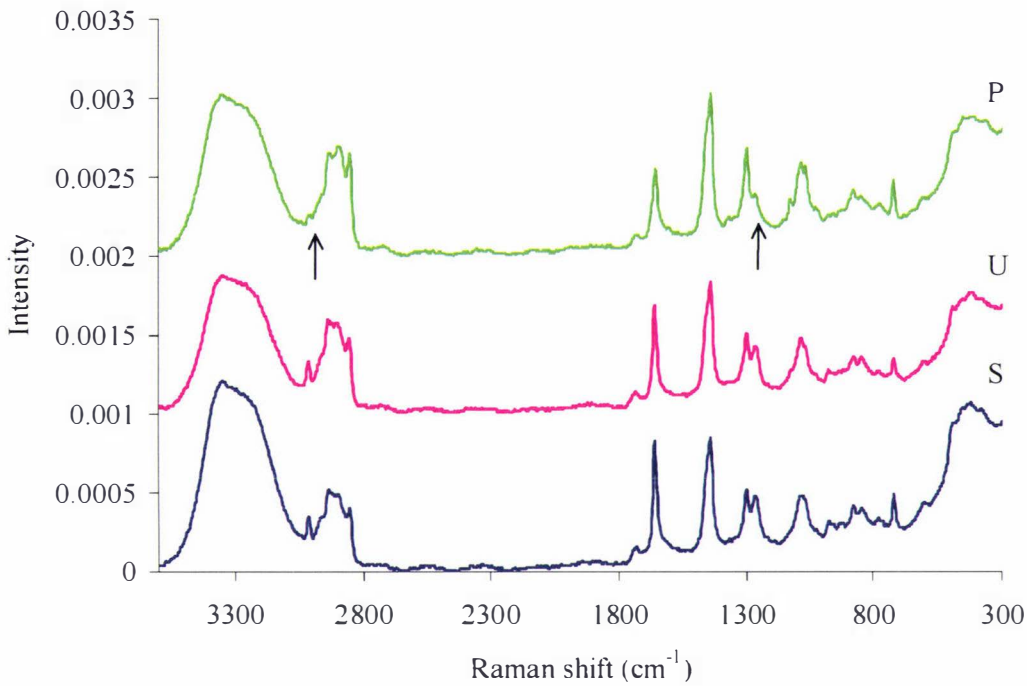



**Figure 5-24 Raman spectra of Phospholac (P), SigP3644 (S) and Ultralec (U). Arrows indicate regions in Phospholac sample (P) that differed from the SigP3644 (S) and Ultralec (U) samples.**

## 5.4 Conclusions

There were a number of significant differences between the liposome dispersions produced from the three commercial phospholipid fractions.

The Phospholac fraction appeared to produce liposomes with a hydrodynamic diameter (as measured by PCS) of approximately 95 nm, slightly larger than the average diameter of approximately 80 nm for the SigP3644 and Ultralec dispersions. All three dispersions had a

very wide particle size distribution, with polydispersity values of between 0.4-0.5. AFFF results showed that the Phospholac dispersions had fewer very small liposomes and an overall narrower particle size distribution than the other two dispersions.

SigP3644 and Ultralec both had phase transition temperatures below 0 °C, while the Phospholac dispersions showed a very broad transition centered at 28-30 °C. This meant that the SigP3644 and Ultralec dispersions were in the fluid phase at all common storage and processing temperatures, while the Phospholac was in the supposedly less permeable gel phase at refrigeration and ambient temperatures. However, although the SigP3644 sample had a considerably higher membrane permeability than the Phospholac sample at 20 °C, there was little difference between the Phospholac and Ultralec dispersions. Despite the literature asserting that permeability increases at the phase transition, there was no significant increase in membrane permeability of the Phospholac membranes at the membrane phase transition temperature (28 to 30 °C). The permeability of the Phospholac fluid phase (40 °C) was similar to that of the gel phase (20 °C), and the permeability of the SigP3644 and Ultralec samples was also not affected by increasing the temperature.

The permeability data are consistent with the bilayer thickness of the different liposome dispersions. The Phospholac and Ultralec bilayers were approximately 20% thicker than SigP3644 membranes, and increasing the temperature from 20 to 40 °C did not result in a significant change in the width of the bilayer.

All three dispersions appeared to be primarily unilamellar, but there seemed to be a small percentage of multilamellar and multivesicular liposomes present. Despite the larger average hydrodynamic diameter of the Phospholac dispersion, this sample had the lowest entrapped volume of between 0.8-2.0ml/g phospholipid, approximately half that of the Ultralec dispersion.

# Chapter 6: Stability of liposome dispersions

## 6.1 Introduction

The stability of a liposome dispersion is usually monitored by the ability of the system to maintain its original particle size distribution and retain any entrapped material.

A liposome dispersion is theoretically at the minimum energy level for the system, with liposomes inherently stable units unless environmental or chemical changes cause a disruption to the system. However, the dynamic nature of the bilayer membrane means that the phospholipids and proteins within the membrane are constantly moving and interacting with other compounds in the environment. Signs of system instability including liposome aggregation, fusion and rupture will occur over varying time periods in virtually all liposome dispersions. Oxidation products can increase the instability of liposome dispersions, and it would seem reasonable to assume that storage in a dark, low-oxygen environment at low temperatures would minimise oxidative degradation and extend the stable life of most dispersions. Microbiological growth can also damage the membrane components either through direct microbial action or through the formation of undesirable by-products.

To ensure long term stability, freezing, drying and freeze-drying may be considered. With all of these techniques, care must be taken to avoid dehydration-induced phase transition and membrane fusion. New (1990b) stated "techniques which prevent membrane fracture upon freezing or drying have not yet been fully perfected". Cullis *et al.* (1987), Kirby (1991), Frézard (1999) and Gibbs *et al.* (1999) have all reported that liposomes can be successfully stored by freeze-drying, although the use of a cryoprotectant seems to be essential.

Among the most commonly used cryoprotectants are mono- and disaccharides. It is thought that these materials protect liposome systems during drying and freezing by forming a stable glassy matrix, preventing phase transitions and crystallisation which would usually damage the membrane (Chen *et al.*, 2001b). Hydrogen bonding between some sugars and polar groups on the lipids may also help prevent fusion (Anchordoquy *et al.*, 1987). An extensive review on the freezing and drying of membrane systems compiled by Wolfe and Bryant (1999) is recommended for a more in-depth coverage of this topic.

The chemical degradation of liposomes may occur *via* the oxidation of unsaturated fatty acid chains or through hydrolysis of the phospholipids (Martin, 1990). This degradation may be monitored in a number of ways, ranging from TLC and HPLC techniques to spectrometric methods. UV absorbance at 230 nm can be used to follow the formation of conjugated dienes during the free radical chain initiation phase of oxidation, and extensive phospholipid degradation may be signaled by a third peak at 270 nm due to the formation of conjugated trienes. Separate tests are used to measure the levels of the two types of peroxides which can be formed during oxidation reactions - hydroperoxides and cyclic peroxides (or 'endoperoxides'). The latter are detected by reaction of their breakdown product at elevated temperatures (malondialdehyde) with thiobarbituric acid (TBA) giving a red chromophore that absorbs at 532 nm. The method for detecting hydroperoxides is based on their susceptibility to reduction by iodide. For more information on liposome stability, refer to section 2.6.8 in *Chapter 2: Literature Review*.

Carboxyfluorescein (CF) is a hydrophilic fluorescent dye (Figure 6-1), the release of which is often used as an indication of liposome stability. Jousma *et al.* (1987) state that it is membrane-impermeable, which means if the unentrapped CF is removed from the dispersion, any CF found in the extra-liposomal phase must be as a result of liposome rupture or fusion. These are both common signs of liposome instability.

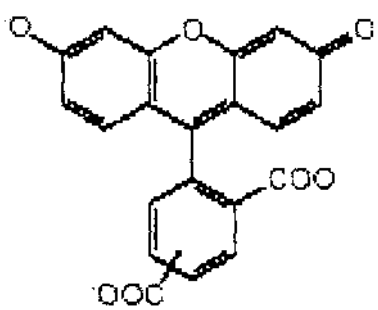



**Figure 6-1 Structure of 5(-6)-carboxyfluorescein (CF). $pK_a$ = 6.4.**

Weidgans (2004) analysed the effect of pH on the fluorescence of CF. It was found that samples at low pH exhibited a much lower level of fluorescence than the same concentration of CF at higher pH. Therefore it is essential that all measurements are performed at a consistent pH value if valid comparisons are to be made.

## 6.2 Materials and methods

Liposome dispersions were prepared using the standard technique (*Chapter 3: Selection of phospholipid fractions and liposome preparation method*) except for the addition of 25 mM 5(-6)-carboxyfluorescein (CF) to the imidazole buffer. To remove unentrapped CF, aliquots of each of the three liposome dispersions were passed through a 100 × 10 mm column containing Bio Gel P-6 DG desalting gel (BioRad, catalogue 150-0738). The eluted fractions containing liposomes were identified by measuring turbidity at 600 nm. These were pooled and diluted to 900 ml with imidazole buffer; 140 ml of each dispersion was adjusted to pH 2, 4, 6, 7, 8, or 10 using 0.1 M HCl and 0.1 M NaOH, then made up to 150 ml with imidazole buffer.

Changes in average hydrodynamic diameter, peroxide value and conjugated diene level were monitored as an indication of liposome stability, as was the release of CF into the bulk aqueous phase. Hydrodynamic diameter was measured using a Zetasizer 4 (Malvern Instruments Ltd, UK) as outlined in section 5.2.2.1 in *Chapter 5: Characterisation of liposome dispersions.* The peroxide value and the level of conjugated dienes were measured before and after microfluidization to provide an indication of the effect of the processing on phospholipid oxidation.

The peroxide value was obtained using a technique based on ISO 3960:2001 (International Organization for Standardization). Briefly, 1 mL of the liposome dispersion was dissolved in 6 mL of acetic acid:chloroform (3:2), and 0.5 mL of saturated potassium iodide solution was added. The sample was mixed for 1 min, with further addition of 6 mL of distilled water. The solution was titrated against 0.1 *M* sodium thiosulfate solution until the yellow color had almost disappeared. Then 0.5 mL of a 1.0% starch solution was added, and titration was continued until the blue color disappeared. The peroxide value was calculated as milli-equivalents of peroxide per 1000 g of sample.

The levels of conjugated dienes and trienes formed during oxidation were determined by a method based on IUPAC Method No. 2.505 and Lethuaut et al. (2002). A 25-µL aliquot of the liposome dispersion was dissolved in 10 mL of isopropanol, mixed for 4 s, and centrifuged for 5 min at 2500 *g* (CentraMP4R centrifuge, International Equipment Company, Needham Heights, MA). The absorbance was read against a blank containing 10 mL of

isopropanol and 25 μL of Milli-Q water at 232 nm (linoleic hydroperoxides and conjugated dienes) and 268 nm (conjugated trienes and secondary products).

To measure the loss of CF, a 0.1 ml aliquot of each sample was diluted to 2 ml using buffer at pH 7, and filled into Centristart I 13239E filters. These were centrifuged at 4000 *g* for 15 minutes in a CentraMP4R centrifuge (International Equipment Company, MA) to obtain a sample of the bulk aqueous phase. Both the bulk aqueous phase and a sample of the non-centrifuged liposome dispersion were diluted 1:10 with buffer, and the CF concentration measured using a luminescence spectrophotometer LS50B (Perkin Elmer, Wellesley, Massachusetts, USA) with $\lambda_{ex}$ = 492 nm and $\lambda_{em}$ = 514 nm.). The difference in fluorescence between the non-centrifuged sample and the bulk aqueous phase corresponded to the entrapped CF.

## *6.3 Conditions for assessing stability*

A variety of conditions were used to assess the stability of the liposome dispersion. These included a range of pH, temperature and time combinations that may be used for storage, and a selection of processing environments commonly encountered in the food industry.

### *6.3.1 Storage*

To examine the stability of the liposomes during storage, pH-adjusted samples were held at 5 °C, 20 °C, 30 °C and 35 °C. Liposome dispersions were assessed weekly.

### *6.3.2 Heat processing*

A selection of heat treatments were chosen to represent common temperature/time combinations used during the processing of foods. These are outlined below. Liposome stability was assessed one day after the heat treatment, and again seven days later.

6.3.2.1 Extended time at elevated temperatures (55 °C for 15 hours)

The pH-adjusted samples were held in a stirred waterbath at 55 °C. After 15 hours, samples were cooled by placing under cold running water, then stored at 5 °C.

6.3.2.2 Pasteurisation (75 °C for 2 minutes)

A number of sample containers filled with liposome dispersion at room temperature were placed in a stirred waterbath at 80 °C. A container was removed every 30 seconds and the temperature measured using a digital thermometer. The samples took approximately 3 minutes to reach 75 °C, and after a further two minutes the temperature did not rise significantly. Aliquots of pH-adjusted liposome dispersions were filled into the same sample containers, and placed in the waterbath for a total of 5 minutes. The samples were then cooled under cold running water and stored at 5°C.

6.3.2.3 High temperature treatment (90 °C for 2 minutes)

A stirred waterbath was completely surrounded with polystyrene and heated to 95 °C. A number of sample containers filled with liposome dispersion at room temperature were placed in the waterbath. A container was removed every 30 seconds and the temperature measured using a digital thermometer. The samples took approximately 3 minutes and 30 seconds to reach 90 °C, and a further two minutes did not cause significant increases in temperature. Aliquots of pH-adjusted liposome dispersions were filled into the same sample containers, and placed in the waterbath for a total of 5 minutes 30 seconds. The samples were cooled under cold running water and stored at 5°C.

6.3.2.4 Ultra high temperature (UHT) treatment (141 °C for 10 seconds)

The samples were heated to 141 °C and held for 15 seconds using a mini UHT plant (Alfa Laval, Sweden), with a flow-rate of 1 litre/minute. The samples were rapidly cooled and stored at 5 °C.

### 6.3.3 Addition of NaCl or $CaCl_2$

Samples were mixed 1:1 with solutions of between 0.01 and 4 M NaCl or $CaCl_2$ and left for 1 hour before determining the average hydrodynamic diameter.

It was possible that differences in the stability of liposome dispersions when exposed to increases in ionic concentration were due to the differences in the mineral content of the phospholipid fraction rather than the actual phospholipids themselves. Dialysis was used to exchange the bulk aqueous phase for each of the dispersions to provide a sample of Phospholac which had the same soluble mineral content as SigP3644, a sample of Phospholac which had the same soluble mineral content as Ultralec, etc. These were then mixed 1:1 with the NaCl or $CaCl_2$ solutions as described above.

### 6.3.4 Freezing and freeze-drying

There is some evidence that the presence of cryogenic sugars on both sides of a membrane improves the effectiveness of the sugar compared with having it on only one side of the membrane (van Winden, 2003). Many sugars (including sucrose and glucose) will diffuse through the bilayer, automatically ensuring that the molecules are present on both sides of the membrane. However, others (such as trehelose) have been shown to be relatively membrane impermeable. Therefore, there may be a difference in the effectiveness of these sugars depending on whether they are added prior to liposome formation (present on both sides of the membrane) or after liposome formation (only present on one side of the membrane).

Varying amounts of sucrose, glucose, trehelose or maltose were added to samples of liposome dispersions that had been produced in buffer in the usual manner. Liposome dispersions were also made where varying amounts of trehelose or maltose were added to the phospholipid dispersion prior to Microfluidization. Approximately 2ml of each sample was filled into eppendorf tubes and then either frozen or freeze-dried. After freezing for 24 hours at -20 °C, samples were thawed in a stirred beaker at 60 °C, and analysed for hydrodynamic diameter and by TEM.

Freeze-drying was performed using a FD0610 freeze-dryer (Cuddon Marlborough Engineering Works, Blenheim, New Zealand). The samples were dried for 72 hours under

vacuum with a chamber temperature of -30 °C and a shelf temperature of 20 °C, and stored in a desiccator until required. To rehydrate, 2 ml of deionised water was added to each tube, then the tube was inverted several times and allowed to stand for 24 hours at room temperature. Hydrodynamic diameter was assessed and TEM micrographs taken within 5 hours of rehydration.

## *6.4 Results and discussion*

### *6.4.1 pH*

The initial changes in liposome diameter caused by pH adjustment are shown in Figure 6-2. There was little change in the average diameter of the Phospholac liposome dispersion (~ 95 nm) across the entire pH range, but both the SigP3644 and Ultralec dispersions showed rapid increases in average diameter in the samples at the lower pH values. The Ultralec dispersion appeared to stabilize at ~ 80 nm above pH 4, but there were significant increases in the average diameter for the SigP3644 dispersions for all samples below pH 7. From pH 7-10, the SigP3644 dispersions had an average diameter of ~ 80 nm.

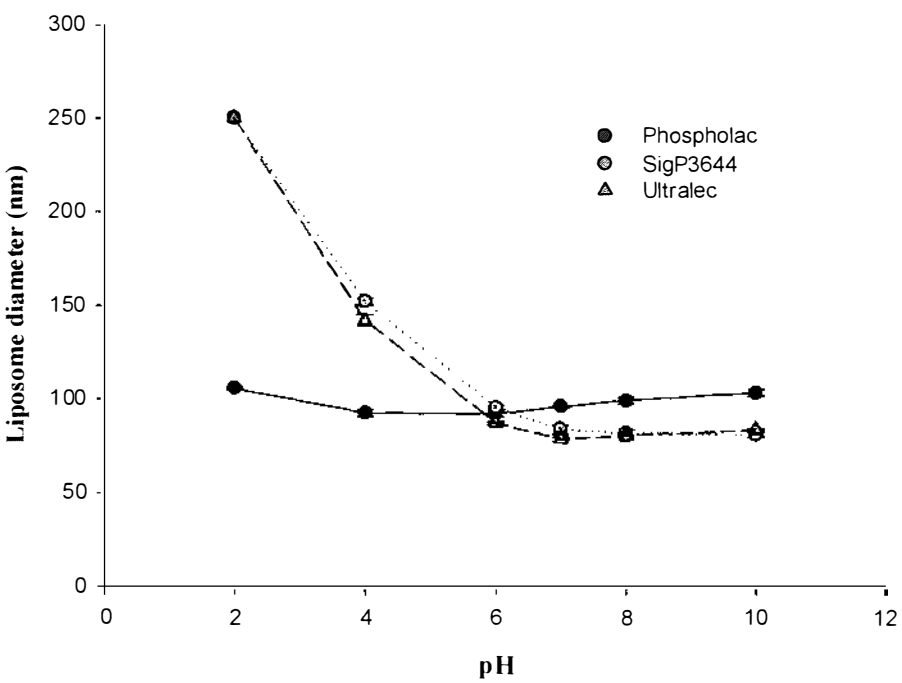



**Figure 6-2 Effect of pH on the average hydrodynamic diameter of liposome dispersions. Samples were measured 24 hours after pH adjustment. Each point is the mean of three measurements with error bars ± 1 standard deviation.**

The increase in liposome diameter at the low pH values for both the SigP3644 and Ultralec dispersions was also reflected in the increase in sample turbidity shown in Figure 6-3. There was also a slight increase in turbidity of the Phospholac dispersion at pH 2. The Ultralec dispersion at pH 2 rapidly formed a solid phospholipid pellet the same yellow colour as the initial Ultralec phospholipid material, with a much smaller amount of sediment also present in the pH 2 SigP3644 dispersion but no apparent sedimentation in the Phospholac dispersion. After 72 hours, the SigP3644 dispersion at pH 2 had also formed a solid pale yellow pellet, but there was still no sedimentation in the Phospholac sample.

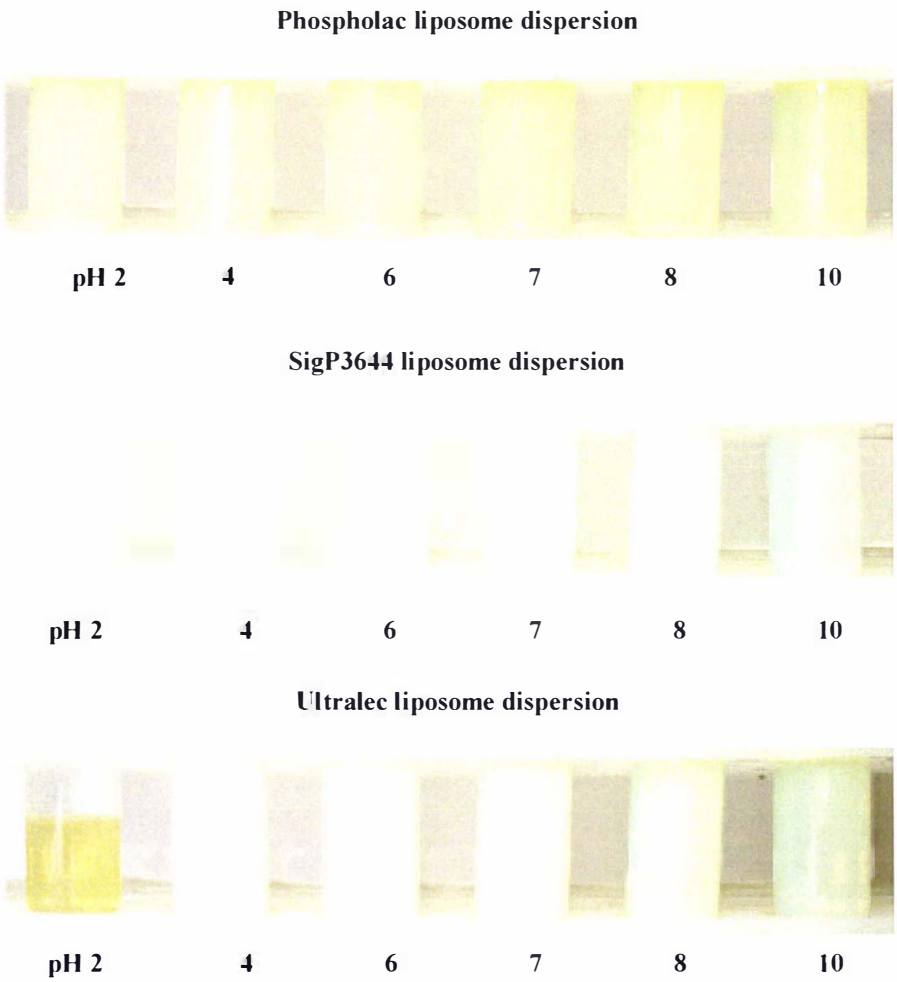



**Figure 6-3 Photographs of liposome dispersions 24 hours after pH adjustment.**

Figure 6-4 shows the effect of pH adjustment on CF entrapment. There appeared to be a slight reduction in entrapped CF at pH 2 for the Phospholac and SigP3644 dispersions and again at pH 10 for the Phospholac dispersion, but there was no effect on entrapment at pH values between 4 and 8. The Ultralec dispersion released more of the CF at the lower pH

values, but was similar to the SigP3644 dispersions at pH 7-10. Although no pH adjustment was required for the samples at pH 7, the measured CF entrapment was between 92-98%, suggesting that there was a small under-estimation of entrapment. This appeared to affect all three dispersions to a similar degree, so no correction was made to the results.

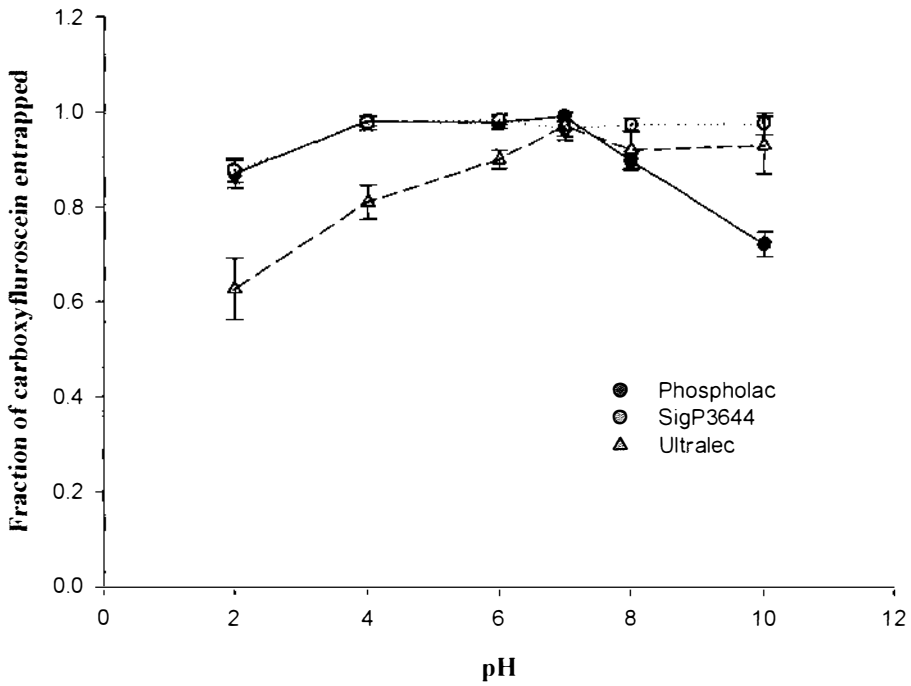
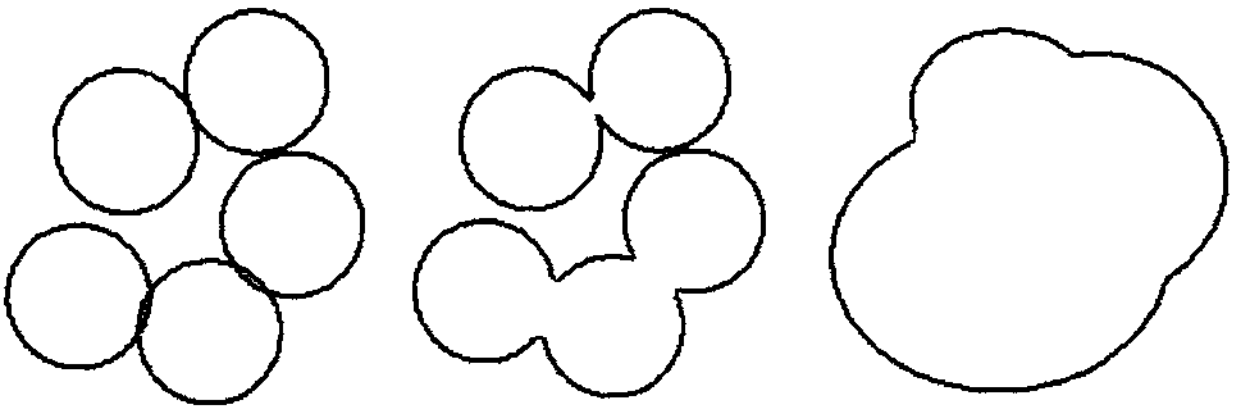



**Figure 6-4 Fraction of entrapped carboxyfluroscein retained 24 hours after pH adjustment of liposome dispersions. Each point is the mean of three measurements with error bars ± 1 standard deviation.**

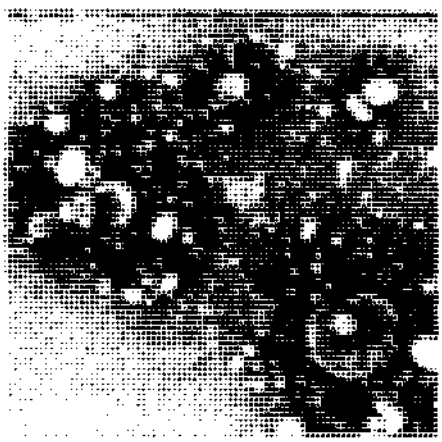
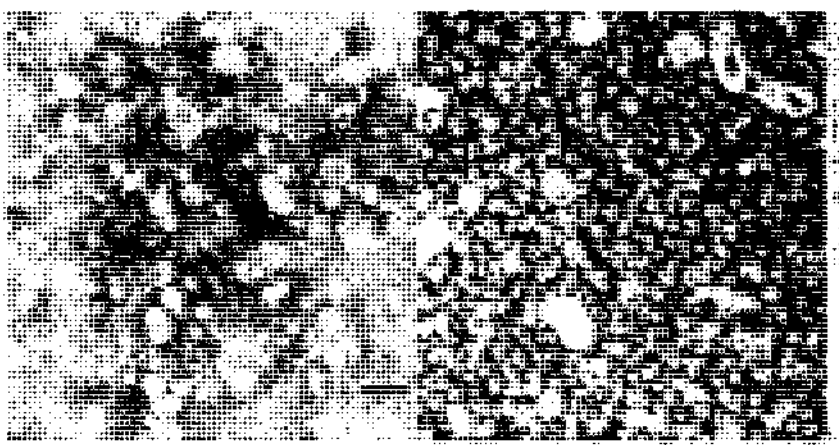
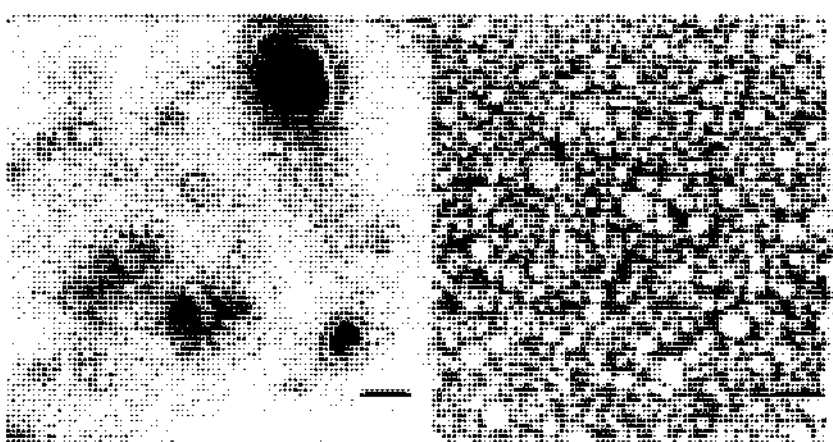
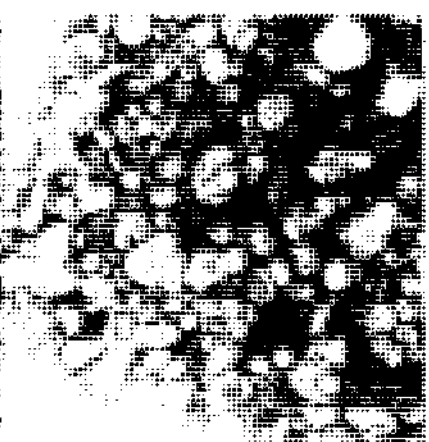
There are several possible causes for these increases in liposome diameter and turbidity. The level of retained CF shown in Figure 6-4 for the SigP3644 dispersion suggests that the liposomes are merely aggregating rather than fusing (Figure 6-5). It is likely that the low pH is neutralising the surface charge as shown by the changes in zeta potential as a function of pH (section 5.3.3), and it is possible that the liposomes are still retaining their individual identity and thus any entrapped contents. However, the photograph of the Ultralec dispersion at pH 2 in Figure 6-4 shows that the dispersion has been destroyed, forming a pellet of phospholipid with a clear supernatant. The chances of sufficient liposomes remaining intact to retain 60% of the entrapped CF (as shown in Figure 6-4) seems unlikely. This is supported by the TEM micrographs shown in Figure 6-6, which indicate that pH has a significant effect on the physical structure of the liposomes.



(a) Aggregation (b) Mild fusion (c) Major fusion

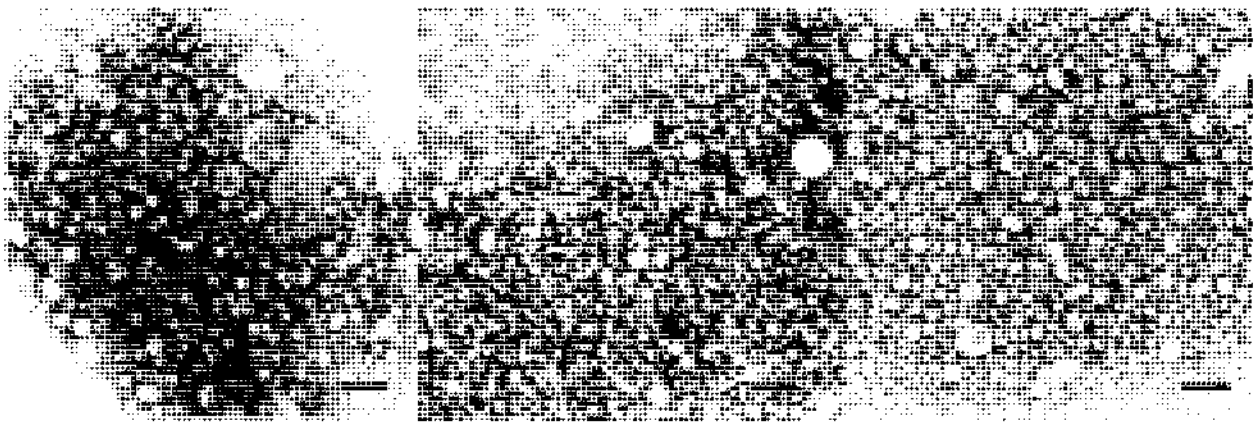
**Figure 6-5 Schematic showing possible causes of significant increases in liposome diameter. Liposomes may aggregate to form large clusters while still retaining their individual identity and any entrapped contents. Under more severe stress, the bilayer membranes between the liposomes may merge or break to produce a smaller number of larger vesicles. During the breaking and reformation of the membrane it is likely that at least some of the entrapped material will be lost to the bulk aqueous phase. Eventually these much larger fused vesicles can also aggregate and sediment.**

The TEM micrographs of all of the dispersions show that the liposome structure appears to be affected at low pH values, although the Phospholac dispersion has retained more of the normal liposome appearance than either of the other two fractions. Based on these observations, the CF entrapment shown in Figure 6-4 does not seem to be a valid reflection of the stability of the liposome systems. This is discussed in further detail in section 6.4.2.3.

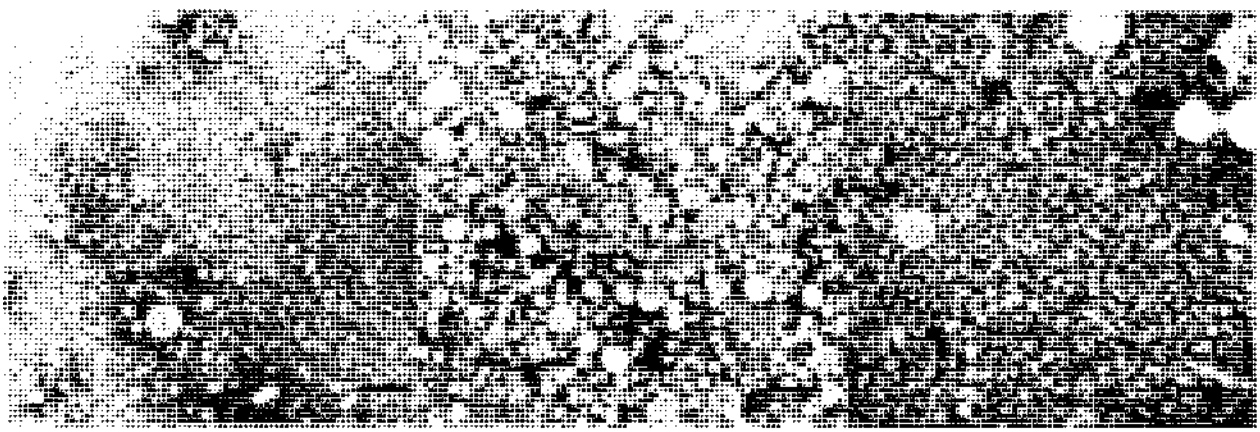
| Phospholac | SigP3644 | Ultralec |
|---|---|---|
| pH 2 | | |
|  | *No micrograph available due to solidification of sample* | *No micrograph available due to solidification of sample* |
| pH 4 | | |
|  | |  |
| pH 6 | | |
|  | |  |

**Phospholac** **SigP3644** **Ultralec**

pH 7



pH 8



pH 10

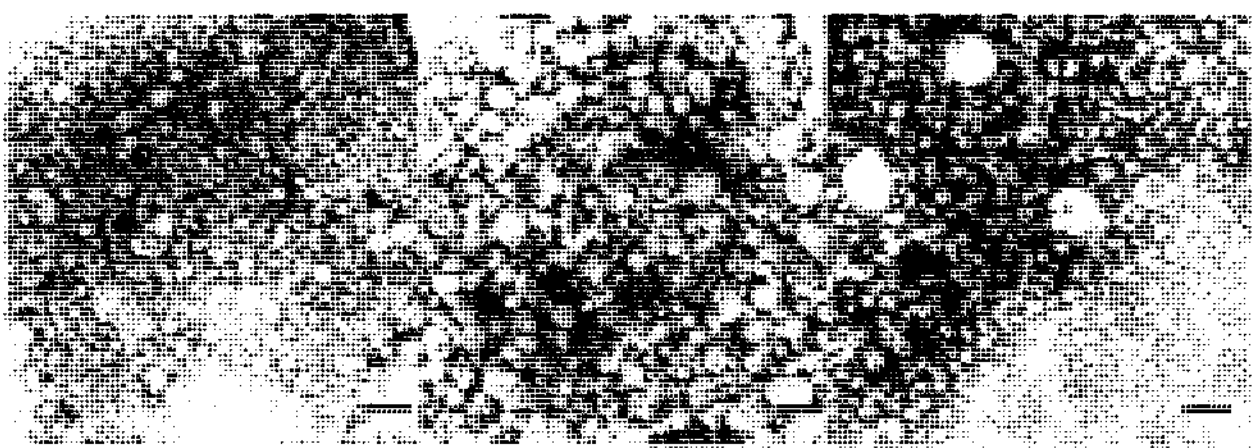


**Figure 6-6 Negative staining TEM micrographs showing the effect of pH on liposome dispersions. All micrographs are the same scale, 48,600 x magnification, bar = 0.3µm.**

## 6.4.2 Storage

### 6.4.2.1 Liposome diameter

Figure 6-7 shows the effect of pH on the average Phospholac liposome diameter during storage at different temperatures. At 4 °C there was an initial slight increase in diameter for samples at pH 2, but no change for samples at pH 4-10. The lower pH samples had a slight increase in size after 60 days, but samples at pH 6-10 appeared to be relatively stable over the 100 day period. Storage at 20 and 30 °C resulted in a similar pattern, but as the storage temperature increased, the increase in liposome size at pH 4 and below appeared to occur more quickly. After 100 days at 30 °C there was a slight increase in particle size even amongst the higher pH samples, with major changes at those pH values for the samples stored at 35 °C. However, samples at pH 6-10 appeared to be stable during storage for up to 60 days even at the higher temperatures.

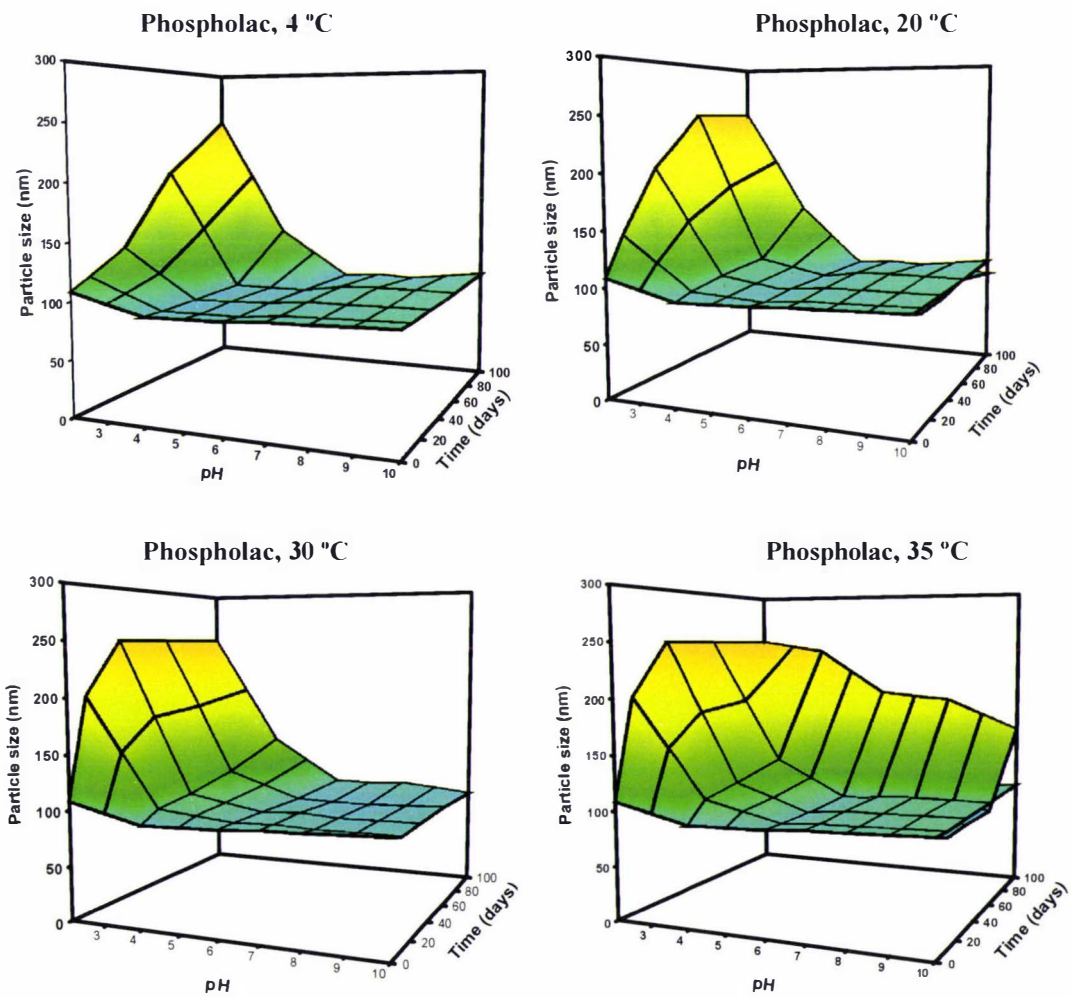



**Figure 6-7 Effect of pH on average hydrodynamic diameter of a Phospholac liposome dispersion during storage at various temperatures.**

The effect of temperature on the storage of SigP3644 liposome dispersions is shown in Figure 6-8. At low pH values, these dispersions were not stable at any temperature even for short periods. However, when samples at pH 7-10 were stored at 4 °C there appeared to be very little change in size over the 100 days. At the higher storage temperatures the average liposome diameter of the higher pH samples also increased, demonstrating significant instability after only 10 days at 20 °C, and almost instantaneously at 30 and 35 °C. Although the samples at pH 10 initially had a smaller increase in average size, a rapid increase in size occurred after 40 days. The dispersions at pH 8 had the best long-term stability at all temperatures.

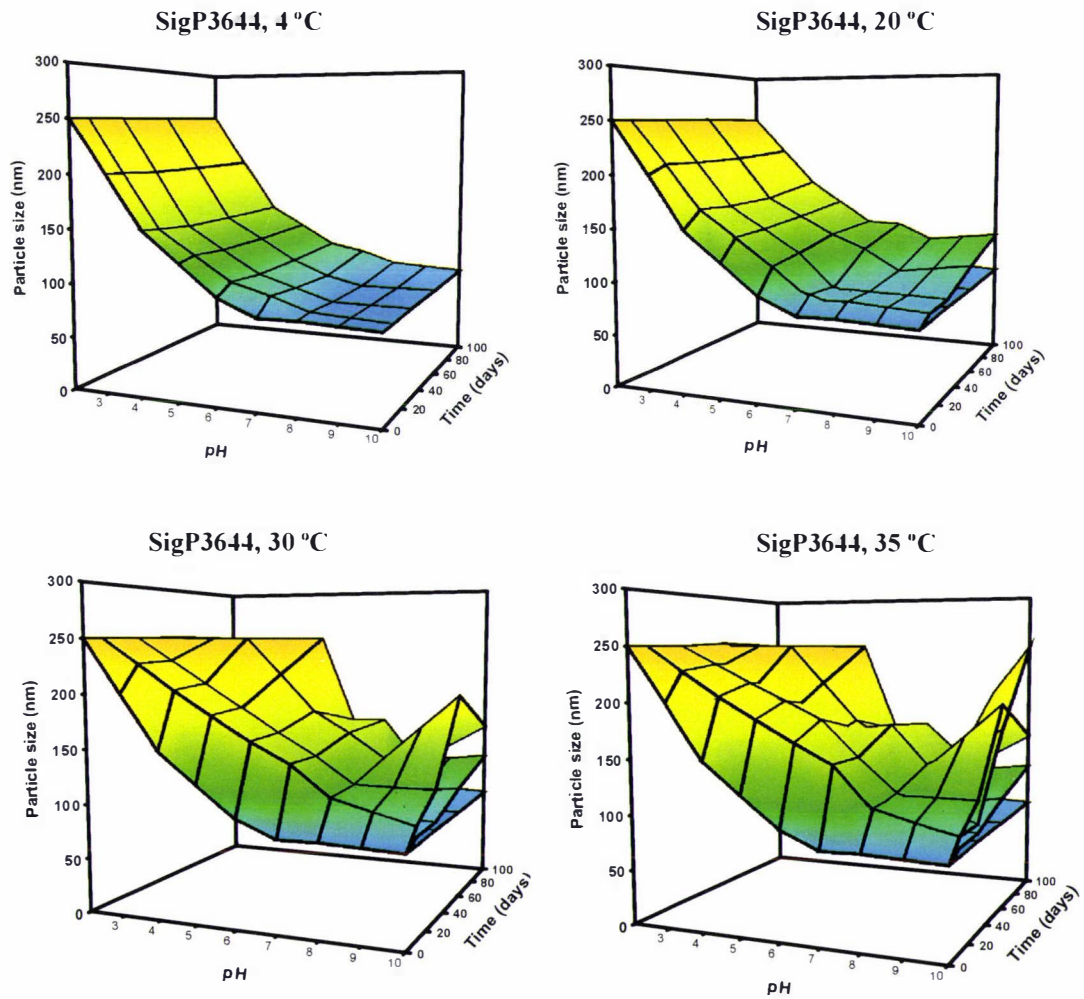



**Figure 6-8 Effect of pH on average hydrodynamic diameter of a SigP3644 liposome dispersion during storage at various temperatures.**

Ultralec dispersions underwent a similar pattern of change in liposome diameter to the SigP3644 dispersions (Figure 6-9). In low pH samples, liposome size increased rapidly, with the highest pH samples showing a delayed increase which occurred more quickly at the higher temperatures. Samples at pH 7 appeared to have the best long-term storage stability.

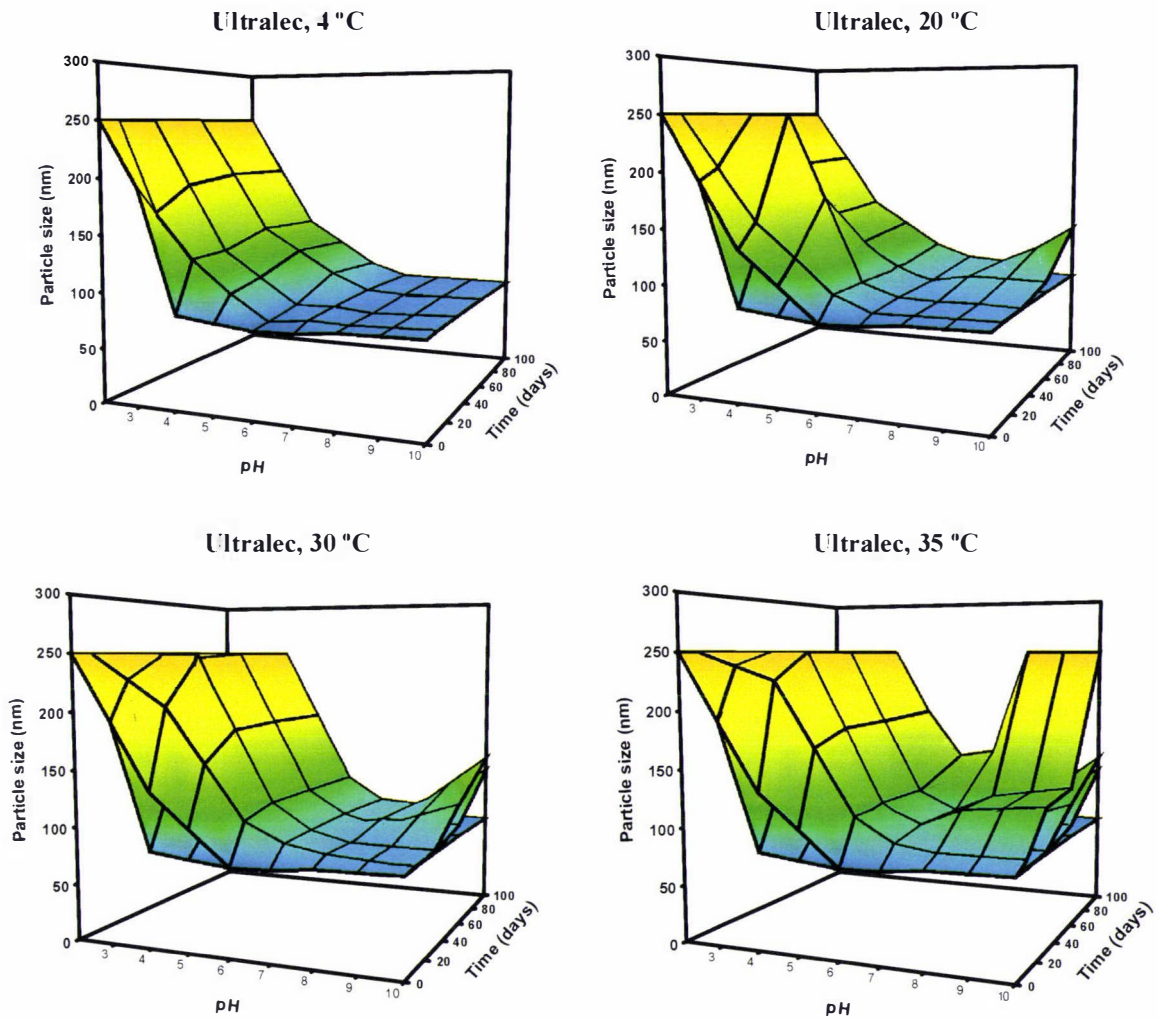



**Figure 6-9 Effect of pH on average hydrodynamic diameter of an Ultralec liposome dispersion during storage at various temperatures.**

To investigate the statistical significance of the changes during storage, a statistical package (WinBugs 1.4) was used to fit a simple curve to the data for liposome diameter vs pH for a particular temperature and time combination. This was then compared with the curve for day 0. Due to the general shape of the data, the curve was based on the exponential shown in Equation 6-1, with the constants explained in Figure 6-10.

$$\text{Size (nm)} = a + b(e^{-c(pH)}) \qquad \textbf{Eq 6-1}$$

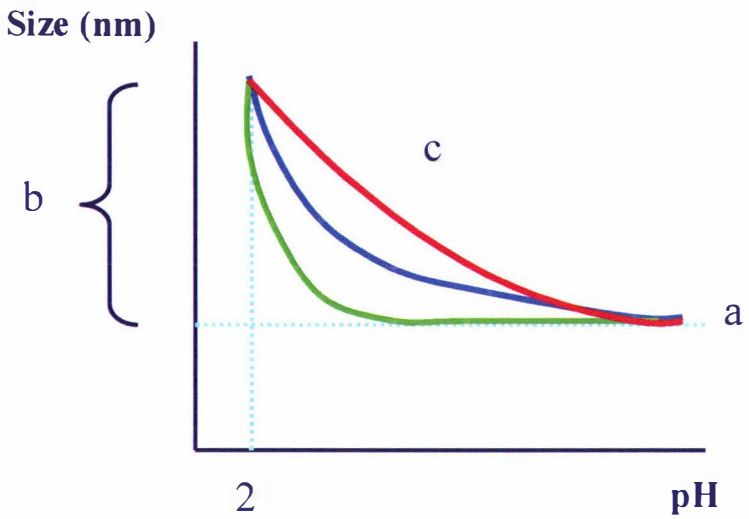



**Figure 6-10 Schematic showing the physical interpretation of the constants for the statistical analysis of the effect of various conditions on liposome diameter. a = function of the value of the asymptote, b = a function of the difference between the liposome size at pH 2 and the asymptote, and c = a function of the shape of the curves. The three curves shown in this figure have the same a and b values but different c values.**

Unfortunately, this simple model was not able to fit the increase in liposome diameter observed at the high pH values for storage of SigP3644 or Ultralec at 30 or 35 °C. The rapid aggregation and solidification of the lipid component of the SigP3644 and Ultralec samples at pH 2 and after storage >20 °C for Ultralec samples at pH 4 meant that these points were also not able to be easily included in the model. There was insufficient data to justify developing a more sophisticated model with an increased number of terms, so it was decided to only use between pH 4-8 in the modelling process.

Unfortunately, this statistical analysis based on a simple model was not able to accurately reflect the observed changes as shown in Figure 6-8 to Figure 6-10 for the SigP3644 and Ultralec samples. However, it is obvious from a visual comparison of these figures that the Phospholac dispersions had a much greater stability during storage than either SigP3644 or Ultralec dispersions in all conditions assessed.

### 6.4.2.2 Modeling of the storage stability of Phospholac dispersions

The relatively consistent pattern of change observed for the Phospholac liposome dispersion indicated that it may be possible to use the basic model developed above (Equation 6-1) to predict the changes in liposome size as a function of pH, time and temperature. Results from the samples from pH 2-10 were included in this model.

The WinBugs 1.4 statistical package was used to compare the various values of (a), (b) and (c) for the different temperature/time combinations and output a relationship between temperature, time and pH that fitted with the changes observed in (a), (b) and (c). The full version of this relationship is given in Equation 6-2 through to Equation 6-.

$$a + d \times e^{-c \times (pH-2)} \qquad \textbf{Eq 6-2}$$

Where:

$$a = a_i + (temp_a \times temp) + (time_a \times \ln tim) + (timesq_a \times \ln tim \times \ln tim) \qquad \textbf{Eq 6-3}$$

$$d = (b_i - a_i) + (t_d \times temp) + (time_d \times \ln tim) + (timesq_d \times \ln tim \times \ln tim) \qquad \textbf{Eq 6-4}$$

$$c = c_i + (t_c \times temp) + (time_c \times \ln tim) \qquad \textbf{Eq 6-5}$$

$$temp = (\text{storage temperature in } ^\circ C) - 20 \qquad \textbf{Eq 6-6}$$

$$tim = (\text{storage time in days}) + 1 \qquad \textbf{Eq 6-7}$$

The values for the constants used throughout these equations are given in Table 6-1.

**Table 6-1 Constants and values for Phospholac model to predict the average hydrodynamic diameter of a dispersion as a function of storage time and temperature.**

| Constant | Value |
|---|---|
| $a_i$ | 94.68 |
| $b_i$ | 140.7 |
| $c_i$ | 5.201 |
| $temp_a$ | -0.1667 |
| tc | -0.07576 |
| td | 2.378 |
| $time_a$ | 1.331 |
| $timesq_a$ | -0.3645 |
| $time_c$ | -0.8565 |
| $time_d$ | -19.51 |
| $timesq_d$ | 8.518 |

A comparison between the measured and predicted results is shown in Figure 6-11. At low storage temperatures the model over-estimates stability for the mid to low pH samples. However, at 20, 30 and 35 °C the differences between the measured and predicted diameters are much less significant.

"*All models are wrong. Some are useful*" (Box, 1979). Although additional work is required to validate this model, it does look promising for allowing the prediction of changes in liposome size as an indication of stability across a range of pH and storage temperatures for up to 100 days.

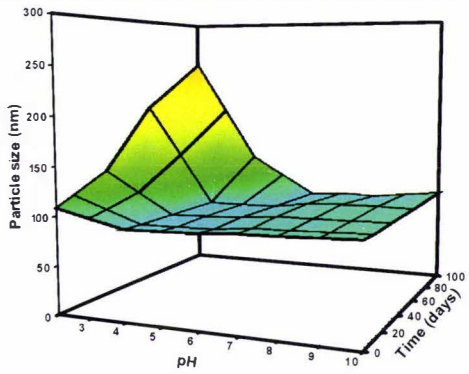
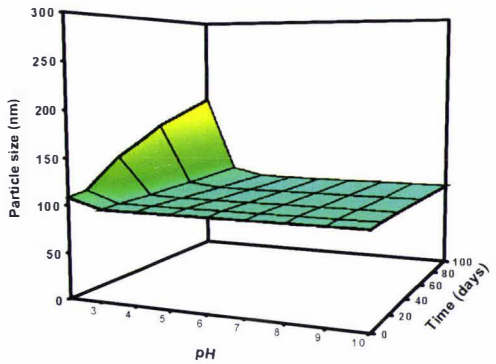
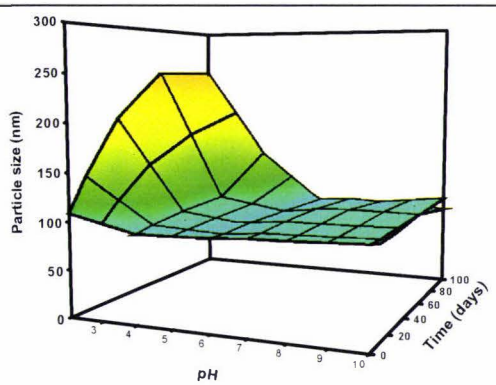
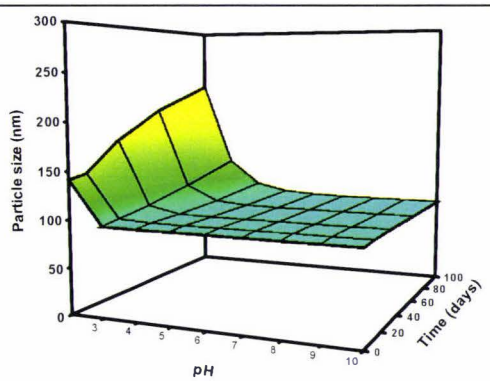
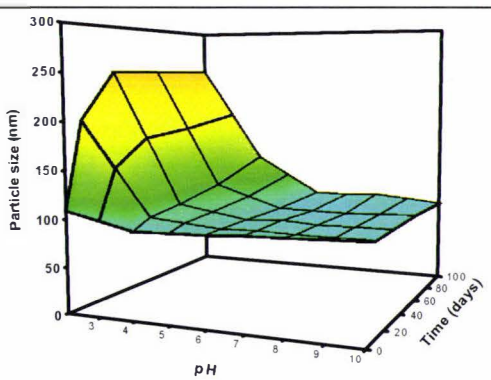
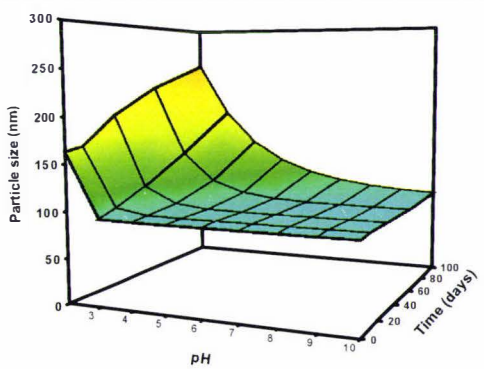
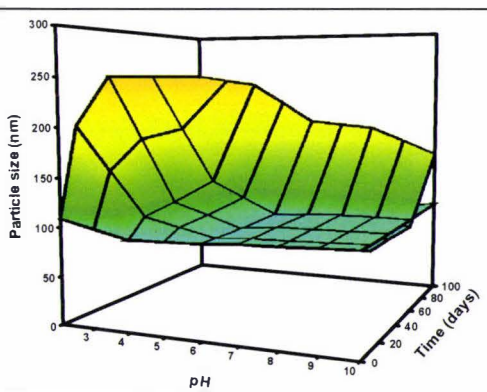
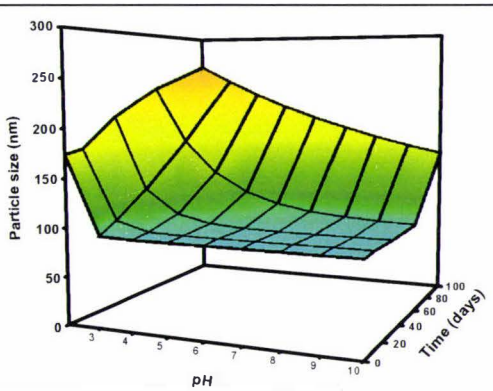
| Storage temperature | Experimental data on changes in liposome diameter | Predicted changes in liposome diameter |
|---|---|---|
| 4 °C |  |  |
| 20 °C |  |  |
| 30 °C |  |  |
| 35 °C |  |  |



**Figure 6-11 Comparison between experimental data on the change in average hydrodynamic liposome diameter for Phospholac dispersions and change predicted by the model Each intersection is a data point..**

### 6.4.2.3 CF entrapment

The CF entrapment of the liposome dispersions at 20 °C are shown in Figure 6-12. It appeared that the majority of this loss occurred during the first week of storage. The loss tended to be greatest at the extremes of pH, with pH 6 showing the highest amount of retained CF. There was some inconsistency within the triplicates, especially for the Ultralec dispersions and at the higher temperatures for all samples, with further replicates demonstrating a similar degree of scatter.

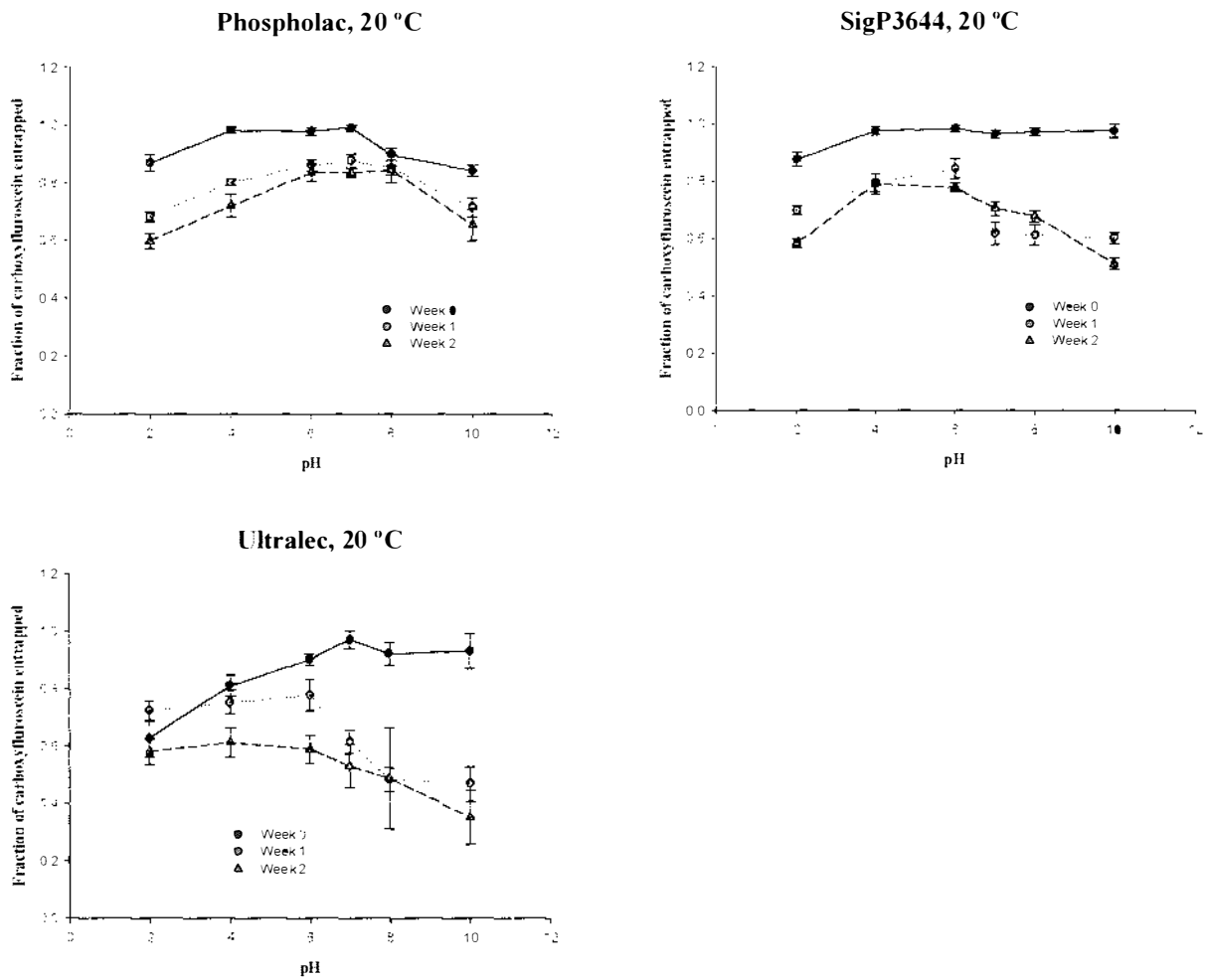



**Figure 6-12 Effect of pH on the ability of liposome dispersions to retain entrapped CF during storage at 20 °C. Mean of three measurements is plotted, with the error bars ± 1 standard deviation.**

Comparing the changes in liposome diameter (Figure 6-8 to Figure 6-10) with the release of CF, the diameter increased most significantly at the low pH values which corresponded with the lowest release of CF. In some of the samples at the higher temperatures, the results appeared to indicate an increase in entrapped CF over time. This observation is not sensible, as the CF should diffuse from the zone of high concentration inside the liposome to the zone

of lower concentration in the bulk aqueous phase. This would cause a reduction in the proportion of entrapped CF over time. The concentration of CF in the bulk aqueous phase will increase over time, and rupture of liposomes would also increase the level of unentrapped CF, but there should never be a lower concentration of CF inside the liposomes than in the bulk phase.

Considering both the apparent increase in CF entrapment over time and the inverse relationship between changes in entrapment and liposome diameter, it appears that there may be some form of binding occurring within the system. There are no reports in the literature of CF binding to phospholipids, but the commercial fractions used in this work contain a significant amount of protein and carbohydrate material. Another possibility is that as small aggregates formed in the less-stable systems, some of the CF has become trapped within these structures. These aggregates may also impede the separation of the sample of the bulk aqueous phase by partially blocking the filter in the Centrisart centrifugal tube.

Another possible explanation is that CF actually does permeate through the liposome membrane. A literature search revealed that it is only the charged form of CF that is membrane impermeable, with the uncharged form freely crossing the bilayer (Lee *et al.*, 1998). Several articles were also found that used the diffusion of CF (in the charged form) across the liposome membranes as an indication of permeability (Kirby *et al.*, 1980; Sagrista *et al.*, 2000; Bayazit, 2003). This suggests that the basic assumption made for this experiment - that any CF in the bulk aqueous phase must have come through liposome instability - is invalid.

Due to the obvious inadequacies with the above method for following liposome stability during storage, measurements of CF entrapment were discontinued after 2 weeks.

#### 6.4.2.4 Lipid oxidation

The oxidation of the lipids during storage was followed by measuring the peroxide value and level of conjugated dienes present in the samples. These measurements provide a chemical means of assessing the stability of the system, and provide a useful addition to the physical changes shown by the measurements of hydrodynamic diameter.

6.4.2.4.1 Peroxide value

The peroxide value of the fresh pH 7 dispersion was measured prior to the pH adjustment and storage of the samples. It was not thought necessary to measure each sample across the pH range, as altering the pH should not have any immediate effect on the peroxide value of the sample. The changes in peroxide value during storage at 20 ºC are shown in Figure 6-13.

The initial peroxide values ranged from 0.08 for the Phospholac dispersion to 0.15 for the SigP3644 sample. However, this initial value for Phospholac may have been incorrect, as the peroxide values measured for pH 7-10 after 8 days were lower than the initial value.

For all three liposome dispersions there was a general increase in peroxide value over time, with a significantly faster rate of increase at the higher temperatures. The overall increase was much smaller in the Phospholac and Ultralec dispersions, with the peroxide values of samples at 35 ºC for 55 days being 4.5 times the initial value. The comparable SigP3644 sample had a peroxide value of 9.7 times its initial value.

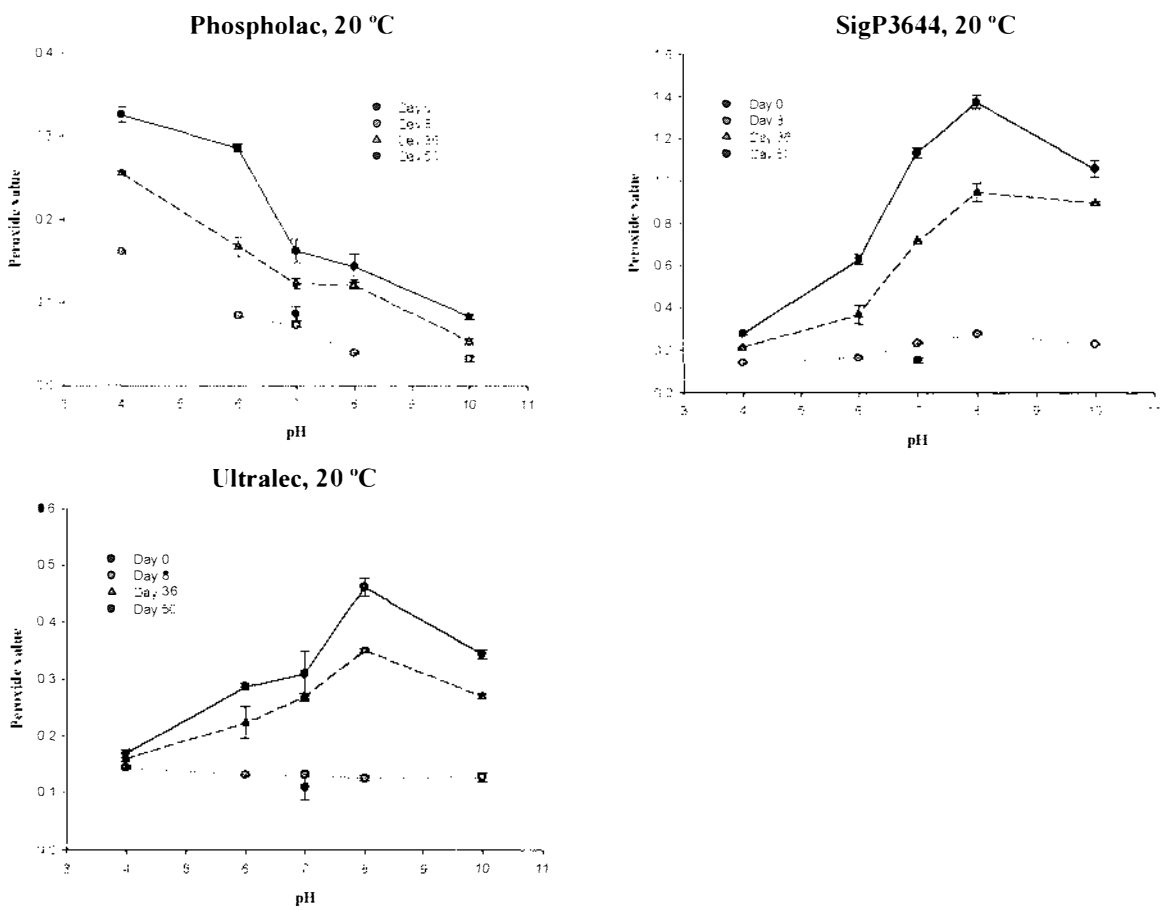



**Figure 6-13 Change in peroxide values for 10% phospholipid liposome dispersion during storage at 20 ºC. Mean of three measurements is plotted, with error bars showing ± 1 standard deviation.**

The effect of pH on peroxide value differs between the fractions. The Phospholac samples tend to have the highest peroxide value at the lower end of the pH range, while the peroxide values for the SigP3644 and Ultralec dispersions were highest at pH 7-8 and 8-10, respectively. This seems unusual as oxidation usually occurs most rapidly at extremes of pH. However, hydroperoxides are unstable molecules and tend to break down over time to form a variety of products including aldehydes and ketones. This means that the total hydroperoxide concentration of a product will reach a peak and then decline as more of the hydroperoxide molecules are degraded and the aldehyde and ketone concentration increases. It is possible that the hydroperoxide content of SigP3644 and Ultralec dispersions at pH 10 was lower than at pH 7 and pH 8 because the peroxides in these samples had already begun to break down.

#### 6.4.2.4.2 Conjugated dienes

The levels of conjugated dienes formed in each of the liposome dispersions during storage at 20 ºC are shown in Figure 6-14. The absorbance at 232 nm of the pH 7 dispersion was initially measured prior to pH adjustment, as it was thought that altering the pH should not have any immediate affect on the level of conjugated dienes present in the sample. There appears to have been a mistake with the initial reading for the Phospholac and possibly the Ultralec dispersions, as it was significantly higher than the subsequent readings. Other than the day zero reading, the absorbance values all increase over time, indicating the formation of conjugated dienes through the oxidation process.The absorbance level for the Phospholac dispersion was between 0.8 and 1.5 for all readings, including after storage at 35 ºC for 55 days. The SigP3644 and Ultralec dispersion had much lower starting values (0.4 and 0.3 respectively), but after storage at 35 ºC for 55 days these values increased to 4.0 for the SigP3644 samples and 1.8 for the Ultralec dispersions. There was a strong correlation between temperature and the rate of increase in conjugated dienes, which is not surprising since the rate of oxidation is known to increase with temperature. There was some indication that the rate of conjugated diene formation increased at the higher pH values during storage of the SigP3644 and Ultralec dispersions. However, the absorbance of the low pH Phospholac samples increased more than the absorbance of high pH Phospholac samples.

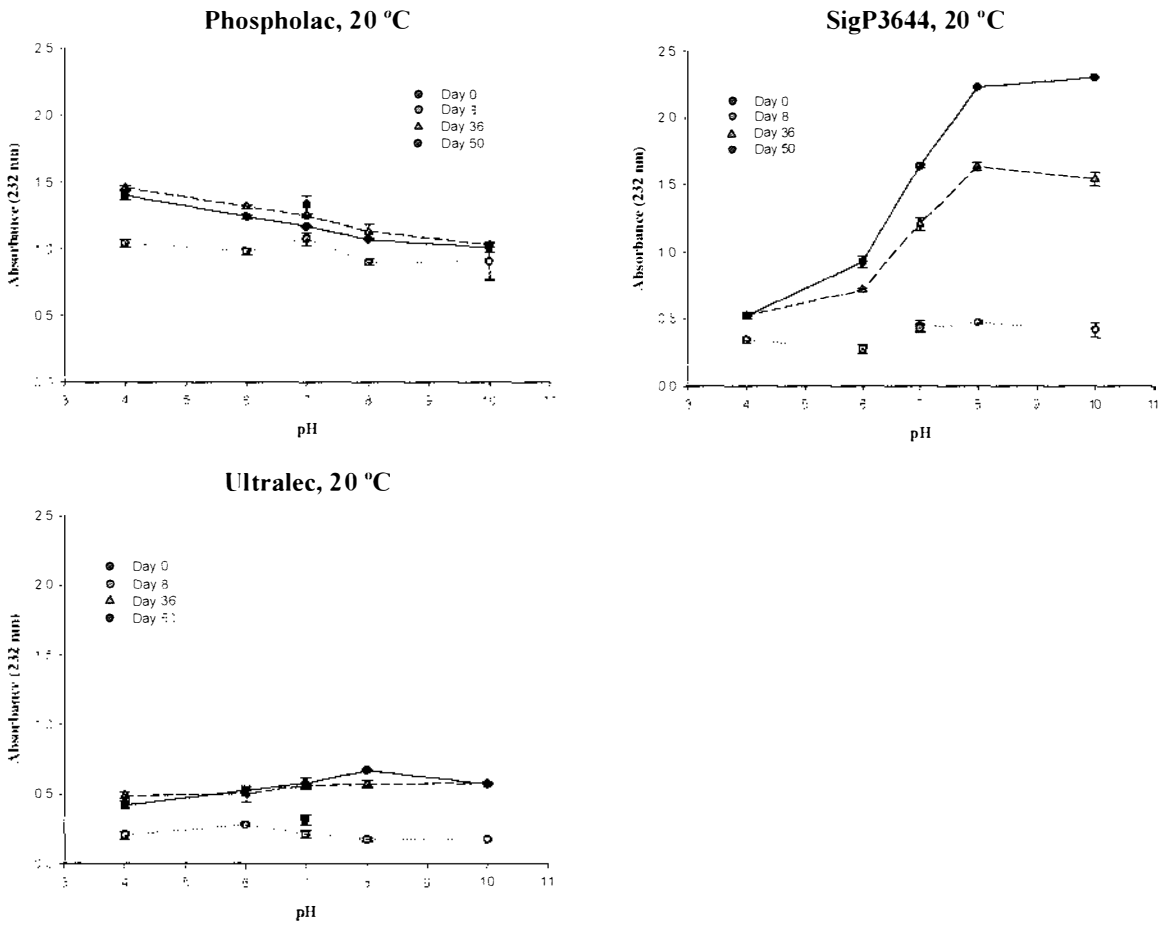



**Figure 6-14 Change in absorbance at 232 nm as a measure of level of conjugated dienes in a 10% phospholipid liposome dispersion during storage at 20 °C. Mean of three measurements is plotted with error bars showing ± 1 standard deviation.**

The high initial value for the Phospholac dispersion may be due to some non-lipid component present in the fraction that also absorbs at 232 nm, but the relatively small increase compared with the other two fractions indicates that the rate of conjugate diene formation is much lower.

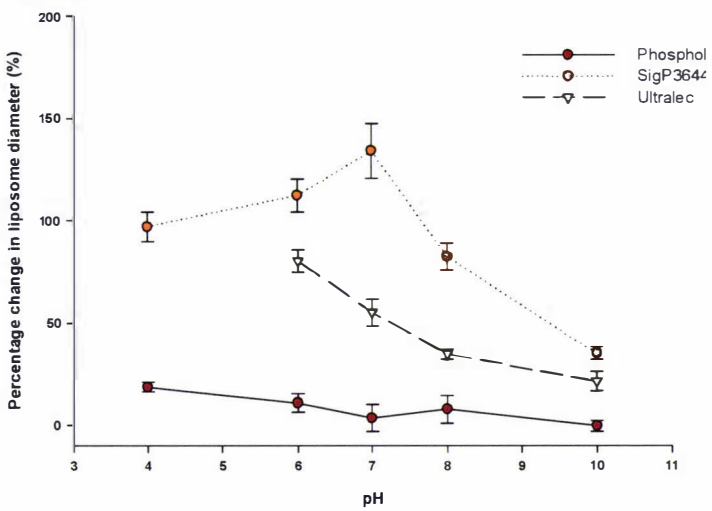
Overall, the peroxide value and conjugated diene results suggest that the SigP3644 phospholipid fraction is more susceptible to oxidation than either of the other two fractions. The Phospholac liposome dispersions have a much lower rate of formation of conjugated dienes, most likely reflecting its primarily saturated fatty acid profile. The increase in rate of oxidation at high pH for the SigP3644 and Ultralec fractions was reflected in both the peroxide value and the formation of conjugated dienes.

### 6.4.3 Heat treatments

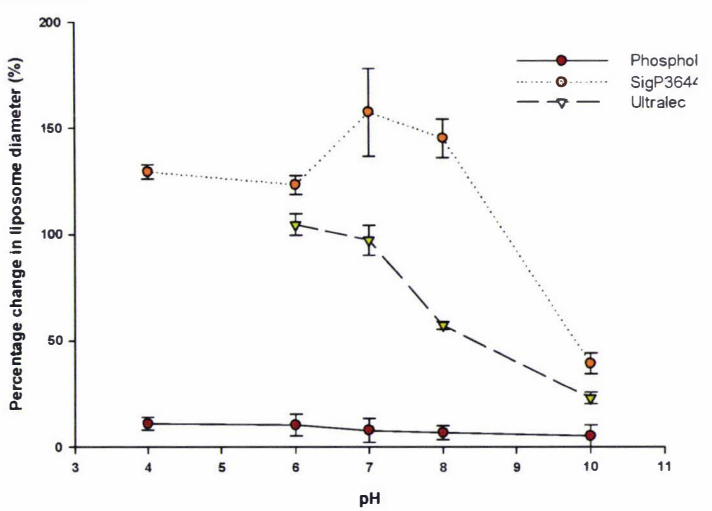
Figure 6-15 shows the effect that a variety of heat treatments had on the average liposome diameter of the phospholipid dispersions at different pH values. In order to allow comparison between the different phospholipid fractions, the results are reported in terms of the percentage change in diameter caused by the heat treatment.

In general, heat treatment at high pH appeared to result in a much smaller change in average liposome diameter than the same treatment at lower pH values. The Phospholac dispersions were consistently much more stable at all pH values than either Ultralec or SigP3644 dispersions, with SigP3644 generally the most sensitive to all of the heat treatments used. However, the Ultralec dispersions at pH 4 became very turbid and formed large lumps at all heat treatments, but no lumps formed in the SigP3644 samples.

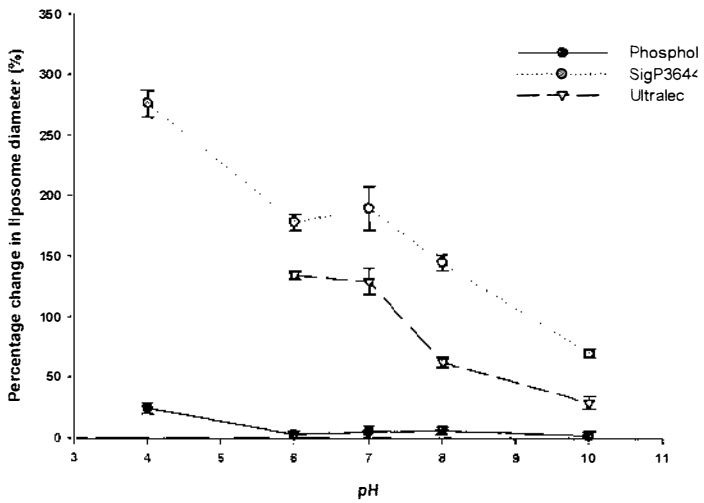
**50 "C for 15 hours.**




**72 "C for 2 minutes.**



**90 °C for 2 minutes.**




**141 °C for 15 seconds.**

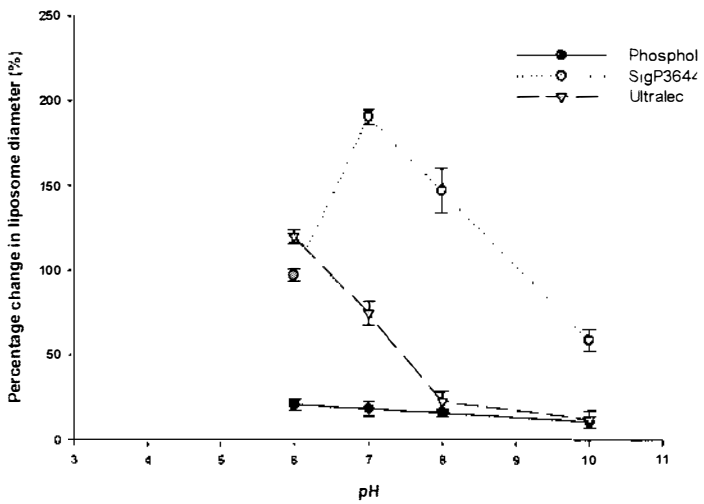



**Figure 6-15 Changes in average diameter of liposome dispersions at a range of pH values after various heat treatments. Mean of three measurements is plotted with error bars showing ± 1 standard deviation.**

There is relatively little published material which considers the heat stability of liposome dispersions, however Chandran *et al.* (1997) stated that liposomes were thermolabile and that the lipids are likely to be hydrolysed during the high sterilisation temperatures. Conversely, Arnaud (1995a) reported that heat sterilisation could be used to attain microbiological stability of selected liposome dispersions, and that it did not appear to affect liposome stability. Zuidam et al. (1993) also looked at the use of heat for the sterilization of liposomes, and found that liposome structure and lipid oxidation values were not affected by autoclaving (121 °C for 15 minutes).

It is anticipated that commercial heat treatments with good mixing of the product and accurate control over the exposure temperature and time would significantly reduce the damage to the liposome dispersions and there would be a smaller increase in liposome diameter. However, the UHT treatment, which was provided by a pilot scale plant had the most severe effect, despite the short exposure to the high temperatures and fast cooling.

### *6.4.4 Ionic concentration*

The effect that changes in ionic concentration had on the average liposome diameter of the phospholipid dispersions is shown in Figure 6-18. In order to allow comparison between the different phospholipid fractions, the results are reported in terms of the percentage change in diameter caused by the addition of the sodium chloride or calcium chloride.

Overall, the Phospholac dispersions were less affected than either the SigP3644 or Ultralec dispersions by the changes in ionic concentration. This difference was most clearly shown with the addition of calcium ions. An increase in liposome diameter of less than 10% was exhibited for up to 1.4 M NaCl addition for the Phospholac dispersion, compared with 0.2 M NaCl and 0.15 M NaCl for the SigP3644 and Ultralec dispersions respectively. Calcium ions caused more rapid destabilization, with 0.25 M $CaCl_2$ addition resulting in a 10% increase in diameter for the Phospholac dispersion and even 0.1 M $CaCl_2$ causing >20% increase for both the SigP3644 and Ultralec samples.

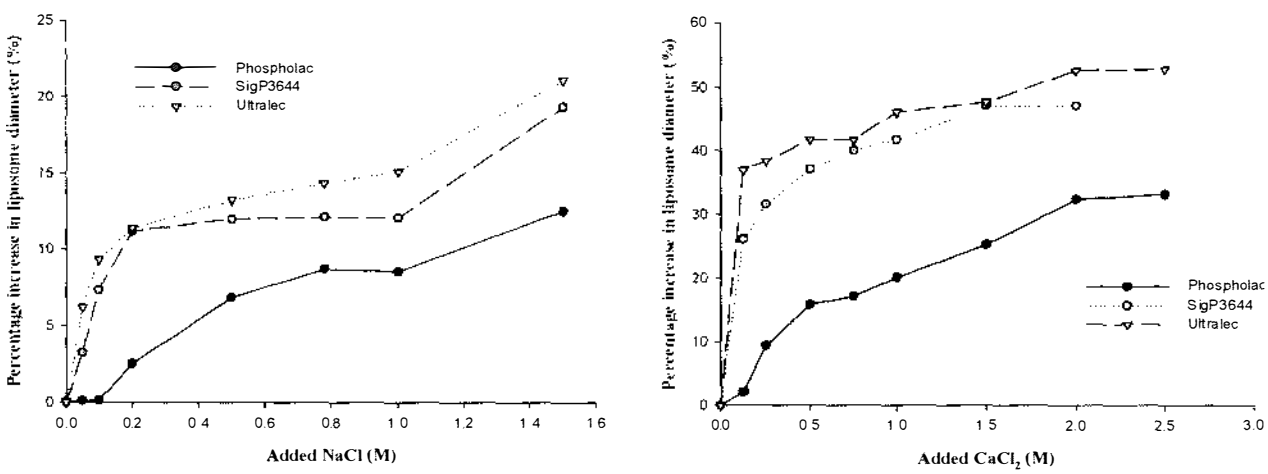



**Figure 6-16 Effect of increasing ionic concentration on the average diameter of liposome dispersions.**

To determine whether or not these differences were due to the phospholipids present rather than the ionic balance of the fractions, samples were dialysed against each of the other fractions to exchange the ionic profiles. These samples then had varying amounts of NaCl and $CaCl_2$ added as previously described. The results for the addition of NaCl are shown in Figure 6-17.

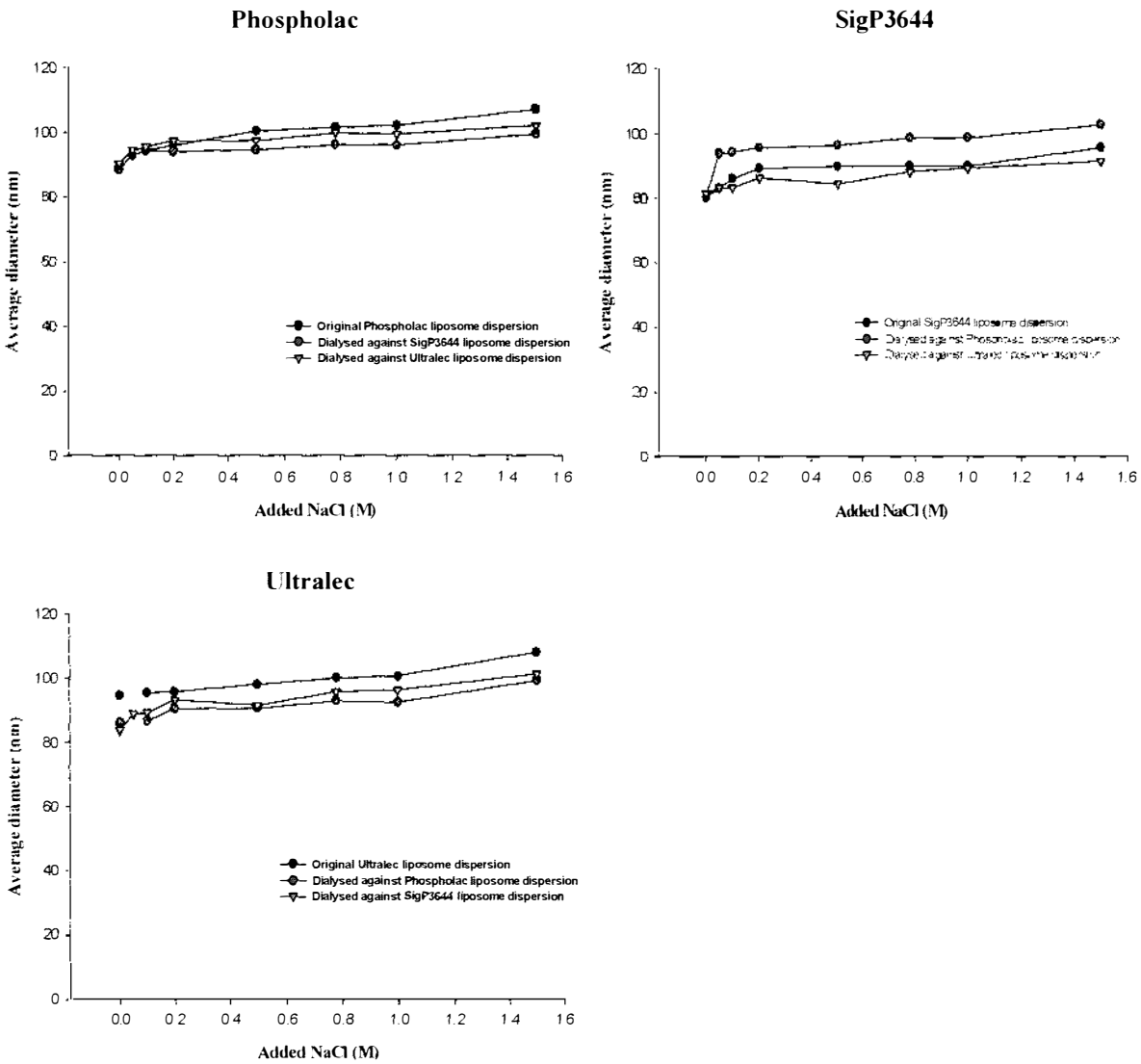



**Figure 6-17 Effect of changing the ionic profile of the liposome dispersion by dialysis against another liposome dispersion on the susceptibility of the dispersion to NaCl.**

The SigP3644 dispersion dialysed against the Phospholac fraction was less stable in the presence of $Na^+$ ions than the same dispersion with its original ionic profile or after dialysis with the Ultralec fraction. The Phospholac and Ultralec dispersions appeared to have a slightly smaller average liposome diameter when their original ionic balance was exchanged for that of either of the other two fractions. However, the change in average diameter due to

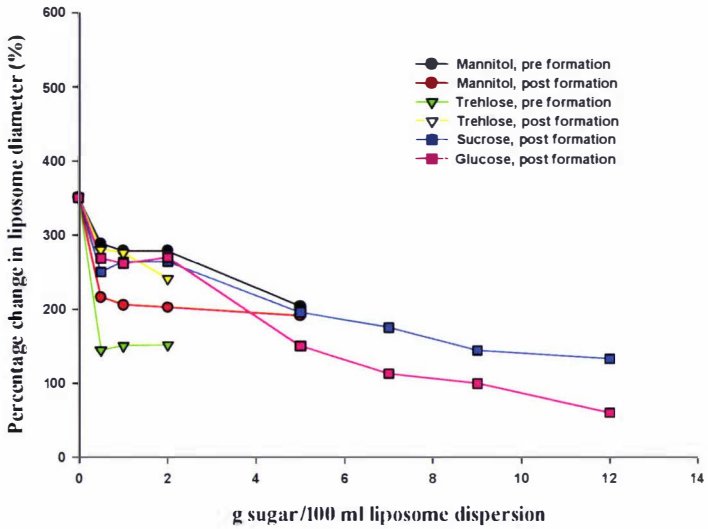
increasing ionic concentration was similar regardless of the ionic balance of the particular phospholipid fraction. These results indicate that the natural mineral content of the fractions was not responsible for the observed differences in the susceptibility of the liposome dispersions to increasing ionic concentration (as shown in Figure 6-17).

### *6.4.5 Freezing*

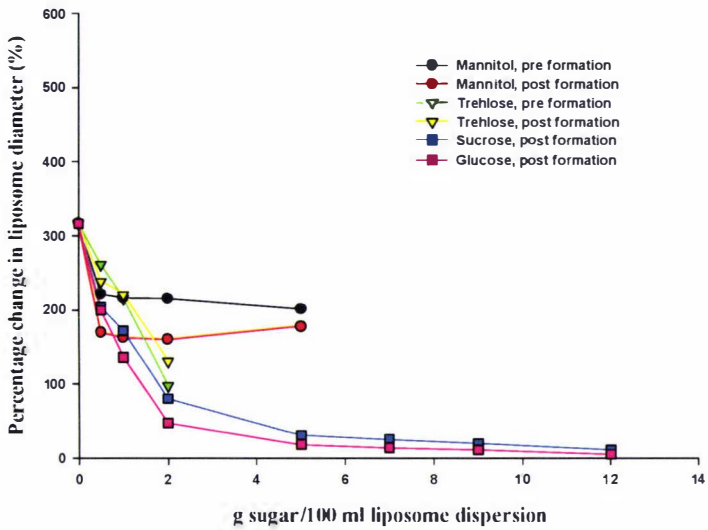
The effects of different cryo-protective sugars on the percentage increase in average liposome diameter during freezing of a 10% phospholipid solution are shown in Figure 6-18. Typical TEM micrographs of the thawed liposome dispersions are shown in Figure 6-19.

With no added cryo-protective sugars, the freeze/thaw process appeared to destroy the liposome structure, causing over a 300% increase in average size (Figure 6-18) and showing virtually no sign of liposome structure in the TEM micrographs (Figure 6-19). The addition of 1% sucrose prior to freezing not only significantly reduced the increase in liposome size of the thawed dispersion but some spherical membranous structures can be seen in the TEM micrographs. Addition of higher concentrations of sucrose further reduced the change in liposome size and a larger number of small particles were present in the micrographs. The addition of trehelose before the formation of the Phospholac liposomes appeared to reduce the increase in average diameter compared with the addition of trehelose after liposome formation, but there was no significant difference between pre- and post-formation addition of trehelose for the SigP3644 and Ultralec samples. Overall, sucrose and glucose seemed to have the best protective effect on a weight/weight basis, although trehelose and mannitol appeared to be at least as effective as sucrose in the Phospholac dispersions. Trehelose is the most commonly used cryo-protective sugar in the literature, but Anchordoguy *et al.* (1987) found that sucrose and trehalose seemed to be equally as effective during freeze-thaw processes. Van Winden (2003) commented that disaccharides such as sucrose, trehelose and lactose are more successful than monosaccharides such as glucose or sorbitol at protecting liposome structure during freezing. However, Figure 6-18 shows that for all three liposome dispersions, glucose was at least as successful as sucrose in preventing large increases in average liposome diameter caused by the freeze-thaw process.

**Phospholac**




**SigP3644**




**Ultralec**

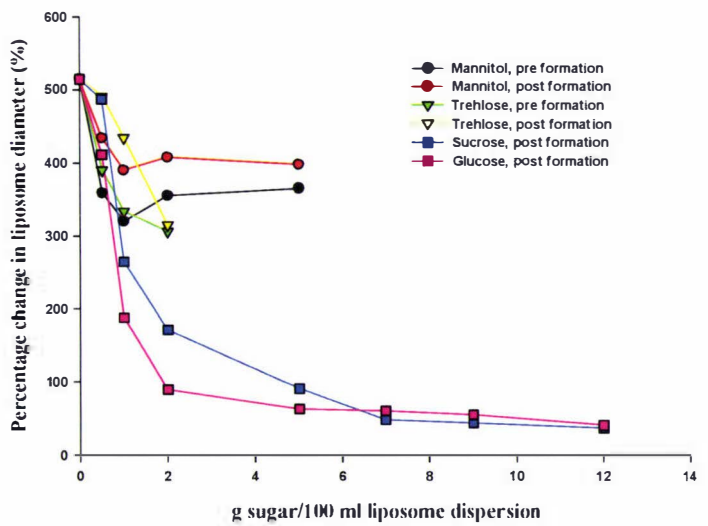
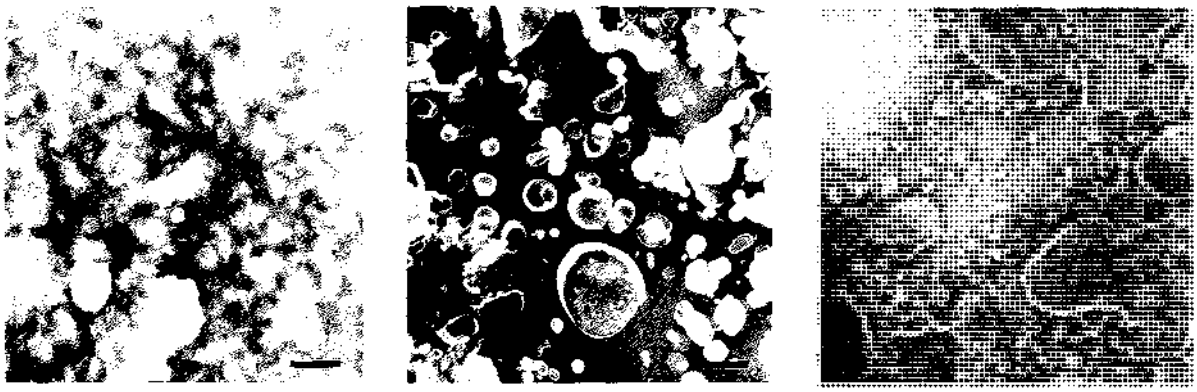



**Figure 6-18 Effect of cryo-protectant sugars on liposome diameter in a 10% phospholipid dispersion during a freeze-thaw cycle.**



**Ultralec liposome dispersion frozen with no sucrose** | **Ultralec liposome dispersion frozen with 1% sucrose** | **Ultralec liposome dispersion frozen with 5% sucrose**

**Figure 6-19 TEM micrographs showing effect of adding sucrose to pre-formed liposome dispersions before freezing. Bar = 1000 nm.**

There was a corresponding decline in the increase in liposome size as the amount of any of the cryo-protective sugars increased, but the relationship appeared to be asymptotic, reaching a plateau at approximately 20% and 40% increase for sucrose or glucose in the SigP3644 and Ultralec dispersions respectively. The asymptotic nature of the curve was not so apparent in the Phospholac dispersions, with increases in the amount of glucose added still having a significant effect on liposome size at 12% glucose. However, the curve for sucrose may have been reaching a plateau at ~120% increase in liposome diameter, much higher than the increases observed for similar levels of sucrose in either SigP3644 or Ultralec dispersions.

These results appear to indicate that even with the addition of cryo-protective sugars, freezing and thawing results in significant evidence of damage to the liposome dispersions. However, van Winden (2003) found that a ratio of >2:1 sugar to phospholipid on a weight/weight basis was required for successful freeze-thawing of liposome dispersions, which is almost twice as much sugar than the highest concentration used during this work. Therefore, further experiments with higher concentrations of cryo-protective sugars would need to be undertaken to determine whether these liposome dispersions are able to successfully withstand the stresses of a freeze-thaw cycle.

### 6.4.6 Freeze drying

The freeze-drying and rehydration of a 10% Ultralec liposome dispersion resulted in the loss of almost all signs of any membrane structure (Figure 6-20), with an average hydrodynamic diameter of well over 1500 nm. Addition of cryogenic sugars such as maltose and trehalose to the dispersion prior to the freeze-drying process reduced the liposome diameter to between 400-600 nm. However, this was still much larger than the pre-freeze-drying average diameter of 85 nm, and the TEM micrographs indicated significant damage had occurred to the liposome structure. Increasing the amount of sugar present did appear to improve liposome stability, but even the addition of 5% trehalose resulted in an average liposome size of 425 nm and the presence of many very large and irregular non-liposome structures.

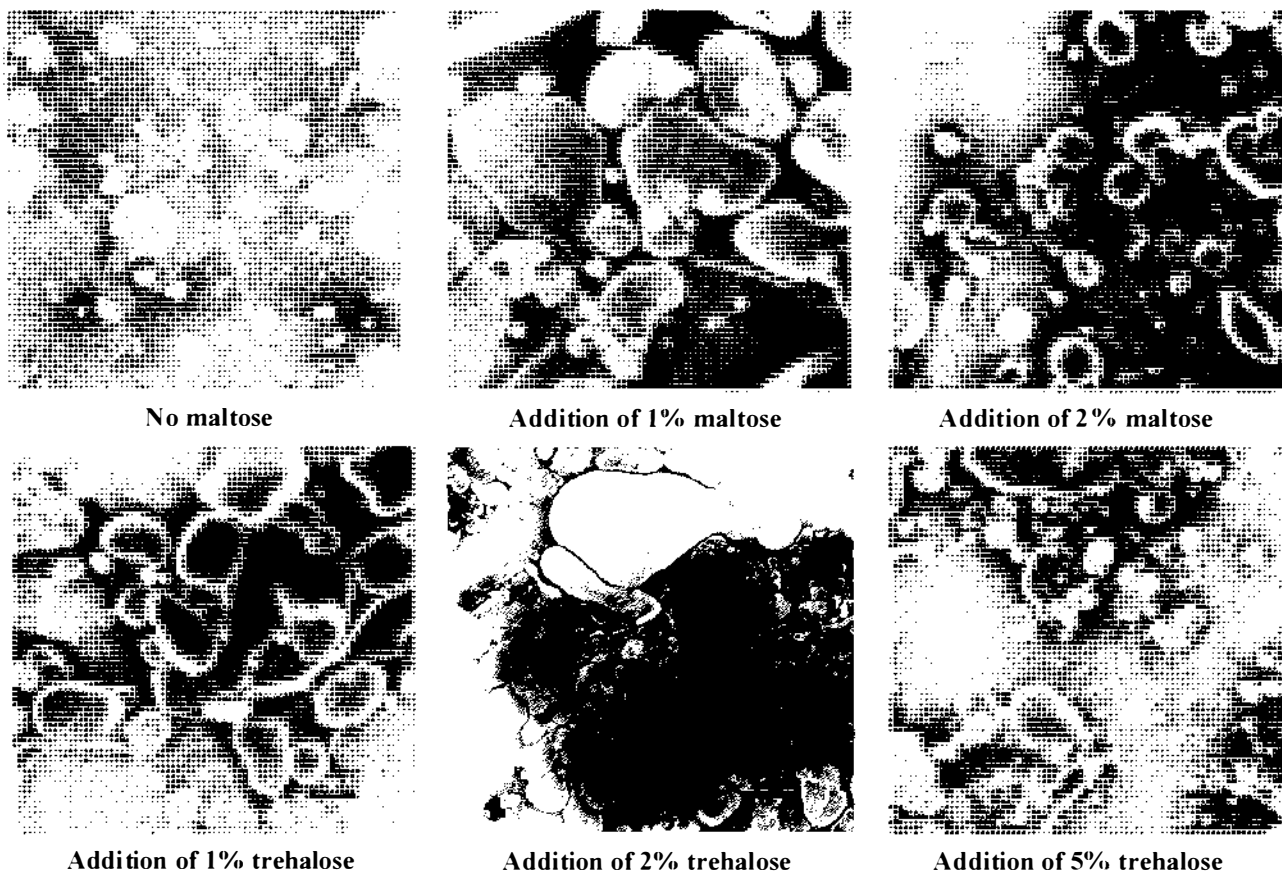



**Figure 6-20 Negative-staining TEM micrographs showing the effect of addition of cryogenic sugars on freeze-drying and rehydration of Ultralec liposome dispersions. Bar = 1000 nm.**

The removal of the free water makes freeze-drying a much more severe treatment than a simple freeze/thaw cycle. Given that significant damage was still caused by the freeze-thawing of liposome dispersions containing cryo-protective sugars at up to 12% w/w, it is

not surprising that there was major damage to the liposome structure after freeze-drying a dispersion containing similar sugar concentrations. Increasing the levels of the cryo-protective sugars would help reduce this damage, but significant further work to optimise the freeze-drying conditions is likely to be required before there is any possibility of using this technique to successfully extend the shelf-life of the liposome dispersions.

## *6.5 Conclusions*

The Phospholac liposome dispersions showed significantly better stability than either of the two soy fractions during storage at a variety of temperatures. The Phospholac dispersions were also more resistant to heat processing treatments ranging from medium temperature for extended periods through to UHT processes. Increases in ionic concentration resulted in much more rapid aggregation and/or fusion among the SigP3644 and Ultralec liposomes than in the Phospholac dispersions. The ability to withstand higher ionic concentrations did not appear to be related to the natural mineral balance of the fraction, but seemed to be due to the differences in phospholipid and fatty acid composition. The fatty acid composition was also likely to be the primary reason for the lower susceptibility to oxidation demonstrated by the Phospholac liposome dispersions, particularly compared with the SigP3644 dispersions.

Although freezing and freeze-drying are commonly referred to as suitable methods for long term storage of liposomes, all three fractions underwent severe damage during these processes, shown by large increases in liposome size and TEM micrographs. There was some evidence to suggest that the Phospholac liposomes were more severely affected by these treatments than the SigP3644 and Ultralec dispersions. The addition of cryogenic sugars reduced the measured average diameter and improved the visual appearance of the micrographs, but even at addition levels of 12% w/w the damage was significant.

# Chapter 7: Entrapment of hydrophilic and hydrophobic compounds in liposome dispersions

## *7.1 Introduction*

During the formation of the phospholipid bilayer, hydrophobic compounds may be incorporated inside the membrane. This bilayer may then form a liposome, enclosing a portion of the aqueous environment inside the vesicle and thus entrapping hydrophilic compounds present in the aqueous phase. Entrapment efficiency is the proportion of the desired compound present in the system that is held within the liposome structure, either in the aqueous compartments or incorporated in the bilayer membrane.

The bilayer membrane is held together by non-covalent interactions, allowing it to carry a wide variety of hydrophobic compounds. The inclusion of such compounds at up to approximately 10 wt. % will not usually disrupt the membrane, although membrane fluidity and permeability may be affected (New, 1990b). For highly hydrophobic materials and liposome preparation methods based on the thin-film technique, entrapment is usually close to 100% regardless of the liposome type and composition. Overall entrapment is thus determined by the total amount of phospholipid present (Weiner, 1995). For molecules of lower hydrophobicity, the location of the compound within the liposome and its entrapment efficiency will depend on its partitioning between the hydrophilic and hydrophobic phases. The dehydration-rehydration method used by one group resulted in entrapment efficiencies ranging from 1 to 34% for proteins of varying hydrophobicities (Picon *et al.*, 1994).

Hydrophilic entrapment for a given liposome system is proportional to the entrapped volume of the liposomes (Weiner, 1995) and the phospholipid concentration. The use of high concentrations of the hydrophilic material increases the active material to phospholipid ratio, making more efficient use of the phospholipid available. The theoretical maximum for entrapment efficiency is 70%, as 30% of the aqueous phase is required to fill the gaps between the liposomes (Martin, 1990). However, this is based on the premise that the liposomes are rigid, spherical vesicles. There is evidence that the bilayer membrane is quite flexible and that liposomes are often non-spherical (section 5.3.2.1, *Chapter 5:*

*Characterisation of liposomes*), which at very high phospholipid concentrations may allow entrapment efficiencies higher than this theoretical maximum.

Reported entrapment efficiencies for hydrophilic materials vary greatly depending on the details of the preparation method and specific hydrophilic compound. The standard thin-film liposome preparation method usually gives a hydrophilic entrapment efficiency of only 1-9%, but repeated freezing and thawing of the solution can increase the efficiency to 35-88%. Mayhew *et al.* (1984) used a Microfluidizer® to encapsulate cytosine arabinoside (Ara-C), and obtained entrapment efficiencies ranging from 5-75% depending on the operating conditions and phospholipid concentration.

The calculation of the entrapment efficiency is usually based on the removal of unincorporated material and the assumption that the remaining material is all entrapped. This requires a method to separate free and entrapped species, and is usually based on either size and/or density differences (New, 1995c; Reineccius, 1995b). A number of different techniques were used during this study, including centrifugation, gel filtration, membrane filtration and dialysis. Details on the advantages and disadvantages of each of these techniques, as well as a comparison between methods reported in the literature, can be found in section 2.7.3, *Chapter 2: Literature review*.

To characterise and compare the entrapment profiles of the three liposome dispersions, a number of model compounds were used. β-Carotene was chosen as a model hydrophobic compound, as its bright orange colour allowed the presence of the molecule to be followed qualitatively by eye and quantitatively by spectrophotometric techniques. The hydrophilic dye carboxyfluroscein (CF) is used extensively in the literature due to its fluorescent nature and lack of membrane permeability. However, due to obviously invalid results obtained when using CF as an indicator of liposome stability (*Chapter 6: Liposome stability*), it was decided not to use CF in further experiments. Glucose, sucrose, and ascorbic acid were selected as model small hydrophilic compounds as they had been investigated by earlier researchers (Kirby *et al.*, 1991; Arnaud, 1995c; Waters *et al.*, 1997), were inexpensive and there were a wide range of options for measuring their concentration. In addition, the entrapment of ascorbic acid would potentially have direct applications in food systems.

## 7.2 Hydrophobic entrapment

### 7.2.1 Materials and methods

#### 7.2.1.1 Liposome production

There are two basic forms of β-carotene available for use in food systems – oil-soluble and water-soluble. The water-soluble form has between 1-10% β-carotene dispersed in a matrix that may be made of compounds such as starch, gelatin and dextran. The oil-soluble form usually has 5-25% β-carotene dispersed in a food-grade oil. More pure forms are available from chemical suppliers, but these are not commonly used in food systems due to difficulties in incorporating the β-carotene into the food products.

The basic method outlined in *Chapter 3: Phospholipid fraction and method selection* was used as a starting point for β-carotene entrapment. Initially the water-soluble form of β-carotene was added to the phospholipid with the imidazole buffer. However, the β-carotene molecules failed to become incorporated within the bilayer membrane, instead remaining in the aqueous phase. Attempts to mix the oil-soluble form into the phospholipid fractions prior to the addition of the aqueous phase resulted in the formation of fine emulsions, with the oil/β-carotene droplets stabilised by the phospholipids.

A 95% β-carotene powder was obtained from Sigma (Catalogue #C9750). Because pure β-carotene is insoluble in water, some form of solvent must be used to combine the carotene and the phospholipid. In the literature, pure β-carotene is usually dispersed in chloroform and the liposome produced using the thin-film method. A variation on this technique was developed as outlined below. Because chloroform is not suitable for incorporation in food systems, experiments were also conducted using ethanol as a food-grade alternative.

The β-carotene was added to a beaker containing the phospholipid and either ethanol or chloroform. This mixture was heated to approximately 60 °C on a hot plate with constant stirring, and held until the phospholipid formed a melt. The solvent was then evaporated over an 80 °C water bath. The aqueous phase was added and stirred using a magnetic stirrer until the lipid film had hydrated. The Microfluidization process then continued as outlined previously.

#### 7.2.1.2 β-carotene analysis

The presence of β-carotene can be determined qualitatively by eye, however quantitative measurements were required to ensure valid comparisons between systems. The concentration of β-carotene in a sample was measured quantitatively using a Waters 2690 HPLC Separations Module and an Alltech Prevail C18 Column (150 x 4.6 mm, 5 μm).

Samples were prepared for HPLC by adding 1 ml chloroform to between 0.1-1ml of the liposome dispersion, then making up to 5 ml with methanol. This solubilised both the β-carotene and the phospholipids, producing a clear solution which was passed through 0.22 μm filters before filling into HPLC vials. The mobile phase was 90:10 methanol:chloroform, and absorbance measured at 436 nm. The primary β-carotene peak eluted at between 8.6-8.8 minutes, although there was a small peak at approximately 6 minutes. Control samples containing liposome with no β-carotene present did not produce any peaks under these conditions.

#### 7.2.1.3 Separation of unentrapped β-carotene

The hydrophobic nature of the β-carotene caused the unentrapped material to form aggregates of varying sizes. Two techniques were used to remove the aggregates. Firstly, 1 ml of the liposome dispersion was passed through a 50 x 200 mm column of Sepherose gel (Pharmacia Biotech, USA). Imidazole buffer was used as the mobile phase, and a clear separation of the unentrapped β-carotene and the liposomes was obtained, as shown in Figure 7-1. However, the wide particle size distribution of the liposome dispersions resulted in their elution over approximately 20 ml, and it was difficult to determine when to start and finish collection. The technique was also very time consuming, with each run taking 2 hours.

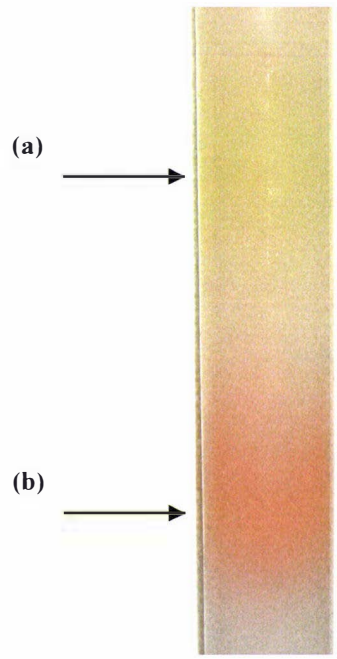



**Figure 7-1 Gel filtration column showing the separation of liposomes with entrapped β-carotene (a) and unentrapped β-carotene aggregates (b).**

In the second technique, 3 ml aliquots of the liposome dispersions were added to 6 ml of buffer solution and centrifuged at 4000 *g* for 4 hours in a CentraMP4R centrifuge (International Equipment Company, MA). The supernatant was removed, and the centrifugation step repeated once more. Centrifugation at this speed removed large β-carotene aggregates, but did not cause significant sedimentation of the liposomes or of the very small β-carotene aggregates. To remove the smaller particulates, the supernatant was then passed through a Millex GS 0.45 μm filter unit (Millipore, MA, USA). A reddish colour indicating the presence of β-carotene could be seen on the upstream side of the filter. While very large liposomes may not pass through the filter, the vast majority of vesicles were significantly smaller than 450 nm and it is unlikely that the exclusion of such a small number of liposome would affect the result.

### *7.2.2 Results*

Figure 7-2 shows the total amount of β-carotene entrapped per gram of phospholipid (using chloroform) as a function of the added β-carotene per gram of phospholipid. As expected, the amount of β-carotene entrapped increased almost linearly with increasing β-carotene addition up to ~ 6 μg/g phospholipid. However, upon addition of > 6 mg β-carotene per

gram of phospholipid the amount of entrapped β-carotene increased more slowly and then levelled off. This plateau value differed between the liposome dispersions, being ~ 6 μg β-carotene per gram of phospholipid for Phospholac, ~ 3.5 μg β-carotene per gram of phospholipid for SigP3644, and ~ 4.5 μg β-carotene per gram of phospholipid for Ultralec. ANOVA analysis found that there were significant differences between the entrapment values for all three fractions at 95%.

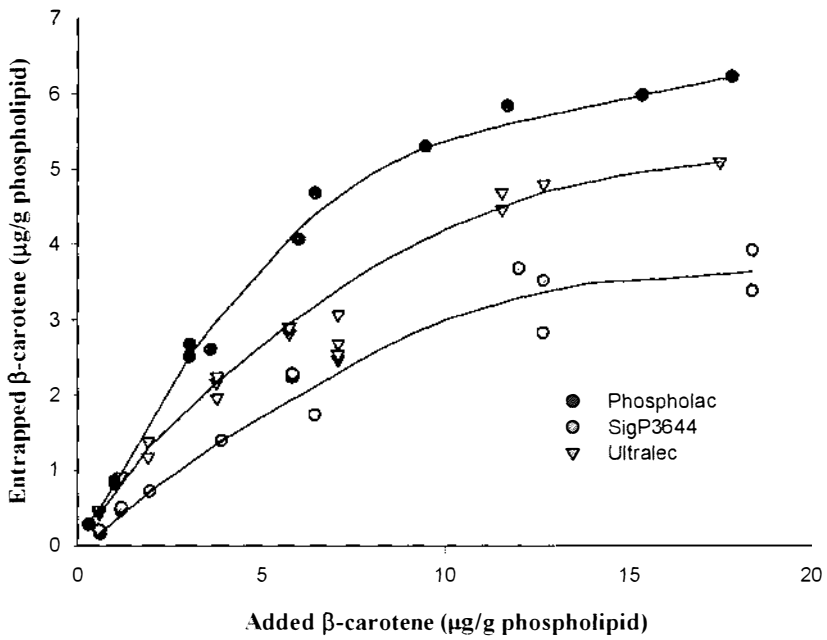



**Figure 7-2 Entrapment of added β-carotene in liposome membranes using chloroform to disperse the β-carotene and phospholipid prior to Microfluidization.**

This data (Figure 7-2) has been used to calculate entrapment efficiencies for β-carotene and the results are shown in (Figure 7-3). The differences in efficiency of β-carotene entrapment were obvious. When very small amounts of β-carotene were dissolved in chloroform along with either Phospholac or Ultralec, almost all of the β-carotene became incorporated into the liposome membrane. As more β-carotene was added, the proportion entrapped decreased. The entrapment efficiency of the SigP3644 liposome dispersion was considerably lower than the other two liposome dispersions; the efficiency did not exceed 0.5 even at very low β-carotene concentrations. The entrapment efficiency of the SigP3644 liposome dispersion was comparatively less affected by the increasing level of β-carotene addition.

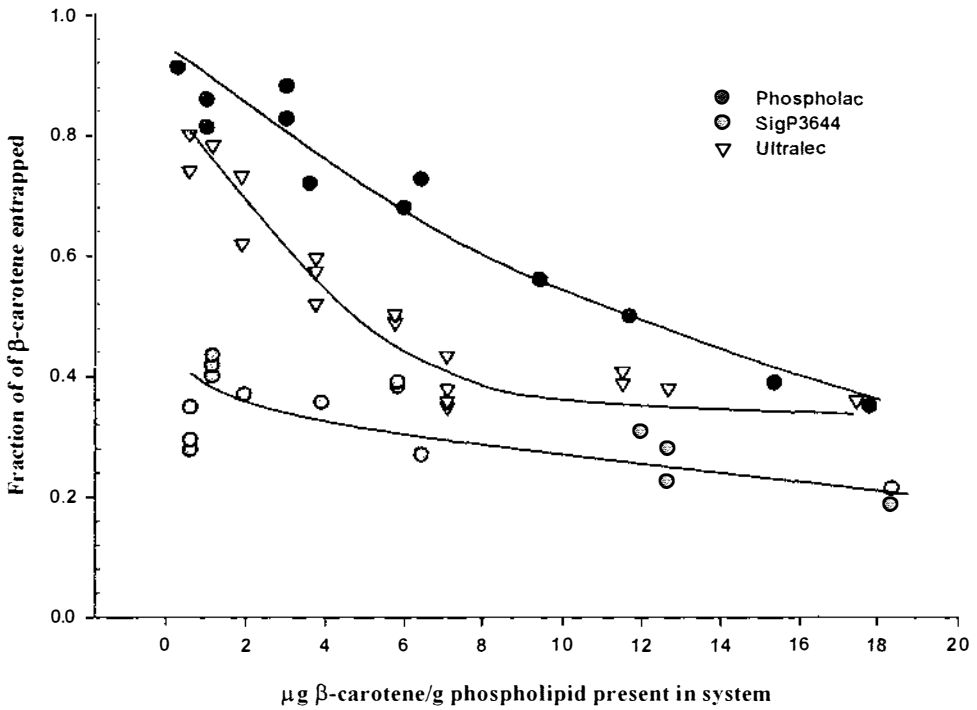



**Figure 7-3 Entrapment efficiency for added β-carotene in liposome membranes using chloroform to disperse the β-carotene and phospholipid prior to Microfluidization.**

The steeper slope of the linear portion of the Phospholac curve shown in Figure 7-2 is reflected in the higher entrapment efficiency below 15 mg β-carotene per gram of phospholipid. At almost all concentrations of added β-carotene used, Phospholac liposome dispersions had the highest entrapment efficiency. However, there was no significant difference between the entrapment efficiencies of the Phospholac and Ultralec dispersions at the highest level of added β-carotene.

Figure 7-4 shows the total amount of β-carotene entrapped per gram of phospholipid using ethanol as the dispersion medium instead of chloroform. It is clear that the use of ethanol significantly reduced the amount of β-carotene incorporated in the liposome membrane for all samples (compare the y-axis on Figure 7-2 and Figure 7-4). The highest amount of β-carotene added was much lower than for the systems using chloroform as the dispersion medium. This was because during sample preparation there were obviously substantial amounts of aggregated β-carotene, and the removal of all of the aggregates was blocking the filters and contaminating the gel column. The general shape of the plot shown in Figure 7-4

is very similar to the portion of Figure 7-2 between 0-6 μg β-carotene per gram of phospholipid. This suggests that had higher concentrations of β-carotene been added, the increase in the amount of β-carotene entrapped using the ethanol as the dispersion medium would be likely to slow down and reach a plateau in the same manner observed when using chloroform.

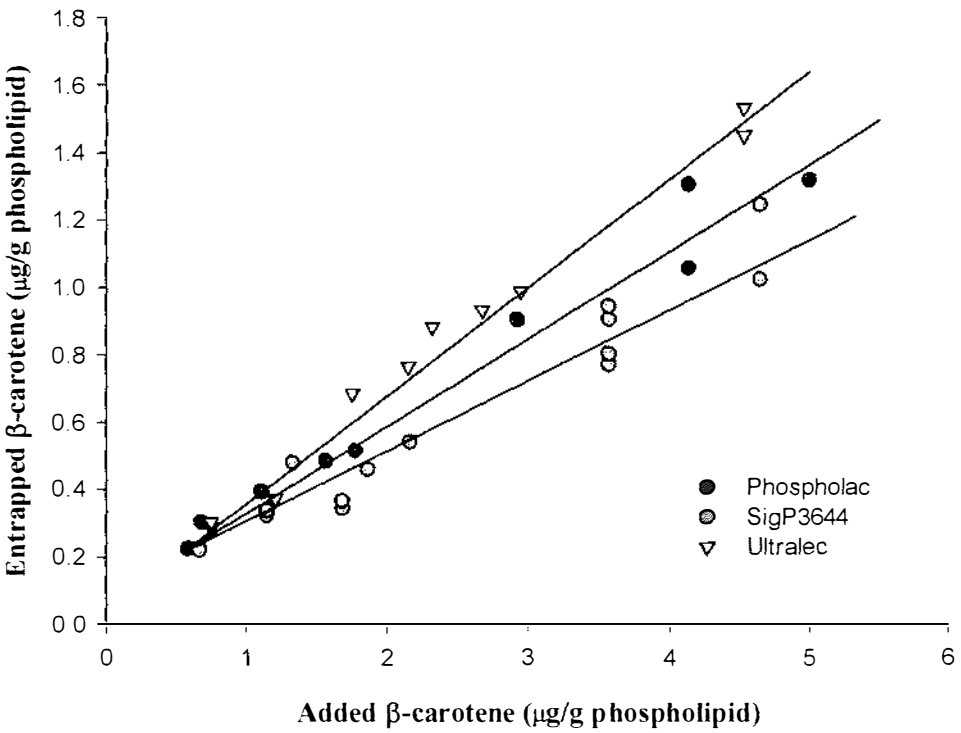



**Figure 7-4 Entrapment of added β-carotene in liposome membranes using ethanol to disperse the β-carotene and phospholipid prior to Microfluidization.**

The entrapment efficiencies for liposome dispersions produced using ethanol are shown in Figure 7-5 Once again there were similarities with the comparative graph using chloroform as the dispersion medium (Figure 7-3). The entrapment efficiency was reduced as the amount of β-carotene added increased, but even at very low levels of β-carotene the efficiency did not exceed 0.5 for any of the liposome dispersions. While this is significantly lower than the 70-90% entrapment shown using chloroform, it is encouraging that between 40-50% of the added β-carotene can be incorporated in the liposome membrane using a food-safe solvent. However, due to previously mentioned problems regarding the removal of aggregated β-carotene, the concentrations of β-carotene used in these experiments are very low compared with those seen in the literature (Strzalka and Gruszecki, 1994; Gabrielska and Gruszecki, 1996; Liebler *et al.*, 1997; Rhim *et al.*, 2000; Lancrajan *et al.*,

2001; Shibata *et al.*, 2001; Socaciu *et al.*, 2002; Cantrell *et al.*, 2003) or those that would be required for most food applications.

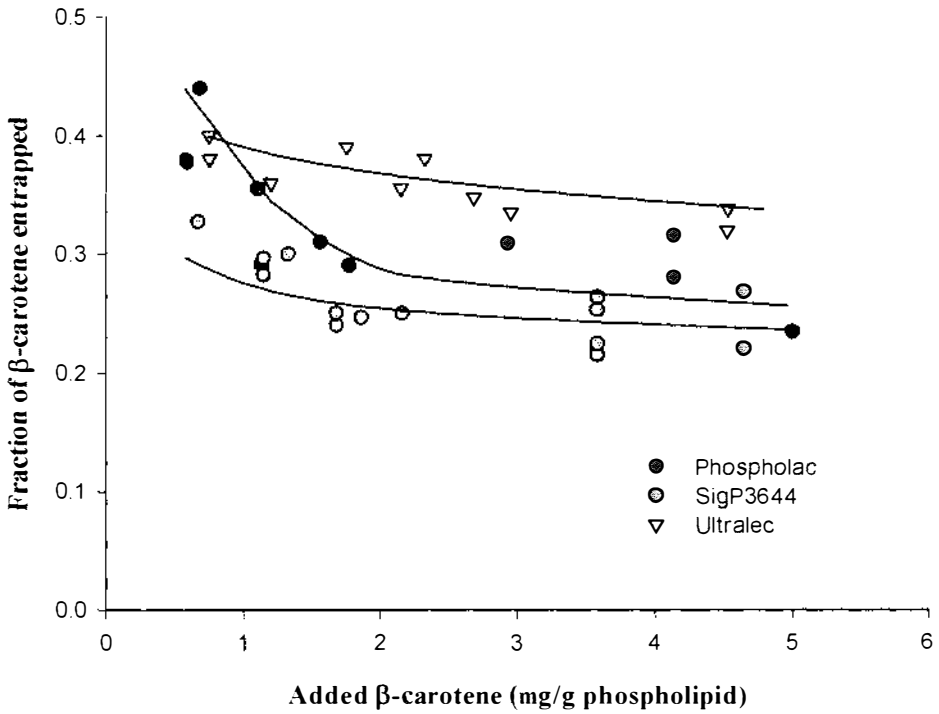



**Figure 7-5 Entrapment efficiency for added β-carotene in liposome membranes using ethanol to disperse the β-carotene and phospholipid prior to Microfluidization.**

### *7.2.3 Discussion*

The incorporation of β-carotene into the phospholipid membrane initially occurs proportionally to the total amount of β-carotene present in the system, with total β-carotene entrapped increasing in a linear fashion. Once a certain β-carotene:phospholipid ratio is obtained within the membrane, it appears that the membrane becomes saturated and there is only limited increase in entrapped β-carotene upon further addition of β-carotene to the system. Phospholac liposome dispersions appeared to reach this point at β-carotene concentrations of between 6-7 μg/gram of phospholipid, while SigP3644 and Ultralec liposome membranes seemed to become saturated at approximately 4 and 5.5 μg/gram of phospholipid, respectively.

The efficiency of entrapment was highest for very low levels of β-carotene, and reduced linearly as more β-carotene was added to the system. For liposome dispersions produced using chloroform, Phospholac and SigP3644 demonstrated a linear relationship between entrapment efficiency and added β-carotene up to 18 μg/gram of phospholipid, while above approximately 7 μg/gram of phospholipid. Ultralec seemed to show a constant entrapment efficiency of 0.4. The plateau in entrapment efficiency may suggest a partitioning of the β-carotene between the solvent phase (chloroform or ethanol) and the phospholipid.

In general, the overall trends were comparable between liposome dispersions produced using chloroform and those produced using ethanol, but the entrapment efficiencies were significantly lower in the ethanol-based systems. This was presumably because of the low solubility of β-carotene in ethanol, less than 0.01g/ml compared with 3g/ml for chloroform (Gordon and Bauerfeind, 1982) For the β-carotene molecules to become incorporated in the phospholipid membrane, they must be solubilised and brought into contact with the phospholipid molecules. If the β-carotene molecules form aggregates they will not become part of the membrane structure. Shibata *et al.* (2001) found that β-carotene has a relatively low miscibility in phospholipid membranes, and is inclined to form aggregates rather than always becoming incorporated into phospholipid membranes.

It must be noted that there was some difficulty in dissolving the SigP3644 phospholipid fraction in the solvents. However, increasing the amount of solvent, temperature or length of dispersion time did not have a significant effect on the level of β-carotene entrapment for this fraction.

The differences in β-carotene entrapment between the three liposome dispersions are most likely to be due to differences in the composition of the phospholipid fractions. The partitioning of the β-carotene between the liposome membrane and the solvent phase will be influenced by the hydrophobicity of the membrane interior, which is determined by the fatty acid composition and presence of other hydrophobic molecules. Amphiphilic molecules including proteins may also affect membrane hydrophobicity.

### 7.2.4 Conclusions

These results suggest that the composition of the Phospholac fraction is able to entrap a higher percentage of the hydrophobic molecule β-carotene than the SigP3644 fraction, using either chloroform or ethanol as the dispersion medium. There may also be some advantages over the Ultralec fraction (i.e. when using chloroform as the dispersion medium), although further experiments are required to determine whether this difference is indeed significant.

## 7.3 Hydrophilic entrapment

### 7.3.1 Methods

#### 7.3.1.1 Liposome preparation

Liposomes were prepared as described previously, with the hydrophilic compound dissolved in the buffer prior to the addition of the phospholipids.

#### 7.3.1.2 Determination of entrapped material

##### 7.3.1.2.1 Glucose

A wide variety of techniques may be used for determining the glucose concentration of a solution. These include Thin Layer Chromatography (Luke, 1971), Gas Chromatography (Luke, 1971; Ribereau-Gayon and Bertrand, 1972), NIR spectrometry (Hartmann and Buening-Pfaue, 1998) and High Performance Liquid Chromotography (Wong-Chong and Martin, 1979) with UV absorption measured between 425 -475 nm. Test strips developed for diabetics to quickly and easily measure their blood glucose concentration have also been used for measuring glucose content of other solutions (Misener *et al.*, 1995).

There are several colourimetric procedures in the literature. One involves the oxidation of glucose by heating in an alkaline ferric cyanide solution, reaction of the ferric cyanide produced with an acid solution of arsenomolybdate, and measurement of the blue colour produced at 750 nm (Bonino *et al.*, 1971). In another, glucose is heated in an ammonium

molybdate solution made in phosphate-phthalate buffer at pH 5.3, and the absorbance of the blue colour measured at 710 nm (Nickerson *et al.*, 1976).

Enzymatic techniques are also common. Budke (1984) describes using an immobilized film of glucose oxidase to convert glucose to hydrogen peroxide which is then measured electrochemically, while Renbing *et al* (1997) used a similar technique but measured the hydrogen peroxide through an iodometric measurement. Miwa *et al* (1984), Everitt and Malmheden Yman (1993) and Steegmans *et al* (2004) reported the use of hexokinase and glucose-6-phosphate dehydrogenase to convert glucose to gluconate-6-phosphate. The NADPH produced during this process is proportional to the amount of glucose converted, and can be determined through the measurement of optical density at 340 nm.

To determine how many processing steps were required to ensure all the unentrapped glucose had been removed, it was necessary to choose a technique that quickly measures glucose concentration. Both diabetic test strips and an HPLC technique were used. For the test strips, a drop of the sample solution was applied to the side of the Accu-Chek strip (Roche Diagnositcs), and capillary action drew the required amount into the strip cavity. The strip was then inserted into the Advantage Complete meter (Roche Diagnostics) which provided a read-out for glucose concentration within 30-45 seconds. In the HPLC technique, a Waters 2690 HPLC Separations Module and an Alltech Prevail C18 Column (150 x 4.6mm, 5μm) were used in combination with a UV detector at 450nm. Deionised water was used as the mobile phase, with a flowrate of 1.5 ml/min. The glucose eluted after approximately 8 minutes.

To provide a standard for comparing accuracy of other techniques, a glucose assay kit (QuantiChrom DIGL-200, BioAssay Systems) and a Roche Cobas Fara II analyser (Hoffmann La Roche, Basel, Switzerland) were used to measure the absorbance at 630 nm resulting from the reaction between *o*-toluidine and glucose. The speed and simplicity of the diabetic test strips was far superior to the other techniques available, although results obtained using HPLC techniques correlated slightly better than the diabetic test strips with the standards measured using the Cobas Fara analyser (Figure 7-6). A combination of the HPLC and diabetic test strips were used throughout the experiments described below.

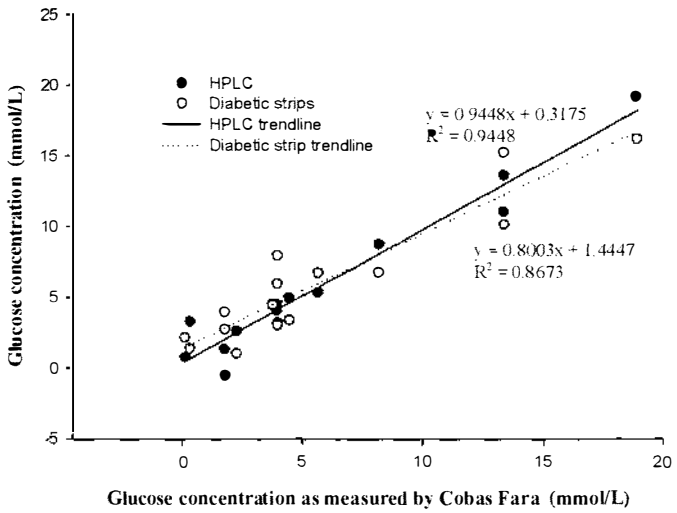



**Figure 7-6 Comparison between results obtained using an HPLC technique and diabetic test strips using the Cobas Fara analyser and an enzymic test kit as the reference method.**

7.3.1.2.2 Ascorbic acid

HPLC is one of the more common techniques for determining ascorbic acid concentration. Detection can be via UV at 245 nm (Liau *et al.*, 1993; Jain *et al.*, 1995) , 250 nm (Rizzolo *et al.*, 1984) or 265 nm (Tanishima and Kita, 1993), electrochemical detectors (Liau *et al.*, 1993) or fluorescence (Iwata *et al.*, 1985; Bilic, 1991; Liu *et al.*, 2000; Wu *et al.*, 2003; Perez-Ruiz *et al.*, 2004; Wang *et al.*, 2005). Other techniques reported in the literature include use of ascorbic acid's interference with the starch-iodine reaction (Sharma *et al.*, 1990; Samotus *et al.*, 1994), capillary zone electrophoresis (Choi and Jo, 1997; Galiana-Balaguer *et al.*, 2001) and the reduction of indophenol (Iggo *et al.*, 1956; Svehla *et al.*, 1963).

It was found that the sensitivity of both the indophenol and the starch-iodine reactions was inadequate for these experiments, so HPLC with UV detection at 245 nm was used. The ascorbic acid used produced two peaks when analysed using HPLC in this manner. The size of the smaller peak increased upon storage of the ascorbic acid solution, suggesting that it correlated to a transformation of the main form of ascorbic acid to a different isomer or to degradation products produced during ascorbic acid oxidation. In addition, imidiazole buffer and phospholipids both absorbed in the 240-270nm region, interfering with the UV detection of the ascorbic acid. Correcting for the absorption of the buffer would be possible using a blank. However, the phospholipid concentration varied between the samples, making correcting for the phospholipid absorption through use of blank or control samples very complicated. Therefore phospholipid concentration was separately determined using the

method outlined in section 5.2.1 (*Chapter 5: Physico-chemical characterisation of liposome dispersions*). .

An enzymatic method was used which was based on the transformation of L-ascorbate to dehydroascorbate by ascorbic oxidase. Two 400 µl aliquots of each sample were taken and added to either 100 µl of water or an ascorbic oxidase solution (Sigma catalogue #A0157). The samples were incubated at 25 °C for 6 minutes, and the absorbance measured at 593 nm. The difference between the two absorbance readings corresponded to the ascorbic acid content of the sample (Moeslinger *et al.*, 1995).

#### 7.3.1.2.3 Sucrose

The sucrose concentration of a solution can be measured by Brix refractometer, HPLC using UV (190 nm) or refractometer detection (Karkacier *et al.*, 2003), gas-liquid chromatography (Wong Sak Hoi, 1983), colourimetric assay (Davies *et al.*, 1995) and enzymatic assay (Vinet *et al.*, 1998).

The method for determining sucrose concentration was based on Rodriguez-Sevilla *et al.* (1999). A 1 ml aliquot of the liposome dispersion was mixed with 4 ml of acetonitrile and filtered through a 0.45 µm Millipore filter (Millipore Corporation, Milford, USA). The sample was passed through a Waters 2690 HPLC Separations Module with a 150 × 4.6 mm Alltech Prevail C18 Column using a mobile phase of 75:25 v/v aceotnitrile and water at a flowrate of 0.9 ml/min. The concentration of sucrose in the elutent was determined using both a refractive index detector and absorbance at 190 nm.

### 7.3.1.3 Removal of unentrapped material

Six different techniques were used to remove the unentrapped hydrophilic material. These were sucrose gradients, dialysis, ultrafiltration (UF) centrifugal filtration, ultracentrifugation and gel filtration.

#### 7.3.1.3.1 Sucrose gradients

Density gradients based on different concentrations of sucrose were constructed in centrifugation tubes, and the liposome dispersion carefully pipetted onto the top surface. The tube was then centrifuged at between 5,000 and 30,000 *g* for up to 4 hours.

#### 7.3.1.3.2 Dialysis

Dialysis is not one of the most common techniques for the purification of liposomes, but it is a very gentle method which has provided good results for the removal of a variety of unentraped materials (New, 1995b; Reineccius, 1995b; Valenti *et al.*, 2001; Devaraj *et al.*, 2002). For dialysis separation, segments of Spectra/Por 132655 Regenerated Cellulose dialysis membranes with a molecular weight cut-off (MWCO) of 6-8,000 were filled with 14ml of liposome dispersion and 1ml of either buffer or a 10% triton solution. Each tube was placed in a beaker containing 100ml imidazole buffer with continuous gentle stirring and the bulk buffer replaced every 6 hours over a total of 18 hours. Samples of the bulk buffer and the retentate were then analysed for the amount of hydrophilic compound present.

#### 7.3.1.3.3 Ultrafiltration (UF)

Samples of the liposome dispersions underwent ultrafiltration in an Amicon stirred ultrafiltration cell (model 8050, Amicon Div., W.R. Grace and Co. Denvers, MA, USA) containing a YM 3 Diaflo UF membrane (3,000 MW cut-off) at a pressure of 300 kPa. The samples were reduced down to a very thick paste before being either analysed for hydrophilic material or re-suspended in buffer for another filtration cycle. It took up to 8 hours to remove as much of the buffer and unentrapped hydrophilic material as possible. This process was repeated until permeate concentrations of the hydrophilic material were close to zero.

Initial tests using a YM 10 membrane (10,000 MW cut-off) showed that there was a small concentration of phospholipid in the permeate, indicating that some molecules must be passing through the membrane. Similar tests using the YM 3 membrane found that the level of phospholipids in the permeate was negligible.

#### 7.3.1.3.4 Centrifugal filtration

To speed up the ultrafiltration technique, the membrane filtration was combined with centrifugal force. Centrisart I 13239E filters utilise centrifugal force to push a UF membrane onto the surface of the liquid, allowing water and small molecules to pass through the

membrane while the larger molecules remain behind (Figure 7-7). The outer tube was filled with 2 ml liposome dispersion and 0.5 ml buffer or 10% triton solution. The inner tube with the filter slid down to rest on the surface of the liquid, and the tubes were centrifuged for 3 hours. Centrifuge speeds of between 3,000 and 4,400 *g* were used.

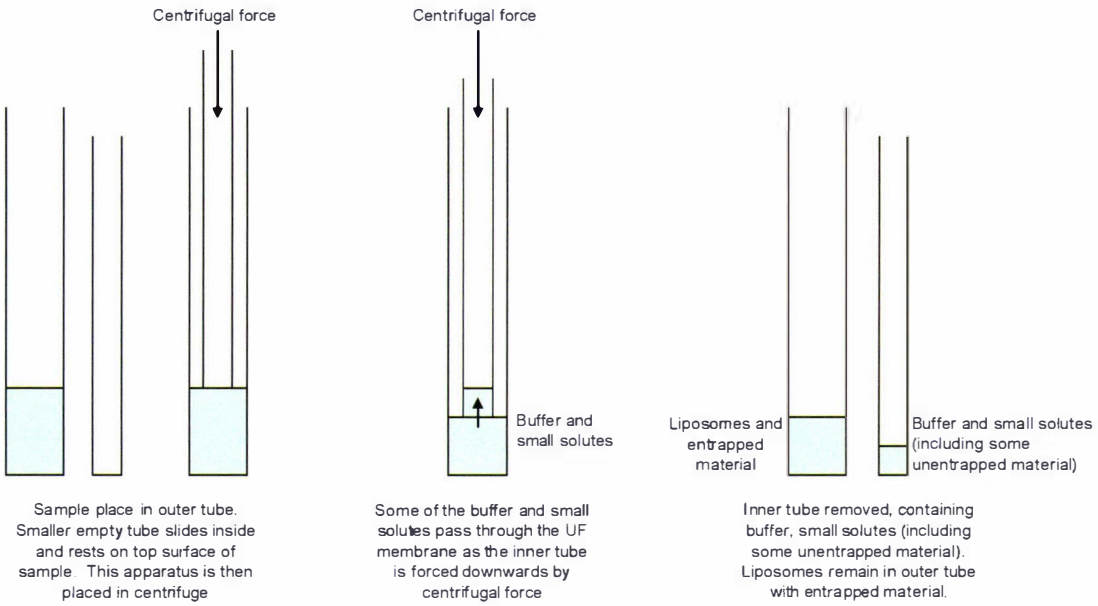



**Figure 7-7 Operation of Centrisart ultrafiltration filters.**

7.3.1.3.5 Ultracentrifugation

Ultracentrifugation is one of the most common techniques used in the literature for removal of unentrapped hydrophilic material. A standard method appears to be centrifugation of the liposome dispersion at 100,000 *g* for approximately 1 hour (Banville *et al.*, 2000; Valenti *et al.*, 2001). However, some authors use up to 160,000 *g* for 3-4 hours (New, 1995b).

Initial experiments found that the centrifugation of liposome dispersions with phospholipid concentrations ≥ 10% failed to completely sediment even after 24 hours at 100,000 *g*. At least two broad layers of a viscous, slightly yellowish liquid were formed above the small pellet, often with a distinct phase boundary between layers of apparently different refractive indexes. Negative staining TEM micrographs confirmed the presence of liposomes in these coloured layers as well as the pellet (Figure 7-8). The images showed that the upper layer contained a larger number of smaller vesicles than the lower layer, along with a few very large vesicles. However, there was not a significant difference in the average hydrodynamic diameter between the layers when measured using PCS. This could be due to the high

scattering caused by the small number of large liposomes. The sediment appeared to contain large proportions of larger-sized vesicles, and this was confirmed using PCS on samples of the pellet resuspended in buffer.

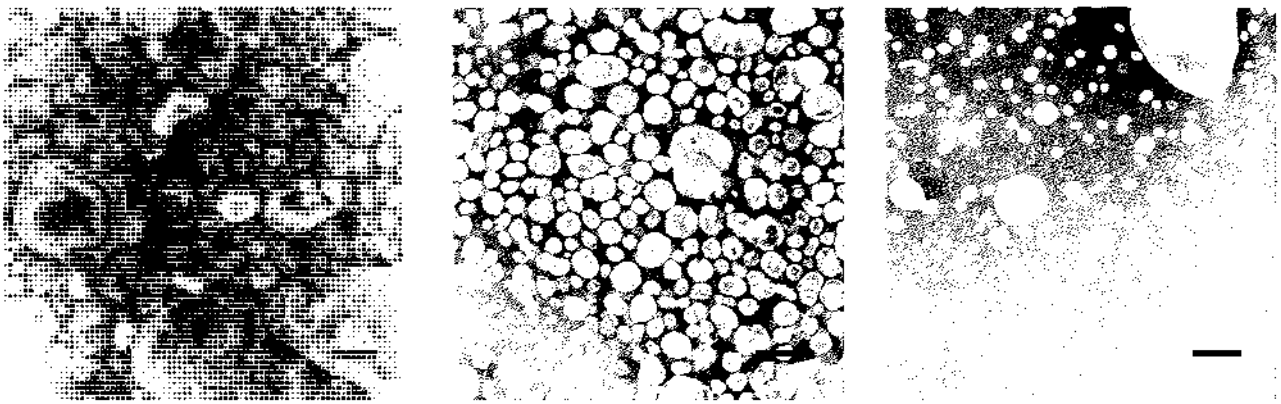



**Figure 7-8 Negative staining TEM micrographs of the layers formed by a 10% Phospholac liposome dispersion after centrifugation at 100, 000 *g* for 24 hours. Bar = 0.2 μm.**

At the top of the centrifuge tube, there was a clear, colourless phase which had a negligible phospholipid concentration, indicating that although the liposomes were not fully sedimenting, they were responding to the centrifugal force. It was possible that the higher viscosity of the layers may have interfered with the sedimentation of the liposomes within each layer. Therefore, fresh samples of the dispersions were diluted 1:5 with buffer and the centrifugation process repeated. This time there were no obvious liquid layers for the Phospholac and Ultralec liposome dispersions, but still a thin layer above the SigP3644 pellet, that did not sediment despite further centrifugation, was present.

The liposome dispersions were diluted 1:5 with buffer, then centrifuged at 100,000 *g* for 8 hours. The total quantity of the supernatant was measured and a small sample retained, and the pellet resuspended in fresh buffer. This process was repeated 4 times for each sample. The concentration of hydrophilic material was determined for each sample of supernatant and for the final pellet. For the SigP3644 sample, the supernatant was removed using a pipette to ensure the thin viscous layer remained with the pellet.

#### 7.3.1.3.6 Gel permeation chromatography

Two gel permeation columns were used. The first was a 50 × 200 mm column of Sepherose gel (Pharmacia Biotech, USA), the second, a 10 ×150 mm column of Bio Gel P-6 DG desalting gel (BioRad, catalogue 150-0738). 1 ml aliquots of the liposome dispersions were

passed through the column, with imidazole buffer used as the mobile phase. The turbidity of the fractions was used to identify the elution of the liposome, and was measured by absorbance of the sample at 600 nm. The indophenol reduction test was used as a semi-quantitative indicator of the elution of ascorbic acid.

Injection volumes of 1 ml of a 10% phospholipid liposome dispersion were used for both columns. The Sepherose column required the use of a pump set at 1 ml/min, and the elution of the liposomes occurred after approximately 75 minutes and was spread over 20-30 minutes (Figure 7-9). The indophenol reduction test showed elution of the unentrapped hydrophilic material occurred between 100-140 minutes, possibly overlapping the elution of the liposomes. The second column (Bio Gel) was gravity-fed, and took approximately 10-12 minutes for the elution of the liposomes and 20-25 minutes for the unentrapped material. There appeared to be a consistent gap of at least 5 minutes between the elution of the liposomes and the unentrapped material (Figure 7-10).

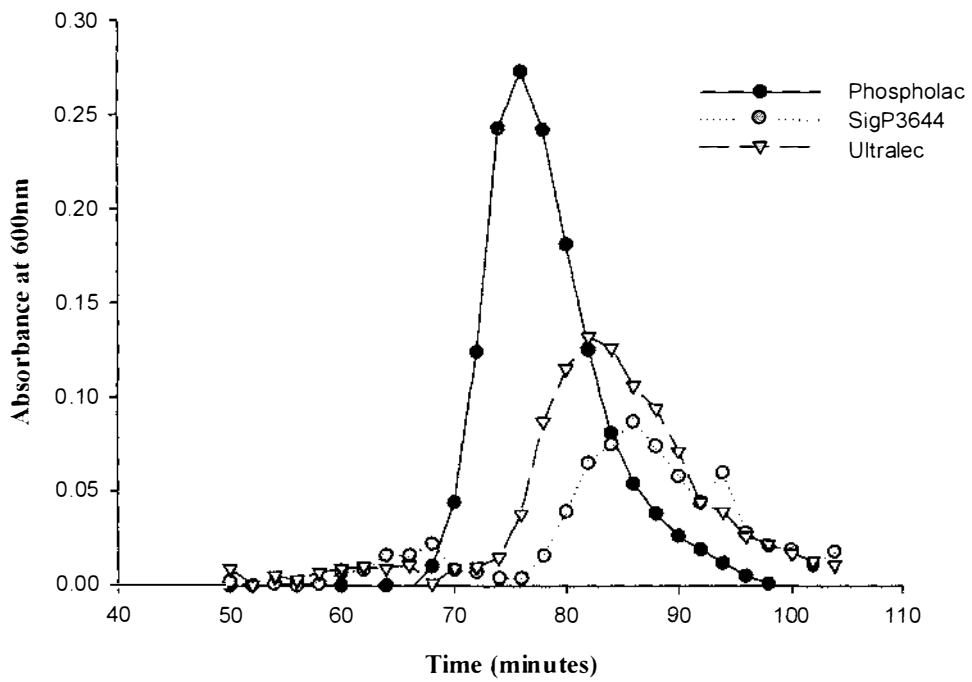



**Figure 7-9 Elution profile of liposomes from Sepherose column as a function of time. Presence of liposomes was indicated by sample turbidity as measured by absorbance at 600 nm.**

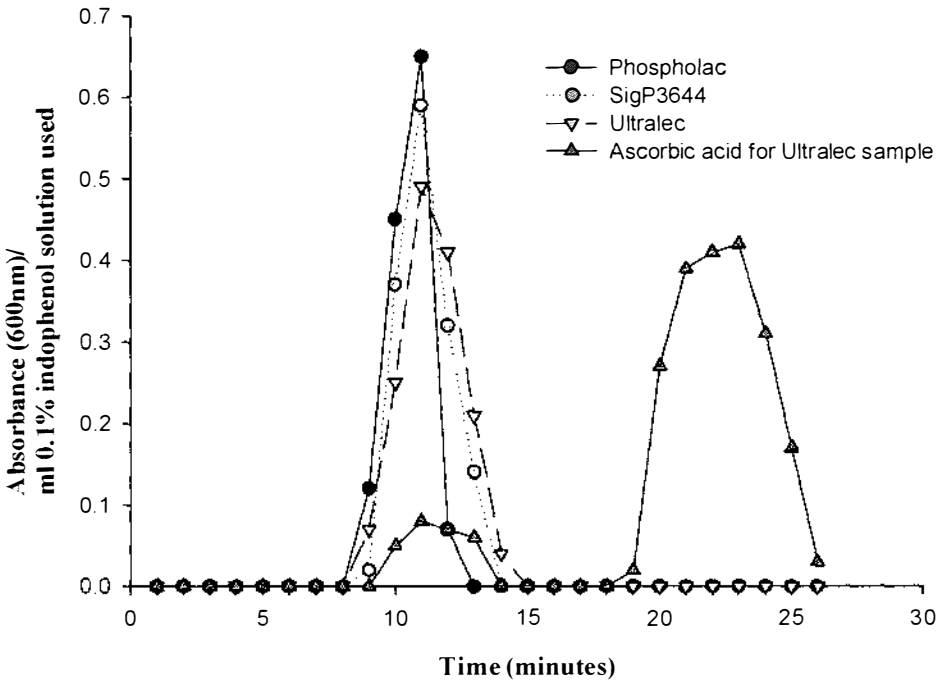



**Figure 7-10 Elution profile of liposomes and ascorbic acid from the BioGel column as a function of time. Ascorbic acid plot shown was from the Ultralec liposome dispersion, with the peak at 9-14 minutes corresponding to entrapped ascorbic acid and the peak at 19-26 minutes corresponding to unentrapped ascorbic acid. The presence of liposomes was indicated by sample turbidity, and the results presented in terms of absorbance at 600nm. Ascorbic acid concentration was determined by reaction with indophenol, and the results plotted as the ml of 0.1% indophenol required to turn collected fractions eluting from the column blue.**

### 7.3.1.4 Membrane permeability

The rate at which sucrose diffused through the membrane for each of the three phospholipid fractions was measured using pulsed-field-gradient NMR (PFG NMR). This technique is discussed in detail in section 5.2.7 in *Chapter 5: Physico-chemical characterisation of Liposomes.*

The PFG NMR diffusion measurements were performed using a Bruker Avance 500 Mhz spectrometer with a 50 G $cm^{-1}$ z-gradient on liposome dispersions prepared in distilled water. The gradient strength ($g$) was varied between 5 and 50 G $cm^{-1}$ in 32 steps, with a gradient pulse width ($\delta$) of 5 ms. Several measurements were taken for each sample, with $\Delta$ values increased from 60 to 150 ms. Models were fitted to the experimental data using the non-linear least-squares regression analyses in Matlab (version 6.5, The Mathworks Inc).

### 7.3.2 Results and discussion

#### 7.3.2.1 Sucrose gradients

This technique was not suitable for separating entrapped and unentrapped sucrose, but was used for liposome dispersion containing both ascorbic acid and glucose. After centrifugation, an opaque white band was formed between 1056 and 1080 g/cm$^3$, with an average particle diameter of between 100-150 nm. Negative staining TEM micrographs confirmed the presence of liposomes in this band. However, the band tended to be relatively broad and diffuse, and it was difficult to separate it from the neighbouring sucrose solutions. Different sized liposomes have slightly different densities, and it is likely that the width of this band is a reflection of the broad liposome size distribution in each of the dispersions (*Chapter 5: Physico-chemical characterisation of liposome dispersions*). The increase in average particle size compared with the average for the original liposome dispersion may indicate that the smaller liposomes were not part of this band. Due to difficulties in isolating the band containing the liposomes and the possibility that this band did not represent a valid cross-section of the liposome population, the use of sucrose gradients was discontinued.

#### Dialysis and ultrafiltration

During dialysis of liposome dispersions containing glucose or ascorbic acid, samples of retentate and bulk aqueous solution were taken at regular intervals. 10% Triton-X was added to each sample to disrupt the liposomes, and the concentration of the hydrophilic material measured as outlined in section 7.3.1.2. Although the hydrophilic content of the retentate was initially significantly higher than the bulk solution, there was a rapid decline in the glucose level of the retentate until there was no significant difference between the retentate and bulk samples. This observation was consistent for liposome dispersions produced from all three phospholipid fractions.

It took up to 12 hours to concentrate 25 ml of a 15% phospholipid dispersion of liposomes into a thick paste using the Amicon stirred cell. The removal of as much buffer and unentrapped material as possible at each stage was necessary to minimise the number of washing cycles required. Usually 3-5 cycles were required before the permeate had a negligible concentration of the hydrophilic material. Unfortunately, after this number of cycles, there was no significant difference between the hydrophilic concentration of the permeate and the retentate.

It is expected that over time, entrapped hydrophilic material will diffuse through the phospholipid membrane and into the bulk phase. This rate of diffusion will depend on the barrier properties of the membrane, but will also be affected by the concentration gradient created through the removal of unentrapped material. As both these techniques required many hours to remove the unentrapped molecules, it may have been that much of the entrapped material was diffusing out of the liposomes during this process.

### 7.3.2.2 Centrifugal filtration

Centrifugal filtration uses centrifugal force to significantly reduce the time required for filtration by dialysis or UF cell. Initial experiments found that the volume of permeate did not significantly increase after centrifugation for period longer than ~ 20 minutes. The reduction in ascorbic acid content of the permeate as a function of the number of washes is shown in Figure 7-11. The amount of ascorbic acid in the permeate decreased to less than 0.5 mg/ml after 7 washes, while the ascorbic acid concentration of the retentate remained between 1.5 and 2 mg/ml. This suggested that there was between 1-1.5 mg ascorbic acid entrapped per ml of liposome dispersion. The ascorbic acid entrapment was calculated for a variety of initial concentrations based on difference between the amount of ascorbic acid in the permeate and retentate after 5 wash cycles (Figure 7-12). The entrapment efficiencies were all extremely low, most of them less than 0.05%.

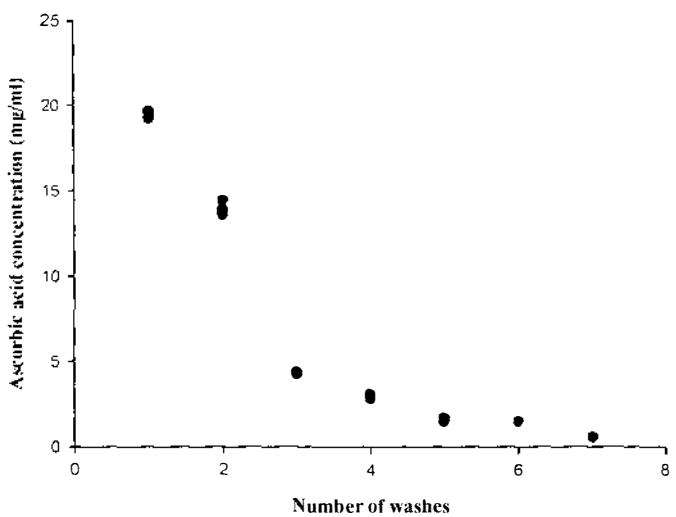



**Figure 7-11 Ascorbic acid content of permeate fractions produced using Centrisart filters and a 10% Phospholac liposome dispersion with an initial ascorbic acid concentration of 50 mg/ml. Each data point is the result of a separate sample.**

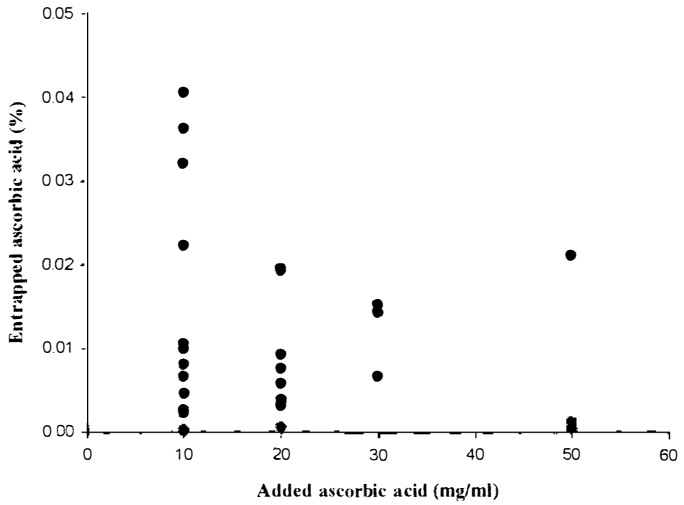



**Figure 7-12 Ascorbic acid entrapment efficiency as determined from the retentate following 5 wash cycles using Centrisart filters and a 10% Phospholac liposome dispersion. Each data point is the result of a separate sample.**

This experiment was then repeated using a liposome dispersion which had been disrupted by the addition of 10% Triton-X, and similar results were obtained to those achieved using intact liposomes. This suggested that although the ascorbic acid was remaining in the retentate rather than being removed with the sequential centrifugation, it may have been no longer encapsulated within the liposomes.

### 7.3.2.3 Ultracentrifugation

After the lack of success of the filtration-based techniques, removal of unentrapped material through the use of sedimentation via ultracentrifugation was tested. Samples of 10% phospholipid liposome dispersions produced from each fraction were centrifuged at 100,000 *g* for 8 hours, the pellet resuspended in buffer, and the process repeated. An example of the decrease in ascorbic acid concentration for samples with an initial ascorbic acid concentration of 0.1g/ml is shown in Figure 7-13. After two centrifugation steps, there was no significant difference in the ascorbic acid concentration of the supernatant and sediment, and after four steps no ascorbic acid could be detected in either the supernatant or sediment. Similar results were obtained for both glucose and sucrose.

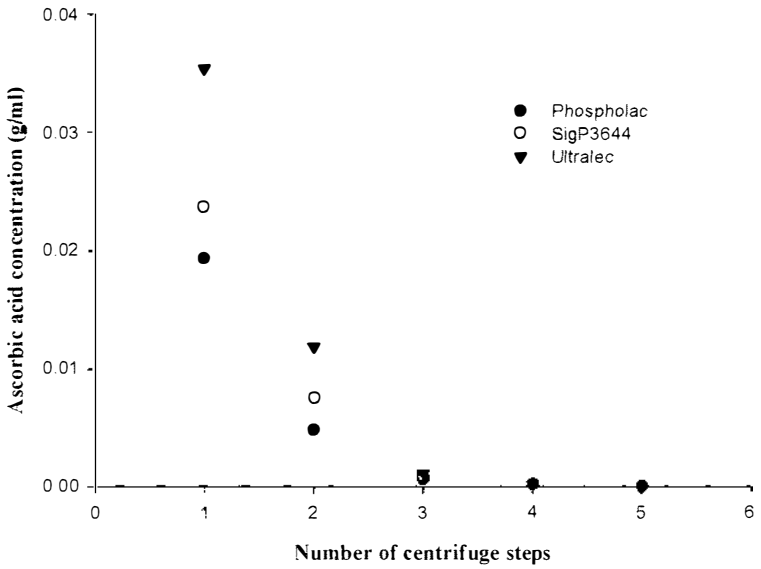



**Figure 7-13 An example of the decrease in ascorbic acid concentration of supernatent following sequential centrifugation steps of a 10% phospholipid dispersion. The initial ascorbic acid concentration for these samples was 0.1 g/ml.**

The negative staining TEM micrograph of the sediment formed after centrifugation of a Phospholac liposome dispersion was shown in Figure 7-8. This suggests that there may be some damage and fusion of the liposomes in the pellet, presumably due to the very high forces experienced during the centrifugation process. Entrapped hydrophilic material is likely to have been released during any fusion or coalescence of neighbouring vesicles. The use of ultracentrifugation is common in the literature, but many of the liposomes contained significant levels of cholesterol (10-50 mol%) which has been shown to increase the rigidity and stability of the membrane. This may allow the liposomes to withstand the high forces of this process without damage to the membrane and subsequent release of entrapped material.

### 7.3.2.4 Gel permeate chromotography

Gel permeation is also commonly used for removal of unentrapped material, but is a much gentler technique than ultracentrifugation. The Sepharose column took 1.5-2 hours from time of injection until the final traces of the hydrophilic materials were eluted. The elution peaks for the liposomes and the hydrophilic compounds had long tails, and appeared to overlap in some cases. The long elution time also resulted in the dilution of the liposomes

from the 1 ml of dispersion injected to between 20-30 ml of collected sample. This reduced the concentration of any entrapped material beyond the sensitivity of any of the techniques used. Changing the flow-rate of the elutent and increasing the length of the column did not significantly improve column performance.

The Bio Gel column was the fastest of all the separation techniques used, and appeared to be the most successful. As shown in Figure 7-14, there was evidence of hydrophilic entrapment. There was an almost linear increase in amount of glucose entrapped as the amount of glucose present in the system increased. This is as expected given that hydrophilic entrapment is directly proportional to entrapped volume. In this system, the total amount of entrapped volume was held constant by the addition of 10% phospholipid to all samples, and only the concentration of glucose within the aqueous phase was changed. However, when the amount of entrapped glucose was converted to entrapment efficiencies (Figure 7-15) there was less than 3.5% of the glucose retained within the liposomes.

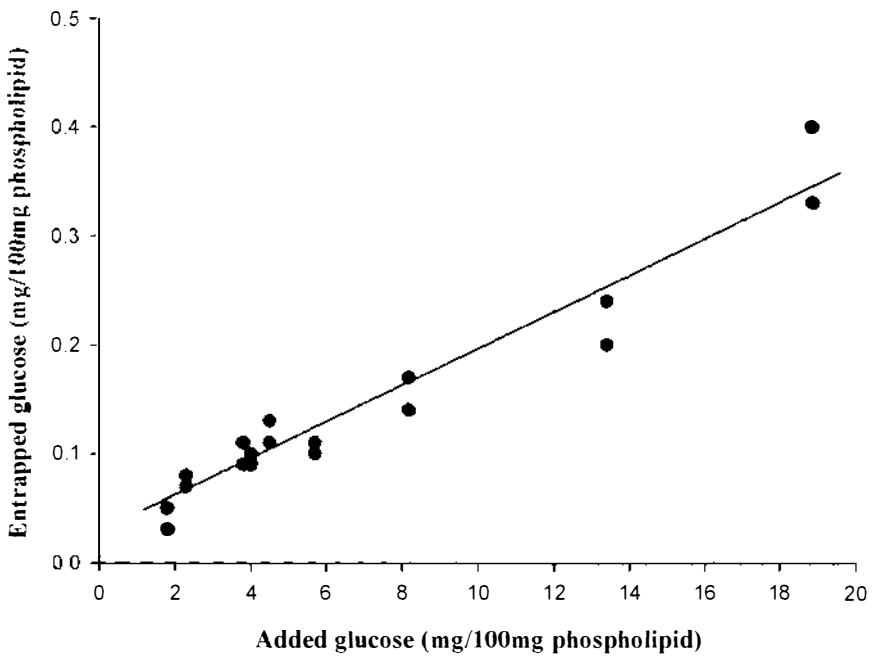



**Figure 7-14 Amount of entrapped glucose in a 10% phospholipid Phospholac liposome dispersion using a Bio Gel column to remove unentrapped glucose. Each data point is the result of a separate sample.**

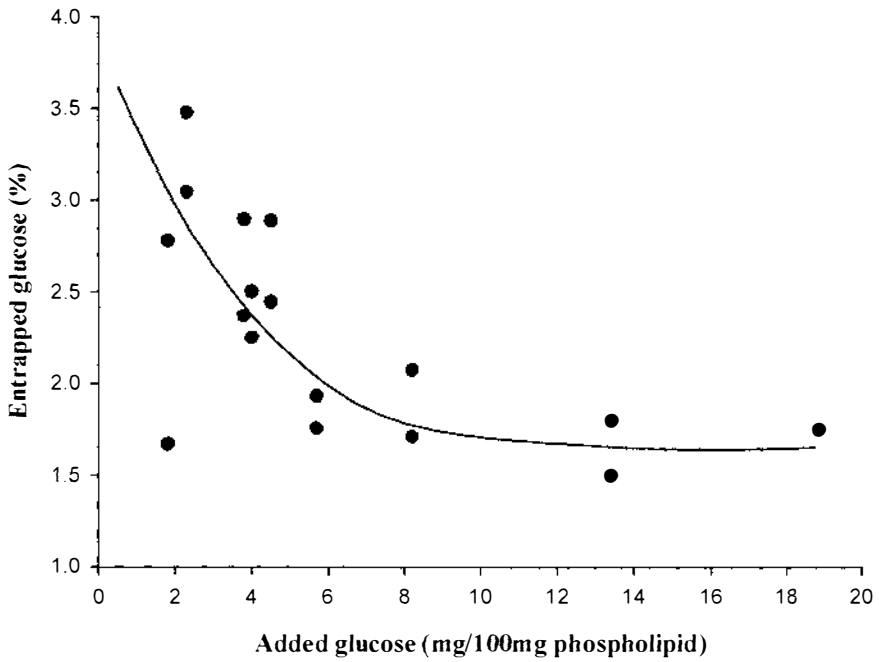


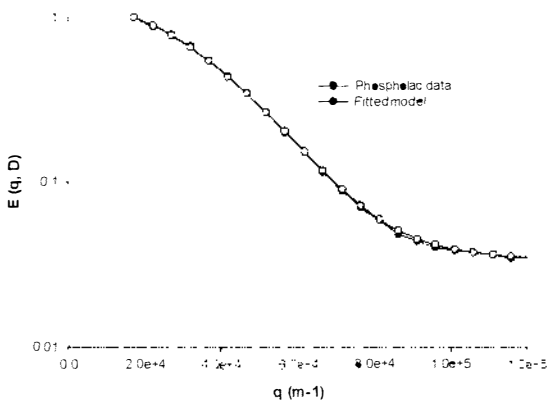

**Figure 7-15 Entrapment efficiency for glucose in a 10% phospholipid Phospholac liposome dispersion using a Bio Gel column to remove unentrapped glucose. Each data point is the result of a separate sample.**

Vemuri *et al.* (1990) reported a hydrophilic entrapment of 35% using liposome dispersions produced via Microfluidization, while Mayhew *et al.* (1984) obtained between 5-75% entrapment of the aqueous space marker cytosine arabinoside depending on the Microfluidizer operating conditions (number of passes and pressure used) and the concentrations of phospholipid and hydrophilic material.

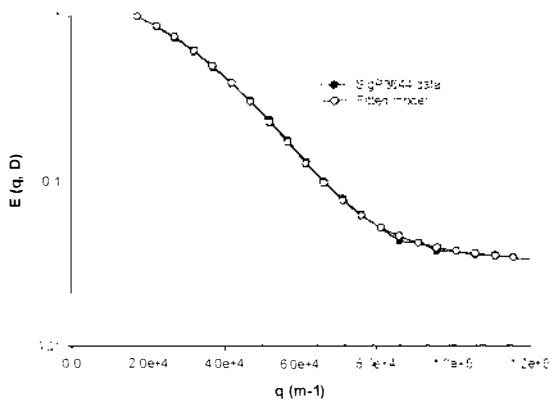
The entrapped volume for Phospholac was estimated to be around 2 ml/g phospholipid (*Chapter 5: Physico-chemical characterisation of liposome dispersions*), which would suggest that a 10% phospholipid solution would entrap approximately 20% of the volume in that sample. Therefore, the entrapment efficiency of any hydrophilic material evenly distributed through the 10% phospholipid solution should be 20%. This is well below the maximum of 3.5% shown in Figure 7-15.

### 7.3.2.5 Membrane permeability

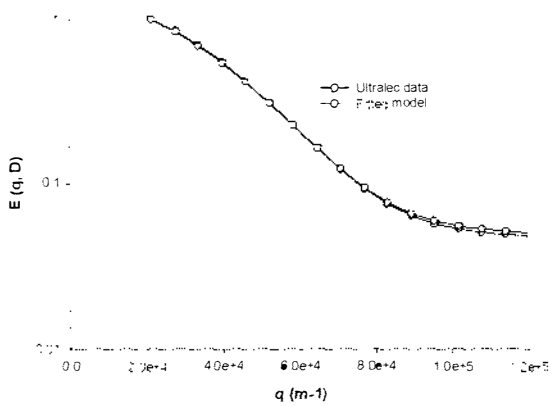
These results suggested that the liposome dispersions either had very low entrapment of hydrophilic material or that the entrapped molecules were diffusing through the membrane at a significant rate. The membrane permeability of sucrose was measured using pulse-field gradient NMR. The experimental data overlaid with the fitted model for each of the three liposome dispersions is shown in Figure 7-16, and it can be seen that the fitted model closely matches the experimental data.




Phospholac liposome dispersion




SigP3644 liposome dispersion




Ultralec liposome dispersion

**Figure 7-16 Experimental data and fitted models for the signal attenuation for the sucrose peak for the different liposome dispersions**

Using the equations presented in section 5.2.10 in *Chapter 5: Characterisation of liposome dispersions,* coefficients were calculated for the diffusion of the liposomes within the system and for the diffusion of the sucrose molecules in the bulk aqueous phase (Table 7-1). The

diffusion of the sucrose molecules in the aqueous phase was approximately $^{1}/_{10}$ of the rate of water molecules, which is not surprising considering the much larger size of the sucrose molecule.

The mean liposome radius produced based on the assumption of a log-normal distribution for liposome size is significantly larger than that obtained by other techniques. Possible explanations for this result are discussed in *Chapter 5: Physico-chemical characterisation of liposome dispersions.*

The mean permeability of sucrose through the bilayer membrane for each of the three liposome dispersions is also shown in Table 7-1. The rate of diffusion for sucrose is approximately $^{1}/_{100}$ the rate for water, but it is still very rapid at $4\text{-}6E^{-08}$ m/s. As an indication of how fast this is, the mean time it takes for all the sucrose molecule inside a liposome to diffuse through the membrane into the bulk phase is less than 2.5 seconds. The membranes produced from the SigP3644 fraction allow significantly faster diffusion of sucrose molecules than membranes composed of either the Phospholac or Ultralec fractions.

**Table 7-1 Summary of sucrose permeability through the liposome membrane at 20 °C.**

| Sample | Bulk sucrose diffusion (D2 & D1) ($m^2/s$) | Fitted to volume-weighted log-normal distribution | | | | |
|---|---|---|---|---|---|---|
| | | Mean liposome radius (r) (nm) | Standard deviation | Fraction of water in liposome | Mean exchange time (seconds) | Mean permeability of sucrose (m/s) |
| **Phospholac** | $1.60E^{-10}$ | 221 | 0.60 | 0.040 | 2.19 | $3.4E^{-08}$ |
| **SigP3644** | $1.80E^{-10}$ | 405 | 0.53 | 0.042 | 2.04 | $6.6E^{-08}$ |
| **Ultralec** | $7.45E^{-11}$ | 252 | 0.52 | 0.048 | 2.21 | $3.8E^{-08}$ |

This rapid rate of diffusion for sucrose suggests that the entrapped molecules are likely to be diffusing out of the liposomes as fast or faster than it is possible to try and separate the unentrapped sucrose from the dispersion. Given that the sucrose molecule is almost twice the size of glucose molecule, it is likely that the rate of diffusion of glucose through the liposome membranes would be more rapid still. Ascorbic acid has a similar molecular weight to glucose, and although the negative charge on the molecule is likely to have an effect on the rate of diffusion, it is unlikely to decrease the exchange time enough to allow

any of the separation techniques described here to remove all the unentrapped ascorbic acid without the diffusion of encapsulated material through the membrane.

Kirby *et al.* (1991) reported successful entrapment of ascorbic acid using equimolar concentrations of cholesterol as well as α-tocopherol, which would have significantly reduced membrane permeability, while Diplock *et al.* (1977) found that inclusion of α-tocopherol in a liposome membrane composed of unsaturated phospholipids significantly reduced the membrane permeability to glucose. It is also possible that differences in phospholipid composition or preparation methods may contribute to the rate of diffusion of small molecules through the liposome membranes.

### *7.3.3 Conclusions*

The successful use of any of the three phospholipid fractions to encapsulate small hydrophilic molecules is severely limited by the rapid rate of diffusion of these molecules through the liposome membrane. The rate of diffusion may be reduced by the use of cholesterol, although plant sterols may provide a more favourable alternative for use in food products.

Due to the rapid diffusion of the hydrophilic molecules through the membrane, it was difficult to accurately assess the effectiveness of the different techniques for removal of unentrapped material. It appeared that combined filtration and centrifugation or the use of certain gel permeation columns may be most appropriate for small-scale separation, but further work would be required to determine suitable techniques for large-scale operation.

# Chapter 8: General discussion

This final chapter provides a summary of the work completed, along with an overall discussion of the relationship between the differences in composition of the phospholipid fractions and the characteristics of the liposome dispersions produced from each fraction. A number of possible areas for further work are also identified.

## *8.1 Summary*

The overall aim of this project was to determine whether the phospholipid component of MFGM could be used to produce liposomes, and to explore whether these liposomes had any advantages over liposomes produced from commercial soy phospholipid fractions. The technique used for liposome production was to be suitable for use in the food industry, and therefore must not require the use of organic solvents and could easily be scaled up to produce large quantities in a relatively small amount of time.

A range of dairy and soy phospholipid fractions were assessed for their ability to form liposomes, and an initial screening experiment used to select the fractions which successfully produced liposomes that were stable during short-term storage. The dairy phospholipid fraction Phospholac 600 and the two soy fractions SigP3644 and Ultralec P were chosen as being most suitable for liposome production. The soy-derived SigP3644 and Ultralec had similar phospholipid profiles, although the more highly-purified SigP3644 had more PC and less PI than the Ultralec fraction, as well as a significantly lower ash content. The dairy-origin of Phospholac was reflected in its more highly saturated fatty acid profile, and the presence of 25% sphingomyelin. The Phospholac and Ultralec fractions contained more protein than the SigP3644 fraction. Phospholac had a high proportion of monovalent cations, while the ash content of the Ultralec fraction was primarily composed of divalent cations.

These three fractions were dispersed in an aqueous buffer and cycled through a Microfluidizer® (a high-pressure homogeniser) to produce liposomes. Increasing the

pressure or number of passes through the Microfluidizer® resulted in a decrease in average liposome diameter, with a larger reduction in liposome size at lower phospholipid concentrations. There was no evidence of the Microfluidization process causing any oxidative damage to the system. A basic model allowing the prediction of average liposome diameter for a given set of conditions was developed, providing a quick estimation of the likely effect on liposome size of any proposed changes to the processing method. In general, liposome dispersions used throughout this work were produced from 10% phospholipid dispersions being passed through the Microfluidizer® 5 times at 1100 bar.

The hydrodynamic diameter (as measured by Photon Correlation Spectrometry, PCS) of the liposomes produced from the Phospholac fraction was approximately 95 nm, slightly larger than the average diameter of approximately 80 nm for the SigP3644 and Ultralec dispersions. All three dispersions had a very wide particle size distribution; although Asymmetric Field Flow Fractionation (AFFF) results showed that the Phospholac dispersions had fewer very small liposomes and an overall narrower particle size distribution than the other two dispersions. However, despite the Phospholac dispersion having the largest average hydrodynamic diameter, it had an entrapped volume of only 0.8ml/g phospholipid, approximately half that of the Ultralec dispersion. This is likely to be due to the overall smaller number of large liposomes in the Phospholac dispersion. Negative staining and Thin-section Transmission Electron Microscopy (TEM) and Freeze-fracture Scanning Electron Microscopy (SEM) showed that the liposomes were indeed within the size range suggested by the PCS and AFFF results. The TEM micrographs also allowed the lamellarity of the dispersions to be investigated. All three dispersions appeared to be primarily unilamellar, but there was also a small percentage of multilamellar and multivesicular liposomes. The unilamellar nature of the dispersions was further supported by the small-angle x-ray diffraction images and additional NMR results using manganese or lanthanide shift reagents.

Differential Scanning Calorimetry (DSC) analysis showed that SigP3644 and Ultralec had phase transition temperatures below 0 °C, while Phospholac dispersions showed a very broad transition with a centre between 28 and 30 °C. This meant that the SigP3644 and Ultralec dispersions were in the fluid phase at all common storage and processing temperatures, while the Phospholac was in the gel phase at refrigeration and ambient temperatures but would also be in the fluid phase at most food processing temperatures. Literature states that the

permeability of the membrane should be lowest in the gel phase, peak at the phase transition temperature, then decrease to a medium level in the fluid phase. It would be expected that the denser packing of the Phospholac membrane in its gel phase at 20 °C would result in a lower membrane permeability compared with the liquid crystal arrangement of the SigP3644 and Ultralec dispersions. However, while the Phospholac dispersion had a much lower permeability than the SigP3644 sample, there was very little difference between the Phospholac and Ultralec samples at either 20 or 40 °C. There was also no indication of any increase in Phospholac membrane permeability at its phase transition temperature (28 and 30 °C).

The presence of high concentrations of cholesterol can reduce or remove evidence of this transition, but the Phospholac fraction contained less than 0.05% cholesterol, too low to have any significant affect on the phase transition. The transformation of the Phospholac membrane from the gel to the liquid crystal phase should have resulted in an increase in membrane permeability as the packing density of the membrane decreases, but the experimental data showed a small decrease in measured permeability. These results were unusual, but appeared to be supported by the measurements of membrane thickness using small-angle x-ray diffraction. The Phospholac and Ultralec bilayers were approximately 20% thicker than SigP3644 membranes at both 20 and 40 °C. Marsh (1996) claimed that the lipid bilayer was 'considerably' thinner in the fluid phase than the gel phase. However, small-angle X-ray diffraction of liposome dispersions at 20 and 40 °C failed to show any significant change in the thickness of Phospholac membranes, despite the transition from gel to fluid state (Table 5-7).

Based on the literature, it would be expected that the presence of sphingomyelin and the higher phase transition temperature due to the primarily saturated fatty acid profile would result in Phospholac liposomes having a lower membrane permeability than the soy fractions. However, there was no significant difference in the permeability of water or sucrose molecules between the Ultralec and Phospholac dispersions at either 20 or 40 °C. This suggests that permeability is not simply dependent on the phospholipid composition of the membrane or the membrane state (i.e. gel or fluid).

The zetapotential was measured across the pH range of 2-10 to investigate the effect of pH on the surface charge of the liposomes, a very important indicator of stability through charge repulsion. It was found that the Phospholac dispersions had the weakest negative charge

across the entire pH range, suggesting that they may be more susceptible to aggregation due to charge neutralisation. However, investigations into the effect of pH on particle size found that despite the lower surface charge, the Phospholac dispersions were more stable at the lower pH values. All three dispersions appeared not to be negatively affected by a pH of between 6 and 10.

Increases in ionic concentration resulted in much more rapid aggregation and/or fusion among the SigP3644 and Ultralec liposomes than in the Phospholac dispersions. This was not expected based on the zetapotential results. The ability to withstand higher ionic concentrations did not appear to be related to the natural mineral balance of the fraction. The fatty acid composition is also likely to be the primary reason for the lower susceptibility to oxidation demonstrated by the Phospholac liposome dispersions.

Stability was also assessed at a range of pH values for a variety of storage and processing conditions. All three of the liposome dispersions showed minimal changes in average hydrodynamic diameter during storage at up to 20 °C for 60 days. However, the Phospholac dispersions showed significantly better stability than either of the two soy fractions during storage between 20-35 °C, especially at low pH values. The Phospholac dispersions also had a smaller increase in average diameter after a variety of heat processing treatments, including 50 °C for 15 hours, 72 °C for 2 minutes, 90 °C for 2 minutes and 141 °C for 15 seconds.

Long-term storage of liposomes is reported to be possible through freezing or freeze-drying of the dispersions. However, all three fractions had large increases in average liposome diameter, and TEM micrographs showed almost complete loss of liposome structure. The addition of cryogenic sugars reduced the measured average diameter and improved the visual appearance of the micrographs, but even at addition levels of 12% w/w the damage was significant.

Entrapment of β-carotene as a model hydrophobic compound was found to be proportional to the solubility of the hydrophobic material in the solvent used to dissolve the phospholipids. There also seemed to be some partitioning of the β-carotene between the solvent and phospholipid phases. The Phospholac fraction appeared to entrap a higher percentage of β-carotene than the SigP3644 fraction, irrespective of whether chloroform or ethanol was used as the dispersion medium, but the difference in entrapment efficiency between the Phospholac and Ultralec fractions was less significant.

Entrapment of small hydrophilic molecules such as ascorbic acid, glucose or sucrose proved difficult to quantify. The development of a new NMR technique allowed the permeability of specific molecules through the liposome membranes to be followed and quantitatively measured. This revealed that these small hydrophilic molecules were diffusing through the liposome membrane at an extremely rapid rate, too fast for the standard methods of measuring entrapment to be used successfully. Reports of successful entrapment of small molecules in the literature appear to always involve the inclusion of large amounts of cholesterol (at ratios of close to 1:1 with phospholipid), a molecule which is known to reduce membrane permeability. Unfortunately, cholesterol is an undesirable additive for food systems. It is possible that the use of other sterols (i.e. plant phytosterols) may result in a similar effect, but this was deemed beyond the scope of this project.

## 8.2 *Effect of differences in the composition of the phospholipid fraction on liposome behaviour*

This section looks at the relationship between the composition results reported in *Chapter 4: Characterisation of phospholipid fractions* and the behaviours of the liposomes covered in *Chapter 5: Physico-chemical characterisation of liposome dispersions*, *Chapter 6: Liposome stability* and *Chapter 7: Entrapment of hydrophobic and hydrophilic material in liposome dispersions*.

### *8.2.1 Phospholipid types*

One of the most significant differences between the dairy and soy phospholipid fractions was the types and amounts of the various phospholipid classes that made up each fraction. These are shown in Table 8-1, with the relative charges of the lipids as a function of pH shown in Figure 8-1.

Phospholac was primarily composed of approximately equal quantities of PC, PE and sphingomyelin, with small amounts of the negatively-charged PI and PS. The soy fractions were mainly PC and PE with slightly lower amounts of PI. Overall, the SigP3644 fraction had a higher PC and lower PI concentration than the Ultralec fraction. From Table 8-1, it

can be seen that (compared with PA) PI maintains a negative charge at all pH values, and PC is positively charged below pH 3.5 and neutral above that pH. The behaviour of PS is more complicated, with the molecule positively charged at pH values below 4 and negatively charged above 4. Similarly, PE is positively charged below pH 3.5 and negatively charged above 8.

**Table 8-1 Phospholipid class of different fractions (% of total fraction). Results obtained through methods detailed in section 4.2.2.**

| Lipid | Phospholac (%) | | SigP3644 (%) | | Ultralec (%) | |
|---|---|---|---|---|---|---|
| | $^{31}$P-NMR | HPLC | $^{31}$P-NMR | HPLC | $^{31}$P-NMR | HPLC |
| Polar Lipids | 72.0 | | 73.9 | | 68.2 | |
| Phosphatidyl choline | 23.6 | 38.4 | 40.9 | 57.3 | 24.6 | 38.9 |
| Phosphatidyl ethanolamine | 20.2 | 10.6 | 25.4 | 11.3 | 22.2 | 9.7 |
| Sphingomyelin | 22.8 | 20.4 | Not detected | Not detected | Not detected | Not detected |
| Phosphatidyl serine | 2.5 | 5.2 | Not detected | 1.1 | 0.5 | 0.9 |
| Phosphatidyl inositol | 2.6 | 3.1 | 3.6 | 1.2 | 13.3 | 17.6 |
| Other phospholipids | 0.2 | 4.8 | 4.1 | 2.9 | 6.9 | 1.2 |

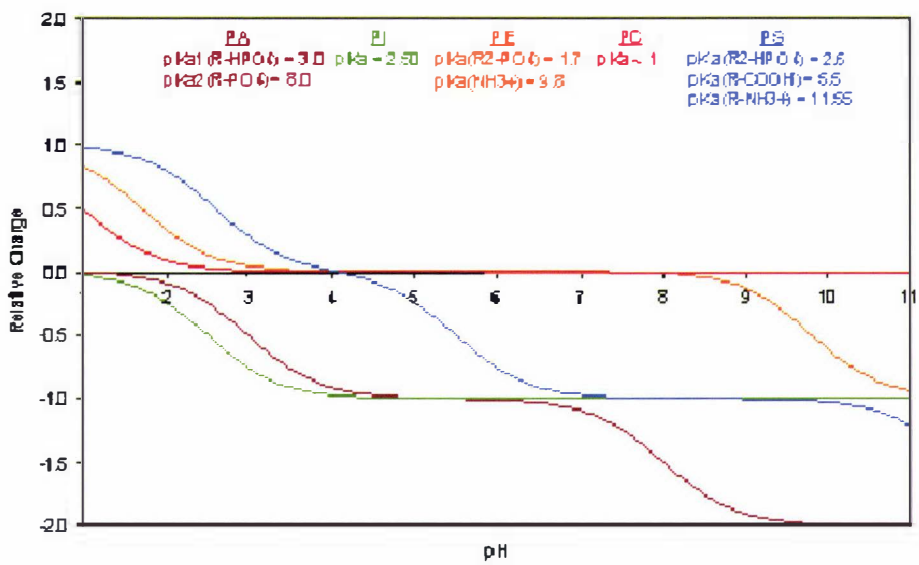



**Figure 8-1 Relative charges of Phospholipids as function of pH (Avanti Polar Lipids, Alabama, USA)**

Figure 8-2 shows that the measured zeta potential for the liposome dispersions changed most rapidly at pH values less than 4. This fits with the behaviour of the main phospholipids present in the fractions (PC, PE and PI), which all demonstrate changes in charge around this pH. It was expected that Ultralec liposomes would have the highest negative charge due to the high proportion of PI, but the measured zeta potentials indicated that the SigP3644 fraction produced the most negatively charged liposome dispersion. Ultralec and Phospholac had a much higher cation concentration than the SigP3644 fraction, which may be responsible for neutralising some of the negative charges on the PI molecules. Similarly, the Phospholac fraction contained relatively little PI and also has a high cation concentration, which may help explain why it was the most positively charged of the three fractions. However, the high proportion of protein present in the Phospholac and Ultralec fractions (Table 8-3) may have contributed to the zeta potential of liposomes from these fractions being overall more positively charged than expected.

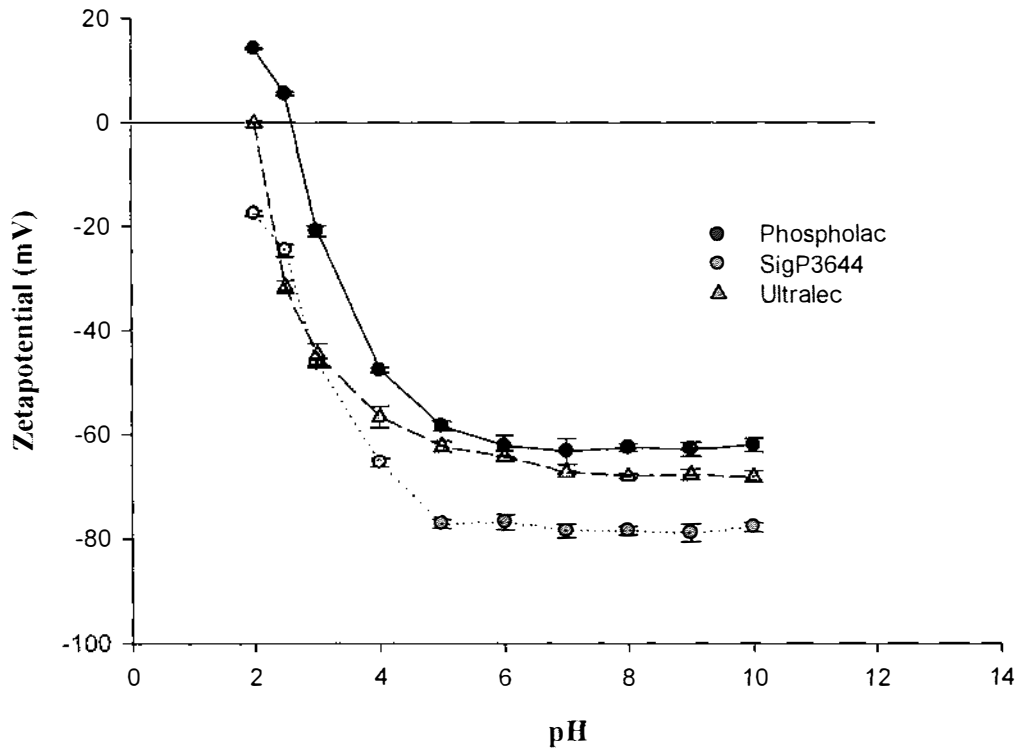



**Figure 8-2 Zeta potential versus pH curve for liposomes produced from Phospholac, SigP3644 and Ultralec**

The shapes of the SigP3644 and Ultralec curves showing the effect of pH on their zeta potential (Table 8-2) are very similar to the effect of pH on liposome size (Figure 8-3). Changes in particle size are indicative of changes in liposome stability, reflecting

aggregation or coalescence. This suggests that there may be a direct relationship between the surface charge of the SigP3644 and Ultralec liposomes and their stability. As the pH decreases, the surface charge decreases (zeta potential becomes less negative), and there is less electrostatic repulsion keeping the individual liposomes apart. The stability of the Phospholac liposomes does not appear to be as dependent on charge repulsion, with the particle size not changing significantly between the pH values of 2 and 10, despite significant changes in zeta potential. This implies the presence of an additional mechanism responsible for stabilisation of Phospholac liposomes.

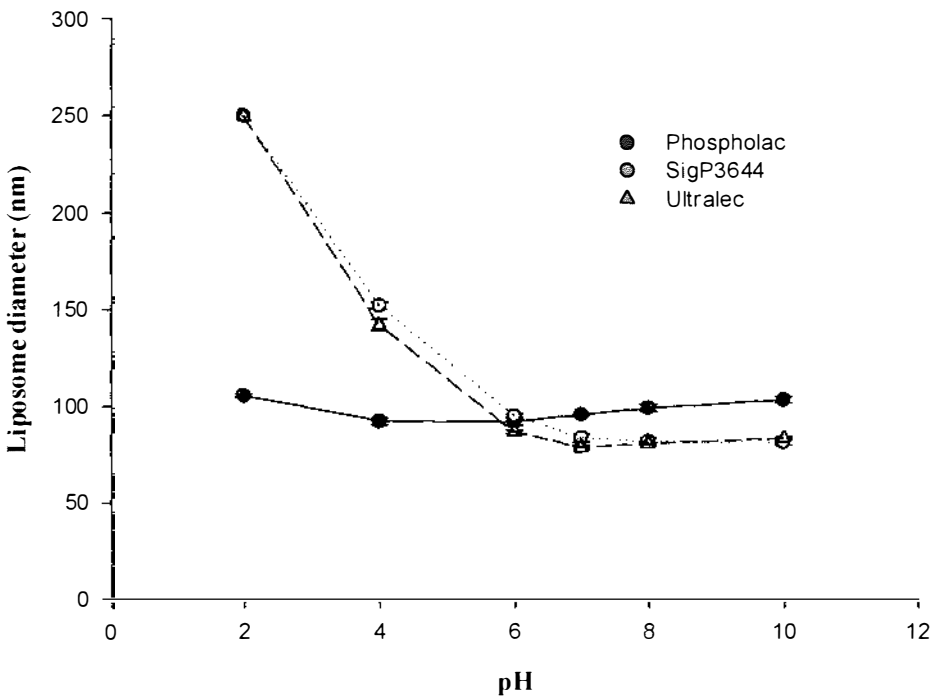



**Figure 8-3 Effect of pH on the average hydrodynamic diameter of liposome dispersions. Samples were measured 24 hours after pH adjustment. Each point is the mean of three measurements with error bars ± 1 standard deviation.**

The most significant difference between the phospholipid compositions was the high level of sphingomyelin in the Phospholac fraction (approximately one third of the polar lipid present). Sphingomyelin has a more structured gel phase than phosphatidyl choline, and sphingomyelin membranes seem to be more stable and have a lower permeability to hydrophilic molecules than PC bilayers (New, 1995c).

Experiments comparing the stability of the liposome dispersions found that the Phospholac fraction showed significantly better stability during storage at between 4-35 °C. The Phospholac dispersions were also more stable during a wide range of heat treatments. Increases in ionic concentration resulted in much more rapid aggregation and/or fusion

among the SigP3644 and Ultralec liposomes than in the Phospholac dispersions, independent of the natural mineral balance of the fraction. These results all support the claimed increase in stability due to the presence of sphingomyelin, and suggest that the high proportion of sphingomyelin in the Phospholac fraction may provide significant advantages in terms of the resistance of the liposomes to environmental stresses.

There was no significant difference in membrane permeability between the Phospholac and Ultralec liposomes at either 20 or 40 °C, although they both had a lower permeability than the SigP3644 liposomes. Perrett *et al.* (1991) found that liposomes formed from proliposome mixtures containing charged lipid were smaller than those formed from neutral lipids, but had a higher entrapped volume. It was suggested that when charged lipids are present there is a decrease in the number of bilayers present in each liposome, possibly due to increased electrostatic repulsion between bilayer surfaces. The higher concentration of the negatively-charged PI in the Ultralec fraction may have contributed to its smaller average liposome diameter as determined using PCS. The Ultralec dispersion also had a higher entrapped volume than either the Phospholac or SigP3644 dispersions. However, as discussed previously, the zeta potential of the SigP3644 dispersion showed it was the most negatively charged, with only a small difference between the Phospholac and Ultralec liposome dispersions (Figure 8-2). In addition, the techniques used to measure lamellarity did not provide any significant evidence of any of the liposome populations containing a higher proportion of multilamellar or multivesicular liposomes than the other dispersions. Therefore, although the PCS average size and the entrapped volume of the liposome dispersions fit with the system outlined by Perrett *et al.* (1991), the lamellarity and zeta potential results suggest that the amount of charged lipids present is not entirely responsible for determining the average size and entrapped volume of a liposome dispersion.

### *8.2.2 Fatty acid profile*

There were obvious differences in fatty acid composition between the dairy- and soy-derived phospholipid fractions. As expected, the Phospholac fraction contained a much higher proportion of saturated and mono-unsaturated fatty acids than the soy fractions, which were primarily polyunsaturated (Figure 8-4).

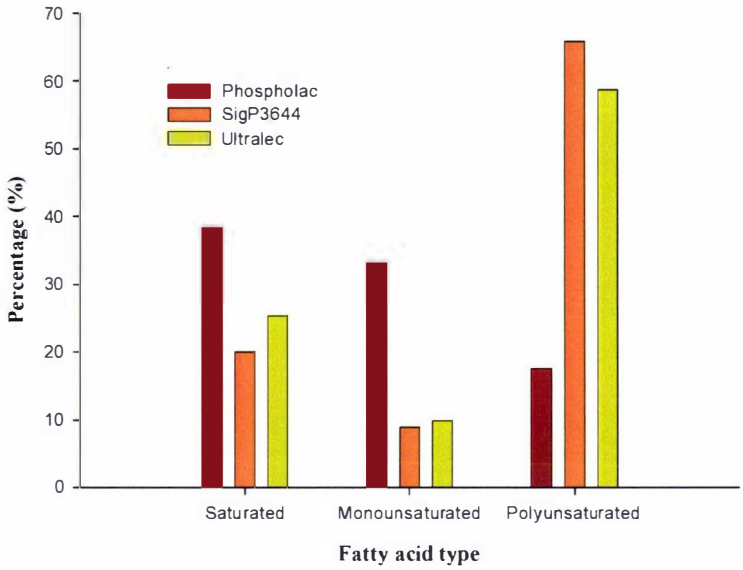



**Figure 8-4 Fatty acid types present in the different phospholipid fractions**

Phospholipids composed of saturated fatty acids have higher phase transition temperatures than their unsaturated counterparts. Membranes in the gel phase (below the phase transition temperature) tend to have reduced membrane permeability and increased membrane stability when compared with membranes in the fluid state (above the phase transition temperature). The phase transition temperature of the Phospholac liposome dispersion was between 20 and 35 °C (Figure 5-14), while the two soy fractions produced liposome dispersions with phase transition temperatures below 0 °C. This means that at room temperature (20 °C) the Phospholac liposome membranes will be in the gel phase, and the SigP3644 and Ultralec membranes will be in the fluid phase.

Small hydrophilic molecules such as water and sucrose were able to diffuse through SigP3644 membranes much more rapidly at 20 °C than through membranes composed of the Phospholac fraction, but there was no significant difference between the Phospholac and Ultralec dispersions at either 20 or 40 °C (Table 8-2). There was also no increase in permeability of the Phospholac membrane at its phase transition temperature (28-30 °C). These results suggest that membrane permeability is not simply a function of the membrane state. It would also be expected that the increased number of double-bond "kinks" in the poly-unsaturated fatty acids would result in a less densely-packed membrane, increasing membrane permeability (Monroig *et al.*, 2003). However, while this may help explain the increased permeability of the SigP3644 membrane there is no evidence of this influencing the permeability of the Ultralec dispersions.

**Table 8-2 Effect of temperature on the membrane permeability of water**

| Sample | Mean permeability of water through the liposome membrane (m/s) | | | |
|---|---|---|---|---|
| | 20 °C | 28 °C | 30 °C | 40 °C |
| Phospholac | $1.6E^{-06}$ | $1.5E^{-06}$ | $1.4E^{-06}$ | $1.2E^{-06}$ |
| SigP3644 | $4.7E^{-06}$ | | | $4.8E^{-06}$ |
| Ultralec | $1.3E^{-06}$ | | | $1.1E^{-06}$ |

Unsaturated fatty acids are more prone to oxidation, damaging the lipid membrane and negatively affecting the overall stability of the liposome system. The low levels of polyunsaturated fatty acids in the Phospholac fraction (Figure 8-4) are reflected in the minimal conjugated diene formation during storage, with the generally saturated nature of the product likely to be responsible for the only small increases in peroxide value. The slightly higher amounts of polyunsaturated fatty acids and the higher lipid concentration (Table 8-3) of the SigP3644 fraction may help explain the increased conjugated diene formation and higher peroxide value compared with the Ultralec fraction. However, it seems unusual that the differences in rate of oxidation are quite large given the relatively small differences in composition. The rate of oxidation of the Ultralec fraction was actually closer to that of the dairy phospholipid than it was to the other soy fraction. The rate of oxidation is also known to be affected by the presence of metal catalysts, but the SigP3644 fraction had the lowest ionic concentration of all three fractions used.

This suggests that while fatty acid composition may affect the susceptibility to oxidation of a liposome dispersion, the complicated nature of the lipid oxidation process means that there are other factors which also contribute to the rate of oxidation within the system. One such factor may be the presence of any naturally occurring antioxidants in the phospholipid fractions. Soy oil has been reported to contain approximately 0.1% tocopherols (Judde *et al.*, 2003), while animal products tend to have very little natural antioxidant activity. It is possible that the additional purification steps undergone by the SigP3644 fraction may have removed a higher percentage of these naturally occurring antioxidants, but the level of antioxidants present in each of the fractions was not measured as part of this thesis.

### 8.2.3 Proximate analysis

The proximate analysis results (Table 8-3) showed that there were significant differences between the three phospholipid fractions for most of the components measured. The variations in moisture and lipid content affect the amount of a particular fraction required to achieve the desired phospholipid concentration, but do not influence liposome behaviour. The role of cholesterol in increasing membrane stability and reducing membrane permeability is well established, with addition rates of up to 1:1 mol% being common. The very small amount of cholesterol present in the dairy phospholipid fraction (0.032%) would not have any significant affect on the Phospholac liposome properties.

**Table 8-3 Composition of phospholipid fractions**

| Component | Phospholac | SigP3644 | Ultralec |
|---|---|---|---|
| Lipid (%) | 53.0 | 67.7 | 61.1 |
| Cholesterol (%) | 0.032 | - | - |
| Nitrogen:Phospholipid ratio | 0.033 | 0.014 | 0.023 |
| Moisture (%) | 5.6 | 3.8 | 3.1 |
| Ash (%) | 14.8 | 5.7 | 13.6 |
| - calcium (mg/100g) | 5.8 | 3.6 | 200 |
| - magnesium (mg/100g) | 1.8 | 6.2 | 240 |
| - potassium (mg/100g) | 2060 | 415 | 1710 |
| - sodium (mg/100g) | 810 | <0.6 | 8.8 |

The Phospholac and Ultralec fractions had a significantly higher nitrogen:phospholipid ratio than the SigP3644 fraction, indicating a higher level of protein in the Phospholac and Ultralec liposome dispersions. The amphipathic nature of protein means the molecules are likely to become incorporated in the phospholipid membrane, with hydrophobic portions located in the interior of the bilayer and the hydrophilic portions in the aqueous phase either inside or outside the liposome.

Small angle X-ray diffraction found that at 20 °C, membranes composed of the Phospholac and Ultralec fractions were 20% thicker than those made from SigP3644. The fact that this difference is occurring between the two soy fractions makes it unlikely that the membrane thickness is simply a reflection of the fatty acid chain-length or the size of the phospholipid

headgroup. An increase in membrane thickness might be caused by a reduction in the packing density of the phospholipid molecules, but there is no obvious reason why this would occur. An alternative possibility is that the protein present in the dispersion has either been incorporated into the membrane during liposome formation or become adsorbed to the surface of the membrane post formation. In biological systems, proteins rapidly adsorb to the liposome membrane (Juliano and Meyer, 1993; Keller, 2001). Steric and charge repulsion forces are likely to cause at least some part of the hydrophilic portion of the proteins to extend out from the membrane surface, resulting in an apparent increase in the thickness of the membrane.

If the increased membrane thickness was due to a reduction in packing density of the phospholipids, it would be expected that the permeability of the membrane would also increase. However, the rate of diffusion of water and sucrose through the liposome membranes was significantly lower in the Phospholac and Ultralec liposome dispersions than in the SigP3644 samples. Shi *et al.* (1999) found that coating liposomes with collagen or chitosan significantly decreased membrane permeability without affecting membrane fluidity. Although there is no literature regarding the use of dairy or soy proteins in this situation, it appears logical that proteins from other sources would have similar effects. Thus the presence of proteins either or both within the hydrophobic interior of the membrane and as an additional barrier on the liposome surface may be responsible for the increased membrane thickness for the Phospholac and Ultralec dispersions, as well as their reduced membrane permeability for small hydrophilic molecules.

The Ultralec fraction had a high level of the divalent cations calcium and magnesium, the Phospholac fraction contained high levels of the monovalent cations potassium and sodium, and the SigP3644 fraction had relatively low levels of all four cations. The stability of a liposome dispersion relies on a number of factors, one of the most important being charge repulsion. As discussed in section 8.2.1, the presence of negatively charged phospholipids can significantly improve liposome stability. However, the presence of positively charged ions can neutralise these phospholipids, reducing charge repulsion and increasing the tendency of the liposomes to aggregate or coalesce. All three fractions had a similar PE content (Table 4-4), but the Ultralec fraction had more than 20 times the level of $Ca^{2+}$ and $Mg^{2+}$ present in the Phospholac and Sigma fractions. While the significance of the effect of the mineral balance on stability depends on how important charge repulsion is as a

stabilising factor in a specific liposome dispersion, this naturally occurring combination of high PE content and high divalent cation levels would suggest that the Ultralec fraction may be less stable than the other fractions. However, despite Ultralec's much higher divalent cation content, the stability of Ultralec dispersions as a function of pH (Figure 8-3) does not appear to be significantly different to SigP3644 dispersions.

Further addition of monovalent cations appeared to result in aggregation or coalescence at lower concentrations in the Ultralec dispersions than either the SigP3644 or Phospholac dispersions, but there was only a small difference in stability between the Ultralec and SigP3644 dispersions upon addition of divalent cations (Figure 8-5). Exchange of the natural ionic balance of each fraction through dialysis did not significantly change these results, providing further evidence that the relative stabilities of the dispersions was not dependent on their ionic profile.

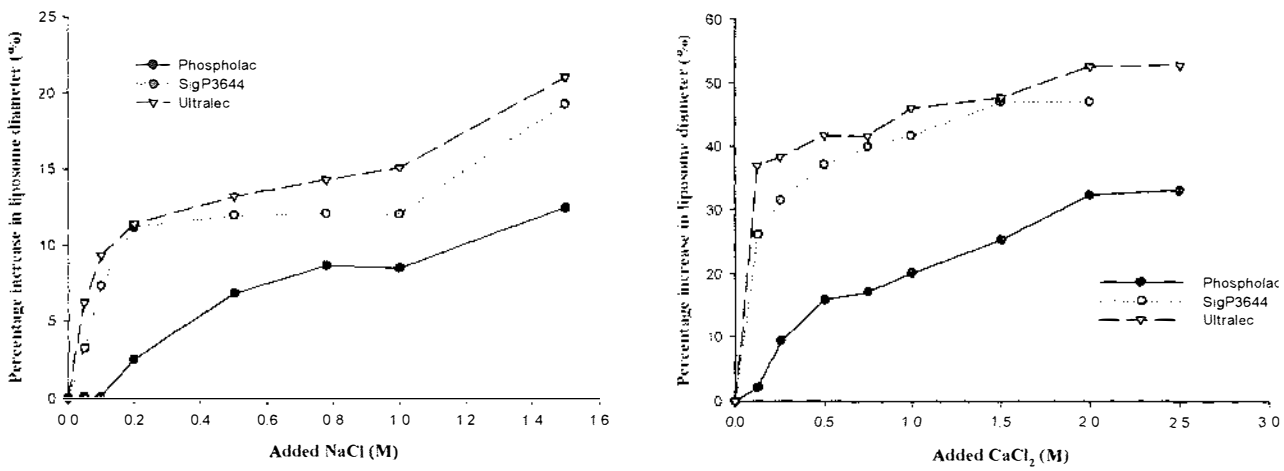



**Figure 8-5 Effect of increasing ionic concentration on the average diameter of liposome dispersions.**

## *8.3 Further work*

### *8.3.1 Purification of phospholipid fraction*

The heterogeneity of the commercial phospholipid fractions provided some complications in terms of the techniques used and interpretation of results. The presence of a relatively large amount of non-phospholipid material may also be a problem when considering possible pharmaceutical or cosmetic applications, where a precisely characterised and very pure raw

material is required. Additional purification of the dairy phospholipid fraction would allow a better understanding of the role of the phospholipid composition, without interference due to proteins, carbohydrates or high mineral levels. Although solvent extraction tends to be a less expensive purification technique, residual traces of organic solvents can create toxicity problems, especially if the product is to be used with high-risk groups, such as nutrition delivery for premature infants. An alternative is the use of supercritical fluid extraction, a relatively new approach to phospholipid purification that avoids the need for organic solvents.

### *8.3.2 Effect of protein on liposome properties*

The rapid permeation of small hydrophilic molecules through the liposome membrane was one of the major problems encountered during this work. One of the more surprising outcomes has been the identification of the possible role of protein in increasing membrane thickness and reducing membrane permeability. Clarification of whether these observations are indeed related to the presence of protein may be obtained through the inclusion of a protease, which would break up the protein molecules without interfering with the phospholipid component of the system. If this step was carried out prior to the Microfluidization process, the resulting liposomes could be characterised in terms of membrane thickness and permeability, and compared with liposomes produced with intact proteins. If it is found that the proteins naturally present in the commercial fractions are affecting the thickness and permeability of the liposome membrane, it raises the possibility that the addition of more protein may help reduce permeability further. Collagen has been successfully used for this in the past, but to date there does not appear to be any work involving the effect of the addition of casein before and/or after liposome formation on the liposome properties.

### *8.3.3 Reduction of membrane permeability through addition of hydrophobic molecules*

It is well established that the inclusion of sterols (usually cholesterol) in liposome membranes increases their stability and reduces their permeability, but the addition of cholesterol to food systems is unattractive from a consumer perspective. Plant sterols, on the

other hand, have been shown to help reduce serum cholesterol, and are being included in a variety of food products as a functional additive. It appears logical to assume that plant sterols would cause similar structural changes to the liposome membrane as cholesterol, but further investigation would be needed to prove this. The use of α-tocopherol to increase membrane stability and reduce permeability is also well-reported (Diplock *et al.*, 1977), and incorporation of the antioxidant within a liposome membrane has been shown in increase its antioxidant effect (Ruben and Larsson, 1984). Entrapment of significant levels of the hydrophobic plant sterols or α-tocopherol without the use of organic solvents may also require some method development or alteration. The development of a technique that allows high levels of hydrophobic entrapment using only food-grade solvents would be useful not only for the incorporation of plant sterols and α-tocopherol, but for hydrophobic actives in general.

### *8.3.4 Hydrophilic entrapment*

If a combination of sterols and proteins are able to sufficiently reduce membrane permeability to allow the encapsulation and retention of very small hydrophilic molecules such as ascorbic acid there are many potential applications in the food industry. However, in the meantime, hydrophilic entrapment is likely to be more successful with larger hydrophilic molecules which will take longer to diffuse through the liposome membrane. The use of a large hydrophilic coloured compound (such as a dye) would provide similar advantages as were seen with β-carotene in terms of being able to visually follow the presence of the dye. Regardless of what sort of hydrophilic molecules were entrapped, once a suitable method was identified or developed to allow the separation of unentrapped material without loss of entrapped molecules it would be interesting to investigate the effect of time, temperature, pH, ionic concentration and the presence of sterols and/or proteins on the release of entrapped compounds.

### *8.3.5 Procedures for achieving long-term stability*

Long-term stability of the liposomes is important if they are to be included in food systems with extended shelf-life, or to allow the liposome to be produced in advance and stored until

addition to the food systems. Freezing, freeze-drying and spray-drying are the obvious means to extend shelf life, with some promising initial results for the freezing of liposome dispersions using sucrose or glucose. Recent reports have suggested that the sugar concentration has to be approximately twice the highest level used during this study, so further trials at higher concentrations would be required to test the feasibility of these techniques for extending the shelf-life of the liposome dispersions.

### *8.3.6 Stability of liposomes upon consumption*

As well as protecting compounds during storage and processing, liposomes may also protect them during digestion and improve the bioavailability of the actives. Investigations looking at the liposome stability in the digestive tract through the use of *in vitro* simulations would provide an initial indication of whether the specific liposome dispersions could survive the passage through the digestive tract intact. If so, bioavailability studies may be undertaken to determine whether there is any noticeable increase in the uptake of the actives due to their encapsulation within liposomes.

## 8.4 Concluding remarks

The research in this thesis has shown that the commercial phospholipid fractions obtained from buttermilk may successfully be used to produce liposomes. The use of a Microfluidizer® allowed liposomes to be manufactured without the use of solvents or detergents, and is able to be easily scaled-up for industrial application.

The liposomes produced from the dairy-derived phospholipid fraction showed considerable advantages in stability over those produced from soy phospholipids. They were able to withstand more severe processing treatments, were stable for longer periods at higher storage temperatures, and were less affected by increases in ionic concentration. It is thought that these observations are due to the high sphingomyelin concentration and more saturated fatty-acid profile of the dairy-derived fraction.

To produce liposome dispersions suitable for the encapsulation of hydrophilic material, further work must be completed to reduce the membrane permeability. However, the improved stability over a wide range of conditions suggests that there may be significant advantages to using dairy-derived phospholipids for the production of liposomes for use in food products.

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