SOLUBILITY ENHANCEMENT OF POORLY WATER SOLUBLE DRUGS USING LIPOSOME TECHNOLOGY

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Summary

Due to their biphasic character, liposomes can act as carriers for both lipophilic and hydrophilic drugs. Despite the enormous amount of work, which has investigated and documented the application of liposomes as carrier systems for the delivery of hydrophilic drugs, their application as solubilising agents for lipophilic drug molecules has received limited attention. The aim of this work is to investigate the various parameters that could control the encapsulation of lipophilic drugs and investigate the influence of the physical properties of poorly water-soluble drugs on bilayer loading.

Initial work investigated on the solubilisation of ibuprofen, a model insoluble drug. Drug loading was assessed using HPLC and UV spectrophotometric analysis. Preliminary studies focussed on the influence of bilayer composition on drug loading to obtain an optimum cholesterol concentration. This was followed up by studies investigating the effect of longer alkyl chain lipids, unsaturated alkyl chain lipids and charged lipids. The studies also focused on the effect of pH of the hydration medium and addition of the single chain surfactant α-tocopherol.

The work was followed up by investigation of a range of insoluble drugs including flurbiprofen, indomethacin, sulindac, mefenamic acid, lignocaine and progesterone to investigate the influence of drug properties and functional group on liposomal loading. The results show that no defined trend could be obtained linking the drug loading to the different drug properties including molecular weight, log P and other drug specific characteristics. However the presence of the oppositely charged lipids improved the encapsulation of all the drugs investigated with a similar effect obtained with the substitution of the longer chain lipids. The addition of the single chain surfactant α-tocopherol resulted in enhancement of drug loading and possibly is governed by the log P of the drug candidate.

Environmental scanning-electron microscopy (ESEM) was used to dynamically follow the changes in liposome morphology in real time during dehydration thereby providing an alternative assay of liposome formulation and stability. The ESEM analysis clearly demonstrated ibuprofen incorporation enhanced the stability of PC:Chol liposomes with drug-loaded liposomes showing enhanced resistance to coalescence during dehydration compared to drug-free liposomes. ESEM was also used to study the effect of salts in the hydration medium, influence of higher transition temperature lipids and liposomal morphology.

The stability of the vesicles was improved by freeze drying the formulations using a new class of compounds, amino acids. The investigations focussed on three basic polar amino acids: histidine, lysine and arginine. The results show that the amino acids provide good protection against vesicle fusion with activity being concentration dependent. The influence of the pre freezing temperature and primary drying rate was investigated with results reflecting the efficiency of amino acids as a promising class of cryoprotectants for liposomes.

Keywords: Lipids, insoluble drugs, ESEM, amino acids, freeze drying

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List of abbreviations

AFM	atomic force microscopy
Chol	cholesterol
CMC	critical micelle concentration
$C_{24}PC$	dilignoceroyl phosphatidylcholine
Da	daltons
ddH_2O	double distilled water
DCP	dicetylphosphate
DEPC	dielaidoyl phosphatidylcholine
DLPC	dilinoleoyl phosphatidylcholine
DMPC	dimyristoyl phosphatidylcholine
DOPC	dioleoyl phosphatidylcholine
DPPC	dipalmitoyl phosphatidylcholine
DSPC	distearoyl phosphatidylcholine
EDXA	energy dispersive X ray analysis
ESEM	environmental scanning electron microscope
FDA	food and drug administration
HCl	hydrochloric acid
HLB	hydrophilic lipophilic balance
HPLC	high performance liquid chromatography
HTS	high throughput screening
LUV	large unilamellar vesicles

min	minutes
nm	nanometers
MLV	multilamellar vesicles
NaOH	sodium hydroxide
PBS	phosphate buffered saline
PC	egg phosphatidylcholine
RES	reticulo endothelial system
SA	stearylamine
SEM	scanning electron microscope
SD	standard deviation
SUV	small unilamellar vesicles
Tc/Tg	transition temperature
UV	ultra violet

Chapter 1

1.1 Developments in disease treatment

Continued development in drug discovery technologies, has provided many new tools to help discovery methods to identify better drugs faster (Di and Kerns, 2003). These new tools stem from advances in molecular biology, genomics, proteomics, combinatorial chemistry, high throughput *in vitro* screening, and molecular modelling (Di and Kerns, 2003). In particular, development of combinatorial chemistry has made a dramatic impact on drug discovery, making it possible to synthesize thousands of new compounds each year. However, for such compounds to become successful drugs they must possess a combination of desired biological activities and an acceptable pharmaceutical profile (including appropriate solubility, permeability and stability). Unfortunately, approximately 40% of the compounds in the discovery/development pipeline fail to reach the market mainly due to this second pre-requisite (Venkatesh and Lipper, 2000). Indeed, of those drugs synthesized via combinatorial chemistry, many of the small molecule (<500 Da) drugs tend to display limited solubility eg <1.5mg/ml in water and the biological milieu and consequently, after administration these compounds can display poor bioavailability often below the therapeutic threshold (Borman, 1998; Kawakami et al., 2002). Further, formulation scientists are often faced with short timelines, limited compound availability and incomplete physiochemical property characterization as hurdles towards formulation development. To overcome these drawbacks, formulation scientists need to be well equipped with techniques to enhance the solubility and bioavailability of compounds so as to support the development of new therapeutic agents (Lee et al., 2003).

1.2 Major hurdles still unanswered—solubility?

Clearly, the introduction of high throughput *in vitro* screening (around 1989) has lead to dramatic increases in the drug discovery process; by screening a huge number of chemical entities based on *in vitro* potency the laborious and time consuming process of synthesizing drug moieties based on "drug-like" properties of lead compounds is circumvented. However, this high throughput screening (HTS) process (Fig 1.2) has resulted in a paradigm shift, with new lead compounds reflecting the properties of the screening library to a greater extent than the "drug-like" properties (e.g. lipophilicity between 0-3, solubility, permeability, stability; Di and Kerns 2003). Further, the common practice of adopting non-aqueous solvents in the screening process can easily mask the poor water solubility of compounds (Curatolo, 1998) resulting in many new entities displaying poor solubility.

1.3 Why is solubility an issue of concern?

No matter how a drug is presented to the body, it must be in a molecular dispersed form (i.e. in solution) before it can be absorbed across a biological membrane (Florence and Attwood, 1998) and hence solubility has an influence on both the *in vitro* and the *in vivo* performance of the drug. For example, poorly soluble compounds might precipitate during *in vitro* assays leading to lower concentrations, which ultimately can be reflected in a reduced activity profile. *In vivo,* low water solubility will result in low systemic exposure leading to lack of *in vivo* efficacy (Di and Kerns, 2003). Itraconazole, a highly insoluble drug used to treat fungal spores

of *Candida innocula* was tested for *in vivo* activity in immuno compromised rats as a solution and nano suspension (to improve solubility) (Wong et al., 2002). Interstingly, following the administration of the solubilised drug in the nanosuspensions at a dose of 40 mg/kg and 80mg/kg, the uptake in the kidney resulted in the complete removal of the spores after an 11 day regimen when compared to the solution which showed significant lower activity (Wong et al., 2002).

1.4 Properties of drugs governing solubility

Factors controlling the solubility of drugs in solution include the nature of the drug substance, its molecular characteristics and the nature of the solvent. For instance, the solubility of a solid at constant temperature and pressure is governed by the free energy associated by the solid and the free energy of the molecules in solution. When the solution free energy is less than the solid free energy, molecules from the solid will dissolve until the free energy of the molecules in solution is equal to that of the free energy of the solid and hence equilibrium is attained (Strickley, 2004). The free energy of a specific solid is a property of the solid and is therefore fixed, but is dictated by the physical form of the drug candidate (crystalline, amorphous, polymorphs) and therefore a factor which dictates solubility. The differences in the lattice enthalpies of the different forms are responsible, with solubility difference between the different polymorphs being less compared to the differences between crystalline and amorphous materials.

Unlike the free energy of the solid, the free energy of molecules in a solution can be influenced by the solvent (and takes into account solute-solvent and solvent-solvent interactions) and the concentration of the solution. For example, investigations into the role of hydrogen bonding between solute-solvent interactions (Sweetana and Akers, 1995) have shown that the solubility of a strong hydrogen donor species like p-nitrophenol increases approximately 4000 fold when the solvent isooctane is replaced with butyl ether, a hydrogen acceptor solvent. Similarly, the solubility of a weaker hydrogen donor carbazole increased by 38 fold in butyl ether, whereas the solubility of anthracene (which has no donatable hydrogens) was influenced by a factor of only 3 when isooctane was replaced by butyl ether. However in most pharmaceutics products, the solvent system is limited to aqueous systems with the options of addition of co-solvents such as ethanol or propylene glycol, which are miscible in water, improving the solubility of some drugs (see section 1.5.4) or pH manipulation to control the ionization of drug molecules. On the other hand, as a great majority of drugs are organic electrolytes, pH is one of the primary influences on their solubility (Fig 1.1). Depending on the ionisation characteristics of the drug (depicted by its pK_a), its solubility in an aqueous environment can be enhanced by appropriate adjustment of pH such that the drug will predominately exist in the ionised form which is readily hydrated. Therefore, weak acids can be solubilised at a pH above their acidic pK_a, and weak bases can be solubilised at a pH below their basic pK_a. For every pH unit away from the pKa, the weak acid/base solubility will increase 10 fold (Strickley, 2004).

$pH = pKa + \log [salt/acid]$ for a weak acid and its salt [Eqn 1.1]

where $pKa = -\log Ka$ and $pH = -\log [H^+]$

Fig 1.1 The pH solubility profile of ibuprofen (source Watkinson et al., 1993)

In addition to the presence of ionisable groups, solute molecular structure in terms of shape and/or surface area can also influence solubility. For example, investigation of Cyclosporin A behaviour in water has shown that the non-polar regions of the drug associate together whilst the polar parts orientate themselves toward the water molecules. This results in higher affinity of the molecule with the surrounding aqueous environment (Ran et al, 2001). The effect of molecular surface area on solubilities is also highlighted by comparison between the aqueous solubility's of pentanol isomers; 2-Methyl-2-butanol which has a surface area of 2.85nm^2 is over 5 times more soluble in water that n-pentanol isomer which has a larger surface area of 3.04 nm^2 (1.40 vs 0.26 mol Kg^{-1} respectively; Amidon et al., 1974).

However *in vivo* performance is not only influenced by solubility, it is affected by various other physicochemical and structural properties including stability and permeability solubility and permeability are interdependent (Di and Kerns 2003)

with many highly soluble drugs displaying low permeability and *vice versa*. Like solubility, the permeability of a compound is dictated by its molecular size and hydrogen bonding capacity and can therefore be manipulated. Increasing hydrogen-bonding capacity and ionic charge will increase solubility, however this will decrease permeability. Therefore, whilst many options of molecular modification are available at the drug discovery stage to improve drug solubility, when choosing between improving solubility or permeability, improved permeability has been recommended, as solubility can be improved through formulation engineering (Di and Kerns, 2003).

Fig 1.2 A schematic representation of the various processes involved in drug development. The role of solubility and the possible routes to overcome poor solubility have been shown. SAR route denotes the structure activity relationship. SPR relates to the structure property relationship (modified from Di and Kerns 2003).

1.5 Chemical modification and formulation approaches to enhance solubilisation

As mentioned, solubility and permeability are the two major components, which influence bioavailability. Increasing the molecular weight and lipophilicity could enhance permeability but will generally reduce solubility. Similarly aqueous solubility could be improved by adding hydrogen-bonding groups and charge, however this can reduce permeability. To overcome the problem, industrial chemists focus on improving permeability by modifying the structure or making pro-drugs whereas solubility is dealt with by investigating formulation techniques (Di and Kerns, 2003). Thus a drug candidate with poor solubility and dissolution rate can still be developed (Huang and Tong, 2004).

1.5.1 Chemical modification: Prodrugs

The pro-drug approach to optimising permeability/solubility involves the chemical modification of a drug moiety into a bio-reversible form to alter its physicochemical properties (Palagiano et al., 1997). The parent compound would be regenerated *in vivo* by enzymatic or hydrolytic mechanisms. The pro-drug structure should display an improved solubility, which can promote enhanced therapeutic action. In addition, the synthesis of prodrugs from the poorly soluble drug, as in the case of propofol, resulted in the generation of compounds with moderate increases in solubility and high aqueous stability (Trapani et al., 1998) with the steric hindrance that may be imparted by the added prodrug groups possibly contributing to this enhanced stability. For example, the synthesis of

propofol esters resulted in an increase in compounds with increased solubility, but the presence of bulky isopropyl groups on the prodrug also masked the site for chemical hydrolysis of the ester group in the prodrug resulting in highly stable structures *in vivo* which prevented the release of the parent molecule (Altomare et al., 2003). Pegylated prodrugs have also been synthesised to incorporate hydrophilic polymer attachments (polyethylene glycol) on to the insoluble drug (including Paclitaxel) thereby not only controlling solubility but also *in vivo* parameters like increased circulation (Feng et al., 2002). Another approach developed in prodrug synthesis is the synthesis of a duplex prodrug which consists of two molecules of the insoluble moieties linked chemically (ester) as reported in the case of naltrexone (Hammell et al., 2004).

However the major drawback in the prodrug method remains with a compromise between water solubility and permeability being required. The prodrug synthesis involves the addition of different structures essentially causing an increase in lipophilicity and hence influencing solubility (Palagiano et al., 1997, Bonina et al., 1992). When delivered orally, this becomes less of an issue with orally administered prodrugs tending to be absorbed in the GI tract after enzymatic action releasing the parent compound. This absorption is however dependent on the generation of high concentrations of the active drug candidate which is absorbed essentially due to the development of a concentration gradient in the GI tract (Heimbach, 2003) and is therefore not appropriate for other routes. The prodrugs also need to demonstrate a good balance on stability. Poor stability would release the parent drug quickly (Feng et al., 2002) whereas highly stable structures

although enhancing solubility would prolong or inhibit the release of the parent molecule (Altomare et al., 2003).

1.5.2 pH adjustment

As mentioned, pH adjustment can be employed to improve the solubility of ionisable drug candidates (Fig 1.1; Table 1.1) and from this the total solubility of a drug may be calculated as the sum of the solubility of the unionized and the ionized species. This can be represented as follows:

$$S_{tot} = S_{HA} + S_{A^-} \qquad \text{[Eqn 1.2]}$$

Where: S_{tot} represents total solubility,

S_{HA} represents solubility of the unionized form,

S_{A^-} is the solubility of the ionized species.

Therefore, the total solubility of a weakly ionisable drug is dictated by its intrinsic solubility, pKa and the pH of the dissolving media (Lee et al., 2003). However, in the formulation of pharmaceuticals the range of pH that can be adopted is often limited, and whilst current FDA approved marketed parenteral products range from pH 2 to 11 (Sweetana and Akers, 1996) for biocompatibility the recommended pH range of between 4 to 8 is adopted for majority of the formulated injectables. Outside this range, there are many examples of marketed parenteral products which have a solution pH outside this range (Table 1.2)Ultimately however, pain at injection site, isotonicity and the risk of drug precipitation after *in vivo* administration must be considered if the pH of the pharmaceutical product is markedly different from the pH of the administration site (Table 1.1). In addition, optimisation of the pH to enhance solubility can stimulate chemical instability in

the drug. A recent report by Kim et al., (2004) involved efforts to enhance the solubility of a lipophilic drug CKD-732 (log P 3.6; pKa 8.5). The administration of the drug as a co-solvent infusion resulted in drug precipitation and irritation; however the presence of ionisable nitrogens on the drug structure offered the potential for pH manipulation to be exploited as a means to overcome the drug's poor solubility. By lowering the pH of the dissolving media to below 6 a 300-fold increase in solubility was achieved, but to achieve the high therapeutic concentration (15mg/ml) required for the drug the pH had to be dropped to below 5. Unfortunately adopting pH values below 5 resulted in acid catalysed hydrolysis of the drug demonstrating further limitations of this solubility enhancing method.

Table 1.1. Summary of formulation approaches to improve solubility (Sweetana and Akers, 1996).

(Sweetana and Akers, 1996).

1.5.3 Salt formation

Salt formation is another commonly employed approach to enhance solubility of weak electrolyte compounds (O'Connor and Corrigan, 2001). Compounds with ionizable groups can form salts with counter ions which can affect the solubility behaviour, dissolution rate and the *in vivo* performance of the drug. Indeed, salt formation is often considered as one of the most practical ways to enhance solubilisation (Bighley et al., 1995). Recent studies by Nielsen et al., (2005) reported the effect of the various counterions on improving the solubility of bupivacaine. A range of counter ions including formate, acetate, chloride ions and glycocholates were studied. The results show that solubility was improved by a wide range from 35 to up to 200 when compared to the poorly soluble parent bupivacaine iodide salt. This improvement in the solubility was attributed to the delocalisation of the positive charge on the quaternary nitrogen atom thereby leading to weaker electrostatic attraction in the solid state (Nielsen et al., 2005)

Salt formation also affects the chemical stability of the molecule. These differences in stability between the salt form and the unionized form, or between the different salt forms, could be attributed to the differences in the micro environmental pH and

the crystal lattice of the salt form. The improvement in the solubility of bupivacaine by forming salts with counter ions of formate, acetate and chloride was related to the balance between lattice and hydration energies with the increase in the hydration energy enhancing solubility whereas no trend was observed with the alterations in the lattice energy (Nielsen et al., 2005). Hence a fine balance specific to the characteristics of the drug and the counter ion needs to be established for a significant improvement in solubility to be achieved (Nielsen et al., 2005).

1.5.4 Co-solvent systems

Co-solvent systems provide a good alternative to enhance drug solubility without compromising the chemical structure of the poorly water soluble moiety and they are useful because they may often provide exponential increases in solubility. Of those employed within the formulation of pharmaceuticals the most commonly employed water miscible co-solvents include glycerin, ethanol, propylene glycol, polyethylene glycol and N, N-dimethylacetamide (Table 1.3). Such systems can provide an exponential increase in drug solubility for weak electrolytes and non-polar compounds (Ni et al., 2002), with the enhancement of solubility being credited to the ability of the co-solvent to weaken the hydrogen-bonding structure of the water molecules. This can be attributed to co-solvents containing both hydrogen bonding and non-hydrogen bonding groups in their structure. The hydrogen bonding group interacts with water thereby incorporating the non-polar and non-hydrogen bonding portions of the co-solvent into the structure of water. This reduces the polar nature of the aqueous media thereby ultimately enhancing the solubility of the non-polar solute (Lee et al., 2003). Therefore overall, the

extent of solubilisation by co-solvents depends on their concentration and polarity and of those most commonly employed the order of solubilisation is reported as PEG> ethanol> propylene glycol> glycerol (Ni et al., 2002).

Table 1.3. Examples of co-solvent systems currently on the market (adapted from Sweetana and Akters, 1996)

However the co-solvent mixtures are not free from drawbacks with reports of poor solubilisation for drug molecules with low log P (1.7; Ni et al., 2002) and toxicity issues including irritation, haemolysis, swelling at the injection site, and drug precipitation all being associated with their use (Sweetana and Akers, 1995). Indeed, co-solvent toxicity and acceptability by the regulatory bodies is a recognised an issue (Sweetana and Akers, 1995). Co-solvent concentrations greater than 60% must be approved by a veterinarian for animal safety before administration (Lee et al., 2003) whereas concentrations of up to 70% have been reported for human consumption (Haim et al., US patent no: 2004011082 (2004)).

1.5.5 Complex formation

Cyclodextrins are cyclic (α-1,4) linked oligosaccharides of α-D- glucose which form a cyclic structure encompassing a hydrophobic central cavity with a relatively hydrophilic outer surface (Miyazawa et al., 1995). The number of the glucose units in the annular ring could vary between 6, 7, 8 and 9 units giving α, β, γ, δ

cyclodextrins. The cyclodextrin central cavity diameter can range between 5 to 8 Å in size thereby enabling the encapsulation of poorly water soluble drugs into the central cavity (Miyazawa et al., 1995). Previous investigations (Lallemand et al., 2003) have demonstrated cyclodextrins enhanced the solubilisation of insoluble drugs such as cyclosporin A 100 fold. This increase in drug solubility is also reported to be linear when the drug forms a 1 to 1 complex with the cyclodextrin (Trapani et al., 2004). However, studies have also shown that not all the different types of cyclodextrins accomplish this effect with both the size and the physico-chemical properties (including amphotericin B, digitoxin) of the drug molecule dictating its entrapment (Miyazawa et al., 1995). Cyclodextrins have also been employed to enhance the chemical stability of drug moieties with instabilities such as drug hydrolysis and photodegradation being overcome, presumably due to the protective, shielding effect of the complex (Loftsson and Brewster, 1996). Stability analysis of the different cyclodextrin complexes has also shown that ionized species have a very low stability constant (the ability of the drug candidate retention in the lipophilic cyclodextrin core) compared to the unionized form of the same drug in non-ionic hydroxy propyl β cyclodextrins however, the stability of the cyclodextrins is pH sensitive with alkaline media stabilizing the ring structure whereas acidic conditions hydrolytically cleave the ring giving linear oligosaccharides (Miyazawa et al., 1995).

The application of cyclodextrins as solubilisation agents is however limited by various factors: firstly the outer surface of the cyclodextrin ring, being hydrophilic reduces the affinity of the complex for biological membranes. This can be overcome by attaching lipophilic chains on the outer circumference but, in turn,

leads to poor loading and fast release of the drug (Duchene et al., 1999). *In vivo* studies have also demonstrated that haemolysis is an adverse effect associated with cyclodextrin administration (Irie et al., 1982). The most commonly employed, β-cyclodextrins which are characterized by a central cavity with a strong affinity for hydrophobic drugs, unfortunately have a relatively low aqueous solubility in their own right, which can result in precipitation in the kidneys leading to renal toxicity (Sweetana and Akers, 1995; Kim et al., 2004). Whilst recent developments of a number of chemically modified cyclodextrins, with an added hydroxyalkyl or methyl groups to the cyclodextrin content, has been shown to overcome the drawback of poor water solubility (Trapani et al., 2004; Kim et al., 2004), their application is still limited by poor drug loading and weak reservoir effect resulting in dissociation of drug-cyclodextrin complexes upon dilution by plasma and extracellular fluids (Mesens and Putteman, 1995).

1.5.6 Carrier systems

Carrier systems, such as micelles, microparticles and liposomes, have been successfully employed not only to improve solubilisation of drug molecules but have added benefits to lower drug toxicity (Lasic, 1998), altered pharmacokinetic pattern (Sadzuka et al., 2000), improved bioavailability (Monem et al., 2000) thus enabling the drug molecule to reach the target site. To thoroughly exploit these advantages, the carrier system is required to fulfil the following criteria:

1. The carrier system should offer effective and stabile drug solubilisation.
2. It should mask the drug from non-target tissues (Sadzuka et al., 2000).

3. The system should be able to deliver the drug across the physiological barriers at the desired site of action (Monem et al., 2000).
4. The system should be non-toxic, non-immunogenic and biodegradable.
5. Formulation of the system should be cost effective and appropriate for large scale production.

There have been an array of delivery systems that have been investigated to achieve the above properties. Although some of the above mentioned properties may be present in a particular delivery system, failure to satisfy all the listed criteria have restricted the application of many of these systems as effective solubilising agents.

1.5.6.1 Surfactants and Micelles

Surfactants are also used in the formulation of poorly soluble drugs as they offer several properties including increasing drug solubility and stability through incorporation within micelles. Micellisation provides a good approach for solubilising a hydrophobic drug in a biodegradable drug carrier surfactant system by physically encapsulating the drug (Singla et al., 2002) and as such micelles can offer improved solubility without necessitating a change in the structure/characteristics of the drug candidate.

Conventional micelles form as a result of self-association of surfactant molecules into aggregates, where the hydrophobic portion of the surfactant orientates itself away from the polar environment and the hydrophilic part would face the aqueous phase (Fig 1.3). This arrangement of surfactants into micelles, which occurs at a

specific concentration (known as the critical micelle concentration; CMC), is primarily driven by the system achieving a state of minimum free energy (Florence & Attwood, 1998). The micellar core formed as a result is essentially a paraffin-like region, which is capable of dissolving water-insoluble drugs (Fig 1.3), essentially due to the lipophilic interactions between the insoluble drug candidate and the hydrophobic area of the surfactant (Attwood et al., 1989). For example, solubilisation of clofazimine (used in the treatment of leprosy) was improved in the presence of both bile salts (such as sodium deoxycholate, sodium cholate, sodium taurocholate) or synthetic surfactants such as sodium dodecyl sulphate (anionic) and cremophor EL (non ionic) essentially due to the formation of micelles (O'Reilly et al., 1994). Similar improvements in solubility were reported by Takino et al., (1994, 1995) for the solubilisation of retinoic acid (logP 6.61). Studies on the solubilisation of indomethacin using the non-ionic surfactant polysorbate 80 along with 25% sorbitol have shown that the encapsulation of indomethacin is improved with the increase in drug/surfactant ratio (Attwood et al., 1989). The entrapment of indomethacin within micelles was also shown to influence the shape of the micelles giving it a spherical appearance in contrast to the oblate ellipsoidal shape in the absence of indomethacin (Attwood et al., 1989).

Fig 1.3 Diagrammatic representation of the formation of micelles from monomer surfactant units. Micelles are formed when the concentration of the monomer units is above the critical micellar concentration. Further increase of concentration results in the formation of cylindrical micelles. Drugs may be solubilised within the micelle structure, with their location within this structure being dependent on their physico-chemical characteristics.

As noted, the self-association of surfactant molecules into micelles occurs at a specific surfactant concentration, known as the CMC, which is affected by both the surfactant molecular structure and the nature of the aqueous media (including temperature and electrolyte concentration/valency). As a result, many conventional low molecular weight surfactants precipitate the drug upon dilution in an aqueous environment (Takino et al., 1995). To overcome the drawback, high molecular weight polymeric micelles have been synthesized which provide enhanced solubilisation and drug retention capabilities (Yokoyama et al., 1998). These polymeric micelles have been shown (Yokoyama et al., 1998) to have a high thermodynamic stability and kinetic stability, which in turn reduces disassembly of the micelles at low concentrations, possibly due to the presence of multiple hydrophobic sites within each polymer molecule (Yokoyama et al., 1993).

Generally, polymeric micelles consist of AB type block co-polymers with the A chains forming the inner core while the B chains form the outer layer exposed to the polar environment. The cohesive forces operating in the inner core consist of hydrophobic interactions, ionic interactions or hydrogen bonding and as with conventional micelles, insoluble drugs can be physically encapsulated into the lipophilic core or linked covalently. However, it has been reported (Yokoyama et al., 1998) that entrapment of the insoluble drugs into these polymeric micelles can be problematic as co-precipitation of the drug along with the polymer in an aqueous environment can result (Yokoyama et al., 1998). Further reported problems limiting the wide-spread application of micellar systems for the delivery of poorly soluble drugs include lack of control over release rates *in vivo* (Takino et al., 1995), and poor stability and shelf life (Lallemand et al., 2003).

1.5.6.2 Polymer based drug delivery system

Polymeric drug delivery systems offer a wide range of system options which are biocompatible, biodegradable and are available in an array of molecular weights offering the feasibility of producing delivery systems with different physico-chemical properties and generally these polymers are broadly classified into two groups. Firstly polymers from natural sources, which include starch (Arthur et al., 1984), collagen, and alginate (Downs et al., 1992) form a good natural material for drug delivery however can have associated problems with quality and quantity. Alternatively the synthetic polymers, which include water soluble materials like poly amino acids (Li et al., 1993) and water insoluble materials like poly lactic acid esters and poly caprolactones, provide an alternate to the natural polymers and offer

advantages in their ability to be synthesised on a large scale at a desired quality. Natural polymers like alginates and gelatin have been investigated for delivery of water insoluble agents but have been reported to generate immunogenic responses due to any endotoxins and contaminants associated during the process of extraction (Sanders and Hendren., 1997). Alginates also need a careful investigation of any cross linking agent that might render them a matrix network.

The available literature on synthetic polymers reveals its high application in designing delivery systems for drug moieties and macro molecules. The commonly encountered polymer based drug delivery systems include nanospheres andmicrospheres. The polymers like polylactic acid, polyglycolic acid, and polycaprolactones offer a wide choice of hydrophilic lipophilic balance (HLB), molecular weight and solubility. The commonly encountered polymer combinations used for solubility enhancing include a combination of polylactic acid and polyglycolic acid.

Many of the above mentioned polymers have also been used to develop micro and nanoparticles/spheres. These systems are now being developed as potential new solubility enhancing agents as they may offer the ability to deliver water insoluble drugs and facilitate sustained release (Burt et al., 1995; Wang et al., 1997). Essentially, nanoparticles/spheres and microparticles/spheres utilize similar polymers however differ in size often necessitating a slight variations in the method of formulation (extrusion/sonication). The polymers heavily investigated in these formulations consist of a mixture of polylactic acid and polyglycolic acid but are characterized with poor surface properties of the formulations, which influence low

drug incorporation and unreliable release patterns. To overcome this drawback, various additives to coat the surface in the form of emulsifying agents have been used including gelatin (Wang et al., 1996), alpha tocopherol, polyethylene glycol 1000 succinate (Mu and Feng, 2003) and phospholipids (Feng and Huang, 2001). In particular, the addition of surfactants improved surface properties but required an optimised concentration of the surfactant for optimal loading and release. Concentrations of the surfactant below or above this optimum value resulted in poor drug retention within the systems (Feng and Huang, 2001).

Currently the main problems associated with these systems include longer-term stability with particle aggregation occurring both in aqueous solutions over time and during freeze drying (Mu and Feng 2003). Further these particulate systems still require further research to optimize the polymer ratio, molecular weight and the need for a surfactant to modify surface properties, however results currently suggest that drug release from these systems is essentially controlled by either diffusion or erosion of the polymer, or a combination of both, with most particulate systems studied showing burst release with a very few exhibiting zero order release (Lallemand et al., 2003). Poor understanding of the release mechanism could possibly explain the inability to control release rate (Lallemand et al., 2003).

1.5.6.3 Surfactant Vesicles: Liposomes and Niosomes

Liposomes are microscopic carriers composed of one or more lipid layers, which have been employed for the encapsulation of both hydrophilic and hydrophobic compounds (Gregoriadis, 1973). The phospholipid molecules posses a polar and a nonpolar group on the same molecule andthis results in them forming closed structures when hydrated with the aqueous phase. These self-assembled liposomes consist of these amphiphilic molecules orientated such that the polar portion of the molecule is in contact with the polar environment and shielding the non-polar part and *vice versa* (Lasic, 1998). Due to this resultant biphasic character, liposomes can act as carriers for both lipophilic and hydrophilic drugs. The solubility and partitioning behaviour of a drug molecule governs its location in the liposome structure (Gulati et al., 1998). Highly hydrophilic drugs (logP<1.7; e.g. arabinosylcytosine) are located exclusively in the aqueous compartment of the liposomes (Tokunaga et al., 1988). Alternatively, highly lipophilic drugs with $logP_{oct}$ >5 are entrapped in the lipid bilayer almost completely, (e.g: cyclosporine; Vadiei et al., 1989). Drugs with intermediate partition coefficients, with logP between 1.7 and 4.0, pose a problem because they partition easily between the lipid and aqueous phases and are lost very easily from liposomes (Gulati et al., 1998).

Similar to liposomes, niosomes are vesicles consisting of one or more bilayers however these systems use non-ionic surfactants rather than phospholipids in the bilayer construction and as such these formulations carry an advantage in that the raw materials employed in the constructs are easily available, cheaper in cost and chemically stable (Carafa et al., 2002). However, recent studies looking at these

systems as solublisation agents for various drugs including lidocaine and dithranol have reported poor drug loading and a faster rate of drug release compared to liposomes (Carafa et al., 2002; Agarwal et al., 2001). The poor drug retention properties of the niosomal systems tested may be due to the lower affinity of the drug molecules with the surfactant and weak structural forces holding the niosomal bilayers together. Further, the single chain surfactants, commonly used in the niosomal systems, also lack the ability to form spontaneous vesicles once deformed and may result in structures which are less able to withstand bilayer stress on storage (Agarwal et al., 2002). Indeed, despite the advantages of niosomes mentioned, these formulations do exhibit physical instability: aggregation, fusion, leakage, sedimentation, and hydrolysis of the encapsulated drug have all been reported to reduce the shelf life of the formulation (Hu and Rhodes, 1999).

1.5 Liposomes: Background and development

In the early 1960s, Bangham and Horne observed under an electron microscope (Fig 1.5a) that purified phospholipids could spontaneously form closed membrane systems when dispersed in water. This prompted them to further investigate the natural membrane like structures. The investigations, which started with purified lecithins and a combination of lecithins and cholesterol opened avenues for a wide application of these natural membrane like structures. These lipid vesicles were subsequently named liposomes.

In one of the initial reports presented by Bangham on these lipid constructs, he summarized his finding briefly as:

- The model membrane systems may be formed from egg lecithin with or without cholesterol
- The model systems are compound structures of bimolecular sheets intercalated by aqueous spaces
- They are permeable to water
- They are osmotically sensitive

By the turn of the 1970s, various ways of liposome preparation, with different charge, size and composition had been discovered and their application as a drug delivery tool was subsequently identified with studies in the '80s emphasizing on the *in vivo* performance of the system and latterly with advances being made in liposome manufacture, scale up, and characterization these systems are now

commercially used for a variety of drugs including Amphotericin B, doxorubicin and nystatin to mention a few.

Fig 1.4 Diagrammatic representation of the structure of liposome. Figure shows the concentric arrangement of the lipid molecules. The hydrophilic moieties are entrapped into the aqueous layers whereas the lipophilic moieties are present in the lipid environment.

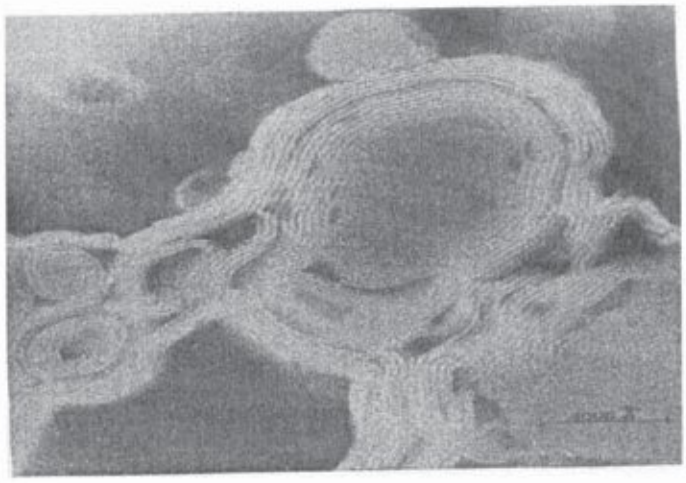

Fig 1.5a The first electron micrograph of ultra sound treated ovolecithin and mixed with an equal volume of 2% potassium phosphotungstate. The picture was one of the first ones to be taken when liposomes were reported by Bangham and Horne.

Fig 1.5b The environmental scanning electron micrographs of the liposomes. The liposomes were observed in the wet mode and no prior sample fixing done. The micrograph shows spherical, drug loaded liposomes at a low pressure of 1.4torr (Mohammed et al., 2004)

1.6.1 Amphiphiles self assembly and liposome formation

The formation of liposomal bilayers can be explained on the basis of three factors: 1. Interaction free energy of the molecule, 2. Geometry of the molecule, and 3. Thermodynamics of self-assembly. The assembly of the lipid molecules into well defined liposomal structures is due to the hydrophobic interactions of the lipid tails which are directed away from the aqueous phase and the hydrophilic interactions of the polar head groups which are in contact with the water phase. This results in the formation of "optimal surface area" where the free energy of the lipid molecules is least.

Geometric considerations include optimal surface area and hydrocarbon chain volume of the surfactants used in the constructs (Israelachvili et al., 1977). The molecular shape of the amphiphile influences lipid self assembly and liquid crystalline phase formation. The two major controlling parameters in amphiphile shape are cross sectional areas of the hydrophobic (A_{np}) and polar (A_p) parts respectively. Structures with high curvature, such as micelles, are formed when the cross sectional area of the polar region of the amphiphile is higher than that of the non-polar region. When the areas are comparable, a stable bilayer is formed (Fig 1.6). Hexagonal inverse micelles are formed when $A_{np} > A_p$ (Israelachvili, 1991).

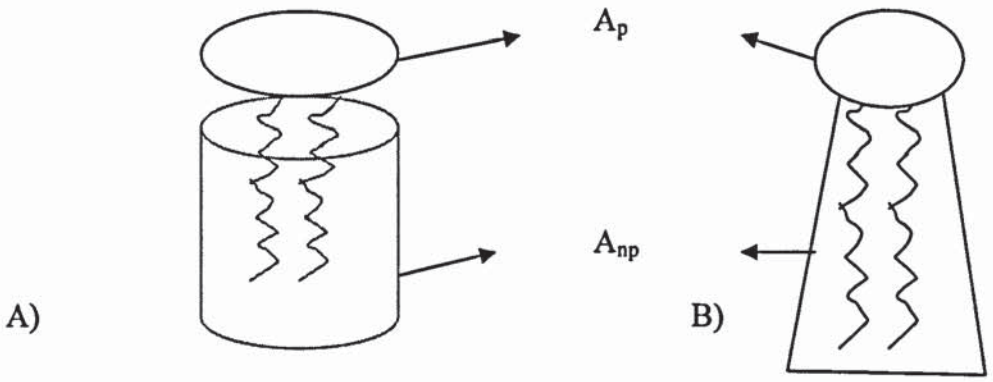

Fig 1.6 A) Lipids with packing parameter of 1 (Anp=Ap) form bilayers. B) Lipids with packing parameter >1 (Anp>Ap) form hexagonal phases.

In addition to their arrangement, liposomal bilayers show various phase transitions that can be used to promote drug release or liposome fusion. Bilayers exist in a solid ordered phase at low temperatures and in a fluid disordered state above a certain temperature, termed the phase transition temperature (Tc). Hence by selective choice of lipids with appropriate transition temperatures the phase of the lipids can be controlled and hence used to control liposome drug release, with higher drug leakage occurring through lipid bilayers in the fluid state rather than those existing in the solid state (Manosroi et al., 2002).

1.6.2 Lipids used in liposome synthesis

As mentioned liposomes are constructed from various lipids, characterized by a packing parameter of n ~ 1.0, to form the liposome bilayers. The amphiphiles most commonly used to prepare liposomes for pharmaceutical purposes are phospholipids and sphingolipids and generally these phospholipids can be divided into four groups:

1. Phospholipids of natural sources: the two main sources for natural phospholipids are egg and soya bean. The egg phospholipids are characterized by the presence of a saturated acyl chain in position 1 and mainly unsaturated chains in position 2 whereas the soya bean derived lipids have unsaturated chains in both position 1 and 2.
2. Modified natural phospholipids: these are natural phospholipids chemically modified by partial or complete hydrogenation to reduce the degree of unsaturation eg: conversion of phosphatidylcholine to phosphatidylglycerol.
3. Semi-synthetic phospholipids: the acyl chains in the natural phospholipids are replaced chemically with other defined acyl chains eg: conversion of phosphatidylcholine (c=12) to dimyristoyl phosphatidylcholine (c=14).
4. Fully synthetic phospholipids: these compounds are prepared via a synthetic route e.g. phosphatidyl serine. (Swarbrick and Boylan, 1994).

The other surfactant used extensively in liposome formulation is cholesterol. A specific physicochemical feature of cholesterol is the presence of a planar steroid ring which imparts a relatively rigid structure to the molecule. The shape of the molecule allows it to fit closely in bilayers with the hydrocarbon chains of the fatty acids. Cholesterol complexes with phospholipids and reduces the permeability to water, cations and glucose. It also condenses and rigidifies the membrane without solidifying it (Florence and Attwood, 1998). This influences the interaction of cholesterol with lipid molecules arranged in the bilayer configuration (Morilla et al., 2002). The interaction of cholesterol with the lipid molecules in the bilayer depends on the structure of their phospholipid head group and the extent of saturation of their lipid chains.

Increased electrostatic interactions on the lipid head group and the presence of other forces eg: hydrogen bonds, reduces the incorporation of cholesterol into the lipid bilayer (Wang and Quinn, 2002). Cholesterol can also influence the self association of the other lipids used in the liposome formulation as seen with Amphotericin B with lower concentrations of cholesterol reducing the encapsulation (Moribe, et al., 1999).

1.6.3 Types of liposomes

Liposomes can be classified based on the structure and size of the lamellae or their method of preparation. The two classification systems are independent from each other (Swarbrick and Boylan, 1994).

1.6.3.1 Multilamellar vesicles

The liposomes are characterised by the presence of multiple lamellae with a volume diameter over 1μm (Fig 1.7). The MLV are characterized by a ratio of ¼ l/mole lipid of aqueous volume to lipid. They are mechanically stable upon long term storage and due to their large size are rapidly cleared by the reticulo endothelial system (RES) and as such can effectively used to target cells of the RES (Sharma and Sharma, 1997). These vesicles, due to their high lamellar capacity, carry the advantage that both water and lipid soluble solutes can be effectively entrapped within their structure; the lipophilic molecules bind to the bilayer whereas the water soluble compounds are entrapped in the aqueous core. In particular these systems

have been shown to be suitable for lipophilic drugs with high values of entrapment of up to 100% being obtained with these vesicles.

MLV can be prepared by hydration of the dried lipid film. The lipids are hydrated at a temperature above the Tc of the lipid to ensure efficient encapsulation, as the lipids are in a more fluid state thereby aiding in the transbilayer movement of the moieties. (Bangham, 1965). In order to minimize large variations in the volume diameter, MLV can be subjected to extrusion where the liposomal suspension is forced through filters with defined pore size giving rise to MLV with mean diameters similar to that of the filter pore size (Mayer et al., 1986). Similarly, MLV can also be prepared by the reverse phase evaporation (REV) method and using this method encapsulation of aqueous soluble solutes with efficiencies of up to 50% have been reported. (Touraki et al., 1995).

1.6.3.2 Large Unilamellar Vesicles

These vesicles are characterized by a single bilayer with volume diameter over 100nm (Fig 1.7). LUV consist of a large internal volume occupied by the aqueous phase (7 l/mole lipid) and are used for the encapsulation of high molecular weight molecules like RNA and DNA (Straubinger et al., 1983). Due to the large aqueous volume water-soluble drugs can be incorporated with an encapsulation efficiency of up to 40% (Gouldpogenite et al., 1985)

1.6.6.3 Small Unilamellar Vesicles

The small unilamellar vesicles vary between 20nm and 100nm in size (Fig 1.7). SUV are most commonly prepared by sonication with a suspension of MLV being subjected to sonication (Hauser, et al., 1971). As with other forms of bilayer manipulation, sonication is carried out above the Tc of the highest melting lipid in the mixture and the suspension allowed to anneal above the Tc for at least 30min (Lawaczek et al., 1976). SUV have been most used to encapsulate small water soluble molecules due to their limited internal volume. Advantages of SUV include that they are homogenous in size, thermodynamically stable and can carry a long circulation half-life *in vivo*. However they also have a low aqueous volume to lipid ratio (0.2-1.5 l/mole lipid) which can limit their drug loading (Sharma and Sharma, 1997).

Fig 1.7 Diagrammatic representation of different types of liposomes A) Multilamellar vesicles B) Large unilamellar vesicles C) Small unilamellar vesicles D) Multivesicular vesicles (Walde and Ichikawa 2001)

1.7 Physicochemical properties of liposomes

The physicochemical properties of the liposomal formulations can be used to alter drug entrapment, stability and biodistribution patterns (Sharma and Sharma, 1997) and the physicochemical properties of liposomal formulations are the main determinants of their targeting efficiency (Fatouros and Antimisiaris, 2002). The *in vivo* performance of the liposomal systems has been shown to be dependent on liposomes physico-chemical properties including size, surface charge and bilayer composition (Gregoriadis and Senior, 1986; Woodle and Lasic, 1992).

1.7.1 Size

Liposomal size gives a broad picture of packing behaviour of drugs, aggregation and *in vivo* fate of liposomes. For example drugs with good packing parameters (eg: hydrochlorthiazide) have been shown to have an increased encapsulation and stability within liposomes when compared to less effectively packaged drugs (Fatouros and Antimisiaris, 2002). *In vivo* behaviour of liposomes suggest that liposomes with size range less than 100nm better tolerate opsonisation whereas liposomes with size over 200nm are readily taken up by the macrophages in the reticuloendothelial cells in the liver and spleen (Allen and Everest 1983). Subsequently, liposomes with a smaller size range and longer circulation time have been shown to better accumulate in the tumour tissue (Papahadjopoulos et al., 1991). This could possibly be due to either a reduction in the uptake by the MPS or increased extravasations through the highly permeable capillaries of the tumour tissue (Papahadjopoulos et al., 1991; Allen et al., 1989; Sharma and Sharma, 1997).

Liu et al., (1992), also studied the effect of vesicle size on the biodistribution of liposomes and demonstrated that liposomes in the size range of 70 to 200nm in diameter better resisted the circulation activity whilst vesicles larger than 200nm rapidly accumulated in the liver and spleen.

1.7.2 Surface charge

The nature and density of the liposome surface charge has also shown to influence vesicle stability, *in vivo* pharmacokinetics and liposome-cell interactions. For example neutral liposomes, due to their low surface potential and hence small electrical double layer, often display poor stability and aggregate whereas liposomes containing charged lipids within their bilayers display less fusion, due to their 'like' charges (and large electrical double layers) blocking aggregation. Liposomes containing negatively charged lipids (e.g. phosphatidylserine and dicetyl phosphate) have been shown to decrease the *in vivo* circulation time of liposomes with an associated increase in RES uptake essentially due to pinocytosis (Allen et al., 1988; Straubinger et al., 1983; Massing and Faxius, 2000). Similar studies have also been demonstrated with cationic vesicles (Allen et al., 1989). In addition, the nature of the surface of liposomes can influence liposome-cell interactions with, for example, the ability of cationic liposomes to promote DNA delivery and cell transfection being ascribed to the fusion of the cationic liposomes with the anionic cell membrane (e.g. Felgner et al., 1994). Therefore it is clear that since these colloidal systems can be stabilized electrostatically or sterically (by covering the surface of the liposome with a hydrophilic coating such as polyethylene glycol) determination of surface charge will provide valuable insight into not only the

surface interaction of liposomes and bilayers, but also into their stability and to a degree their pharmacokinetic and fusogenic profiles (Chrai, et al., 2002).

The encapsulation of neutral drug molecules into the liposomal bilayer have also been reported to causes changes in the liposome surface structure and charge (Fatouros and Antimisiaris, 2002). Addition of griseofluvin has been shown to result in alteration of the phosphatidylcholine head groups at the surface of liposomes whereby the orientation of choline and phosphate group is affected. Subsequently this re-arrangement of the lipid structure produced a change in the surface characteristics, with the liposomes becoming negatively charged when the choline group plane lies below the phosphate group plane and become positively charged in the opposite case (Fatouros and Antimisiaris, 2002).

1.7.3 Liposome bilayer composition

Again similar to the surface characteristics, the liposome bilayer composition has been shown to influence not only the storage characteristics of the formulations but also the *in vivo* performance of the system. In particular the phase transition temperature of the lipid bilayers has a wider impact on drug retention (both in the case of hydrophilic and hydrophobic drugs) within liposomal formulations (e.g. Senior and Gregoriadis, 1982; Mohammed et al., 2004 respectively). As mentioned in Section 1.6.3.1 the transition temperature of the lipids with respect to surrounding ambient temperature will dictate the physical state of the lipids with lipids being in the ordered gel state held at a temperature below that of their the transition temperature. Alternatively, subjecting the liposomes to temperatures

over the transition temperature of the lipids increases the thermal motion of the acyl chain at the junction with the hydrophilic head thereby causing a tilt in the orientation of the lipids causing the lipids to assume the fluid state. As a result, liposomes composed of low transition lipids are more susceptible to drug leakage than are liposomes made of higher transition lipids. However, liposome fluidity can also influence its interaction with biological factors and cells with liposomes made from higher transition temperature lipids (e.g. DSPC) have shown lower optimisation (Gregoriadis and Senior 1980) and uptake by the RES (Gabizon and Papahadjopoulos, 1988).

Early developmental studies of liposomal drug delivery (Gregoriadis and Davis, 1979) also demonstrated that inclusion of cholesterol within a liposome formulation to an optimum of 50% mol/mol (1:1 lipid molar ratio) increased the stability and reduced the permeability of liposomal bilayers with respect to water soluble drugs and it has subsequently been shown that the inclusion of cholesterol within liposomal bilayers increases the packing densities of phospholipid molecules (Semple et al., 1996). This condensation of the membrane is thought to result from the accommodation of cholesterol in the molecular cavities formed by surfactant monomers assembled into vesicles (Devaraj et al., 2002). The ability to modify the characteristics of liposome bilayers has also been shown to be an important factor in the biodistribution of the liposomes *in vivo* with increased circulation time and a decrease in the RES uptake being reported with cholesterol containing liposomes (Kirby and Gregoriadis, 1980).

1.7.6 Liposomal drug delivery system: advantages

Liposomes have now been used for more than 20 years for the delivery of a variety of agents including chemotherapeutic agents, imaging agents, antigens, immunomodulators, chelating agents, haemoglobin and cofactors, lipids and genetic material (Storm and Crommelin, 1998; Manosroi et al., 2004). Increasingly liposomes are now also being looked at as potential systems for increasing the performance of poorly soluble drugs. In this role they offer a variety of assets over and above their ability to enhance solubility.

Reduced toxicity: Whilst a range of solubility enhancing agents have already been mentioned, many of these systems have a range of related toxicity issues. For example the drug of choice for the systemic treatment of fungal infections is amphotericin B which is generally formulated into detergent micelles due to its poor aqueous solubility. However the micelles have been demonstrated to be unstable when administered systemically which can resulted in severe neuro and nephrotoxicity. Liposomal administration of amphotericin has been found to be advantageous both in terms of stability and reduced side effects (Lasic, 1998). Alternatively, drug precipitation has been shown to occur after addition of co-solvent systems to blood (Flynn, 1984) and dissociation of drug-cyclodextrin complexes can occur on dilution by plasma and extracellular fluids (Mesens and Putteman, 1995). Similarly solubilising agents such as that used in the commercially available intravenous dosage form of cyclosporin A are also limited by a broad toxicity profile which includes nephrotoxicity and hepatotoxicity (Thiel et al., 1986; Tibell et al., 1993). Similarly taxol, an insoluble drug is marketed as a cremophor/ethanol suspension and is administered by slow infusion due to

cremophor toxicity. Liposomal incorporation of taxol helped overcome the toxicity, allowing liposomal taxol to be administered as a bolus intravenous injection for cancer chemotherapy.

Altered pharmacokinetic properties: Liposome biodistribution can be controlled by modification of their physico-chemical characteristics (see section 1.7). For example, preliminary *in vivo* investigations demonstrated that unlike the micellar system tested, liposomes were able to modify the pharmacokinetic and tissue distribution patterns of the drug and improve drug delivery to the desired site of action (Lee et al., 1999). Liposome formulations incorporating pilocarpine hydrochloride have also been shown to increase bioavailability and delivery of drug to the intended site of action (site specific delivery) when compared to the solution form of the drug. The increase in drug availability was attributed to the effective adsorption of the liposomes to the corneal epithelium when compared to the free drug for the treatment of glaucoma (Monem et al., 2000).

Duration of action: The use of liposome formulation can also significantly control the release of drug molecules: topical delivery of a lipophilic model drug oestradiol has shown that administration of the drug in the form of liposomes enhanced and controlled the release of the drug to the skin epidermis in comparison to the saturated solution of oestradiol or solution of the drug with the lipids (El Maghraby et al., 2000). Similarly cytosine arabinoside, which is cleared rapidly *in vivo*, can be administered in liposomal formulations for a sustained and controlled release over a longer period of time to treat leukemia (Allen et al., 1992).

Protection: Encapsulation of drugs within liposomes can also offer protection against drug degradation, for example liposomal administration of derivatives of Mitomycin C exhibited increased stability with the liposome system preventing chemical and enzymatic attack *in vivo* (Tokunaga et al., 1988).

1.7.7 Limitations of liposomes

Despite the wealth of conducted research and sustained interest in liposomes as a drug delivery system, only a few liposome based formulations are available on the market. This has been associated with a variety of issues which include short circulation times, delays in developing appropriate sterilization techniques, poor long-term stability, low drug encapsulation, particle size control and batch reproducibility (Sharma and Sharma, 1997). The problem of short half-lives has been addressed successfully with the control of particle size and the addition of various polymers including PEG (Allen et al., 1989). This has resulted in a prolonged and sustained release of the encapsulated moiety. Sterilization has been achieved either by sterile filtration in some cases or by the whole manufacture of large batches in a sterile unit (Massing and Fuxius, 2000). Stability issues comprise both physical and chemical stability; physical instability includes drug leakage and particle aggregation whereas oxidation of unsaturated alkyl chains limits chemical stability. Physical stability has been improved using lyophilisation techniques and addition of charged lipids to the formulation and chemical stability has been overcome by the use of saturated lipids or addition of antioxidants. Another issue related to stability is the knowledge of the various parameters, which interact with each other in the multi component system. The interaction of the drug candidate

with the lipids and the interaction of the lipids with the buffering environment also need to be addressed. A typical example is the destruction of vindesine in the liposomal formulations at the physiological pH (Massing and Faxius, 2000). Liposomal formulations also need to exhibit decent standards of drug loading to elicit the desired therapeutic response. The development of active loading whereby variations in pH are exploited to enhance entrapment of hydrophilic molecules has proven to be successful as in the case of current marketed formulations of doxorubicin (Massing and Fuxius, 2000). However to enhance the encapsulation of the hydrophobic drugs, either a new method for drug encapsulation needs to be developed or a thorough investigation into the various physicochemical properties of both the drugs and the lipids used in the formulation needs to be deciphered.

1.7.8 Liposomes : *in vivo* behaviour

Early investigations into the stability of the liposomes in the blood plasma have shown that cholesterol free unilamellar liposomes lose some of the lipid from the formulation to the high-density lipoproteins (Krupp et al., 1976). However Gregoriadis and Davis, (1979) demonstrated that small multilamellar vesicles enriched with cholesterol reduced any lipid leakage in the presence of serum, plasma and whole blood. Studies investigating the *in vivo* behaviour of liposomes after intravenous administration have been carried out and the formulation behaviour and the short comings in targeting can be deciphered: small liposomes (approx 100nm) can easily avoid any uptake by the RES whereas larger liposomes suffered an increase in RES uptake (Allen et al., 1989) however, to overcome this drawback, the liposomal surface can be modified.

Indeed, liposome activity *in vivo* is largely affected by modifying the surface characteristics of the formulation with liposomes with a large hydrophobic surface area being phagocytosed directly whereas hydrophilic surfaces adsorbed opsonins which render the surface lipophilic and recognisable by the MPS cells (Senior, 1987). Whilst the role of hydrophobicity and MPS uptake is still unclear (Litzinger and Huang, 1992) coating of the liposomal surfaces with hydrophilic polymers, such as PEG, has been shown to increase circulation times of liposomes potentially by providing a steric stabilization barrier, which escapes the uptake by the MPS system (Harper et al., 1991). A range of polyethylene glycol (PEG) derivatives have been proposed as a good approach to render a liposome surface hydrophilic with the inclusion of PEG polymers into the liposomal bilayer promoting reduced drug leakage (Blume and Cevc, 1990), prolonged circulation time half life (Allen and Hansen, 1991), and reduced MPS uptake (Woodle et al., 1992). For example, following an intra venous and intra peritoneal injection of the PEG coated liposomes, the liver and spleen uptake of the liposomes was reduced by 15% and 10% respectively (Allen et al., 1991). Target specific ligand can also be attached to form "stealth liposomes" for targeted uptake by specific tissues (Sharma and Sharma, 1997).

Another approach applied to avoid the MPS uptake was the incorporation of ganglosides in combination with cholesterol. Allen et al., (1989) examined the influence of the inclusion of the monosialoganglioside GM_1 into liposomal formulations. The liposomes exhibited a longer circulation half life in mice, decrease in the uptake by the MPS and a reduction in mass transfer from the liposomal formulation to the high density lipoproteins. However the high cost of

the ganglioside, and the inability to obtain them in large quantities have restricted their further application (Allen and Hansen, 1991).

1.7.9 Application of Liposomes in disease conditions

Liposomes have been widely employed in the treatment of parasitic, fungal and bacterial infections (Juliano, 1989; Ostro and Cullis, 1989). Antimonial drugs for the treatment of Leishmania species, which are intracellular parasites, have been used. However, the application of these drugs is associated with hepatic, gastro intestinal and cardiac toxicity (Webster, 1985). Fortunately due to the passive targeting of liposomes to the MPS, liposomes can target these drugs to the appropriate region and have been used to overcome the toxicity profiles with liposome encapsulated the antimonial drug have shown to provide increased efficacy with a substantial reduction in toxicity (New et al., 1978).

Liposomes have also been used to deliver anti fungal agents such as Amphotericin B. Amphotericin B is the most effective treatment for systemic fungal infections. However its use is limited by a wide range of side effects which include fever, nausea, nephrotoxicity and central nervous system side effects (Lopez-Berestein, 1988). To overcome the limitations, Amphotericin B was encapsulated into liposomal formulations which resulted in a reduction in renal and other associated toxicities of amphotericin (Mehta et al., 1984). This prompted the development of the formulation for clinical trials. These studies showed that out of the 46 patients who received the formulation, 24 were completely cured and 4 had partial responses (Lopez-Berestein, 1989). Similar results were also observed for

liposome encapsulated streptomycin which resulted in elimination of all detectable bacteria from Brucella *canis* infected mice (Fountain et al., 1985).

Liposomes have also been investigated for the delivery of anti cancer drugs. The ability of liposome vesicles to penetrate the leaky vasculature and deliver the agents at the site of action has been exploited (Jain, 1987; Jain and Gerlowski, 1986). Preliminary studies involving the delivery of liposome encapsulated ^{67}gallium citrate have shown that the tumour uptake was nearly 10-13% of the administered dose (Ogihara et al., 1986) however the major breakthrough in this area was with the delivery of liposome entrapped doxorubicin. Doxorubicin application is limited by cardio toxicity, oral ulceration, acute nausea and vomiting (Phillips et al., 1975, Lum et al., 1985). However encapsulation of doxrubicin into liposomal formulations resulted in a decrease in toxicity while preserving the anti tumour activity of the drug (Rahman et al., 1986, 1990; Mayhew et al., 1983) and liposomal formulations of this drug are now commercially used (Caeylx®/ Doxil®).

The observation that liposomes could permeate leaky vasculature in both tumours and inflammatory regions has prompted research into its application for treating arthritic conditions. Inflammation in arthritis is characterized by hyper permeability and the presence of phagocytic cells (Zvaifler, 1973). Love et al., 1989 investigated the biodistribution of ^{99}Technetium encapsulated in negatively charged SUV. The results indicated that 10 times more liposomes accumulated in the inflamed paws than the control rats. Williams et al., (1986), also reported similar

results demonstrating the ability of liposomes to target and deliver drugs to areas of inflammation.

1.7.10 Liposome success story

The liposome story dates back to 1960s with the discovery of multilamellar vesicles, and the exhaustive investigations into the liposomal characteristics has led to the development of a significant list of products. The first liposomal formulation to be approved by the US FDA was the anticancer drug doxorubicin in 1994 with the trade name of Doxil (marketed as Caelyx in the UK). The therapeutic index of the drug doxorubicin when administered in the absence of a liposome formulation is very low and demonstration of the efficacy of liposome delivered doxorubicin, which provided a 500 fold increase in the AUC of the liposomal formulation when compared to the free drug (Massing and Faxius, 2000) led to its approval in the USA and subsequently all commercial markets. Another prominent anthracycline glycoside to be later approved was daunorubicin for the treatment of Kaposi's sarcoma in AIDS. Then followed a series of compounds that were formulated successfully into liposomes, overcoming their associated drawbacks. Some of these liposomal formulations which have been approved and some under clinical trials are shown in Table 1.4.

Product	Drug	Formulation	Target
Abelect™	Amphotericin B	Conventional Liposomes	Systemic fungal infection
Allovectin-7	HLA-B7plasmid	DNA-lipid complex	Metastatic renal cancer
AmBisome™	Amphotericin B	Liposomes	Systemic fungal infection
Amphocil™	Amphotericin B	Lipid complex	Systemic fungal infection
Amphotec™	Amphotericin B	Lipid complex	Systemic fungal infection
Annamycin	Annamycin	Liposomes	Breast cancer
Atragen™	Tretinoin	Liposomes	Kaposi's sarcoma in AIDS
DaunoXome™	Daunorubicin	Long circulating liposomes	Kaposi's sarcoma in AIDS
Doxil™	Doxorubicin	Liposomes	Breast and prostrate cancer
Nyotran™	Nystatin	Liposomes	Candidiasis
TLC-99	Doxorubicin	Liposomes	Metastatic breast cancer
Ventus™	Prostaglandin E1	Liposomes	Acute respiratory distress syndrome

Table 1.4 Liposomal formulations currently on market

1.8 Aims and Objectives of the work: Incorporation of hydrophobic drugs into liposomes

Due to their biphasic character, liposomes can act as carriers for both lipophilic and hydrophilic drugs. The solubility and partitioning behaviour of a drug molecule governs its location in the liposome structure (Gulati et al., 1998). Highly hydrophilic drugs (logP<1.7) are located exclusively in the aqueous compartment of the liposomes, eg: arabinosylcytosine (Tokunaga et al., 1988). Highly lipophilic drugs with $logP_{oct}$ >5 are entrapped in the lipid bilayer almost completely, eg: cyclosporin (Vadiei et al., 1989). Drugs with intermediate partition coefficients with logP between 1.7 and 4.0 pose a problem because they partition easily between the lipid and aqueous phases and are lost very easily from liposomes (Gulati et al., 1998). Despite the enormous amount of work which has investigated and documented the application of liposomes as carrier systems for the delivery of hydrophilic drugs, their application as solubilising agents for the lipophilic drug molecules has received limited attention. Most of the liposome research involving encapsulation of drug molecules was focused around the role of cholesterol in drug retention (Wang and.Quinn, 2002; Morilla, et al., 2002; Moribe, et al., 1999), stability studies and physico-chemical characterization with little emphasis on the other factors (e.g. pH and salt concentration of hydration medium, alkyl chain length, charged lipids and influence of drug loading on stability) that could control and optimize entrapment and stability. The influence of a variety of drug candidates was investigated including molecular weight, log P, packaging and molecular charge. Although previous research has shown that the poor water solubility of drugs such as cyclosporin A could be improved by forming lipid complexes and introducing single chain lipids (Leigh et al., 2001) and introducing

acyl chain lengths on the drug molecule (Mayhew et al., 1996) a through appreciation of the factors dictating bilayer drug loading and release is required if these systems are to be fully exploited as solubility enhancing agents.The aim of this work is to investigate the various parameters that could control the encapsulation of lipophilic drugs and investigate the influence of the physical properties of the poorly water-soluble drugs on bilayer loading. The drugs investigated were chosen from three different classes of compounds:

Acidic drugs: ibuprofen, flurbiprofen, indomethacin, sulindac, mefenamic acid

Basic drugs: lignocaine

Neutral drugs: progesterone.

A systematic approach was followed. Initial experiments were done with ibuprofen to optimize the formulation techniques. The various parameters investigated were as follows

1) Influence of drug properties (molecular weight, log P, structure, pKa, pH, nature of the drug) on bilayer loading.
2) Influence of bilayer characteristics on drug encapsulation (lipid chain length, lipid unsaturation, antioxidants, positively charged lipids, negatively charged lipids, pH).
3) Freeze drying of the formulation using sugar (trehalose) and a range of amino acids (arginine, lysine and histidine) as cryoprotectants. Duration of freezing, drying, effect of cryoprotectant concentration was investigated.

4) Environmental scanning electron microscopic studies of the liposomal formulations studied using wet samples. The role of hydration medium concentration, drug loading and cryoprotection was deciphered.

Chapter 2

2.0 Materials and methods

2.1 Materials

2.1.1 Chemicals

Stearylamine (SA), dicetylphosphate (DCP), cholesterol (Chol), sephadex G-50, indomethacin ^{14}C, sodium hydroxide pellets, phosphate buffered saline (PBS) tablets, arginine, histidine, lysine, sucrose, trehalose, α-tocopherol, flurbiprofen, sulindac, lignocaine, progesterone, mefenamic acid and ibuprofen were obtained from Sigma-Aldrich Company Ltd, Poole, UK. Acetonitrile (HPLC grade), chloroform and Optiphase 'Hisafe'3 were purchased from Fisher Scientific, U.K. All the chemicals used were of analytical grade.

2.1.2 Lipids

Egg phosphatidylcholine (PC), dimyristoyl phosphatidylcholine (DMPC), distearoyl phosphatidylcholine (DSPC) were purchased from Lipid Products, Epsom, Surrey, U.K.

Dilignoceroyl phosphatidylcholine (C_{24}PC), 1,2- dioleoyl PC (18:1 *cis*; DOPC), 1,2 dielaidoyl PC (18:1 *trans*; DEPC) and 1,2 dilinoleoyl PC (18:2- 9 *cis*, 12 *cis*; DLPC) was purchased from Avanti Polar lipids, Alabaster, USA.

2.2 Methods

2.2.1 Preparation of multilamellar vesicles

Multilamellar vesicles (MLV) were prepared based on the established hand shaking method (Bangham, 1965). Briefly, the lipid components were dissolved in a 9:1 solvent mixture of chloroform and methanol with the required amount of the drug. A mixture of chloroform and methanol was used to obtain complete solubility of the compounds, which are sparingly soluble in either solvent. The solvent was evaporated on a rotary evaporator (Buchi rotavapor-R) to obtain a dry film which was flushed under a stream of nitrogen for three minutes to maximise complete removal of solvents. The film was hydrated with phosphate buffered saline (PBS; pH 7.4) by vortexing for 3 min and the formed vesicles were retained for a further 30 min at room temperature to complete the hydration process. Hydration of the dried lipid film was carried out above the lipid transition temperature (T_c) of cholesterol and the lipid so that the lipid is in the liquid crystalline phase for MLV formation. Drug free liposomes were prepared by the same method but with omission of the drug from the lipid mixture.

To establish the effect of charged lipids, the liposomes were prepared as above together with the addition of varying amounts of the charged lipid to the neutral formulation. 1-4 μmoles of the cationic lipid stearylamine or anionic lipid dicetyl phosphate was added. All the lipids were previously dissolved in 9:1 chloroform: methanol mixtures. A similar protocol was also used to study the effect of single chain surfactant alpha-tocopherol by replacing the charged lipids with various amounts (0.5 - 3μmoles) of α-tocopherol.

2.2.2 Preparation of small unilamellar vesicles (SUV)

The suspension of MLV (1 ml) was sonicated (Soniprep 150 sonicator) at $>T_c$ (with frequent intervals of rest) using a titanium probe (exponential micro-probe end diameter 3 mm) slightly immersed into the emulsion. This converted the milky suspension to a translucent to clear suspension of small unilamellar vesicles (SUV) of around 150 nm in diameter. All the samples were sonicated for 8 min at a power rating of 6 units. Sonication was performed such that the suspension was agitated vigorously. After sonication, the preparation was left to stand at the corresponding T_c for 30 min.

2.2.3 Determination of drug entrapment efficiency in MLV

The drug loading of liposomes was determined by assaying for non-incorporated drug present in the hydration medium and wash media after separation of liposomes by centrifugation (Beckman J2 Centrifuge) at 27200 g for 30 min. The supernatant containing free drug was analysed by UV spectroscopy (Unicam Helios). The wavelengths for the drugs studied are as follows:

Ibuprofen: 221 nm (Paavola, et al., 2000) (appendix 1a, 1b)

Flurbiprofen: 248 nm (appendix 1c)

Sulindac: 324 nm (appendix 1d)

Indomethacin: 320 nm (appendix 1e)

Lignocaine: 262 nm (appendix 1f)

Progesterone: 248 nm (appendix 1g)

Mefenamic acid: 284 nm (appendix 1h)

All the wavelengths were initially confirmed by running a UV scan mode (Unicam Helios) for absorption peaks.

Drug entrapment efficiency was therefore calculated as follows:

Amount encapsulated (A) = Initial amount of drug added – drug remaining in supernatant after centrifugation

% Encapsulated = (A / total amount added initially) * 100

2.2.4 Determination of encapsulation efficiency of ibuprofen using HPLC

The encapsulation of ibuprofen into the bilayers was also analysed by high performance liquid chromatography (HPLC) using a modified method of Averginos and Hutt (1986) after separating the drug-loaded pellet from the supernatant as described in 2.2.3. The pellet was dissolved in methanol and analysed at 222 nm wavelength. The isocratic mobile phase was phosphate buffer: acetonitrile (70:30) at a pH of 7.4. The injection volume was 15 µl at a flow-rate of 1ml/min and a sensitivity of 0.01. The standard curve for the known concentrations of ibuprofen in methanol was linear over a concentration range of 0-30 µg/ml with $R^2 = 0.999$. The elution time for ibuprofen was 6.6 min.

2.2.5 Determination of encapsulation efficiency of indomethacin using radio labelled [^{14}C] indomethacin

MLV were prepared as described in 2.2.1, spiked with radio labeled [^{14}C] indomethacin. The entrapment efficiency was measured after separation of the free

drug from the liposome-encapsulated drug by gel column separation. The gel was prepared by leaving Sephadex G50 (15 g) to swell in distilled water (100 ml) at room temperature with occasional stirring for 3 h. The columns were prepared by plugging moist glass wool at the bottom of the column, and filling with the swollen sephadex gel. The column was then equilibrated with the mobile phase, which was PBS (7.4) for 30 min. 1 ml of the sample was then added dropwise to the center of the column. The samples were collected at regular time intervals and the fractions analysed after dilution with 5 ml of Optiphase. The amount of encapsulated drug was estimated by liquid scintillation counting.

The amount of encapsulated drug was estimated as follows:

$$\% \text{ Incorporation} = \frac{\text{dpm in pellet}}{\text{Total dpm in sample}} \times 100\%$$

All results were confirmed by measuring recovery of –[^{14}C] indomethacin according to the equation:

$$\% \text{ Recovery} = \frac{\text{dpm in pellet + dpm in combined supernatants}}{\text{Total dpm in sample}} \times 100\%$$

Recoveries in all preparations were in the range of 85-98% of ^{14}C indomethacin initially added.

2.2.6 Sizing of MLV and SUV dispersions

MLV were sized by laser diffraction on a Malvern Mastersizer X at 20°C by diluting 20 µl of the dispersion to 4 ml with doubly filtered (0.22 µm pore size) ddH_2O. Results are expressed as volume mean diameter.

The SUV were sized on a Zetaplus, Brookhaven Instruments, U.K. 20 µl of the SUV suspension was diluted to 5 ml using ddH_2O and the measurements recorded at 25°C. Each sample was the average of three readings and each reading was a mean of measurements recorded for 3mins.

2.2.7 Surface charge measurements of MLV

Zeta potential was determined by photon correlation spectroscopy using a Zetaplus (Brookhaven Instruments) in 0.001 M PBS at 25°C. 20 µl of the dispersion was diluted to 4 ml and the samples analysed. The reported measurements were the mean values of 10 readings.

2.2.8 Drug release from MLV

The release rate of the encapsulated drug from MLV was determined by incubating drug loaded liposomes in 50 ml PBS at 37°C in a shaking water bath. At intervals of 0.5, 2, 4, 20, 24 h, 10 ml was withdrawn and centrifuged at 27200*g for 30 min. The supernatant was analysed spectrophotometrically and the amount of drug released was assayed by comparison with a calibration curve of the drug in PBS.

2.2.9 Determination of influence of hydration medium pH on encapsulation

The MLV were prepared as described in section 2.2.1. The effect of hydration medium pH on the encapsulation of ibuprofen was investigated at various pH values

above and below the pKa (5.2) of ibuprofen. The pH of the hydration medium for the different formulations was adjusted using 0.01 M HCl.

2.2.10 Partitioning studies

To illustrate the partition of free drug into unloaded liposomes, MLV were prepared as described in 2.2.1 but without the added drug. Free drug (1.25mg) was dissolved in 50ml of PBS and the MLV dispersion added to the medium. The set up was in a shaking water bath at a temperature of 37°C set at 60 shakes per minute. 10 ml of the sample was withdrawn at 0.5, 2, 4, 20, 24 h time periods and centrifuged. The supernatant was then analysed spectrophotometrically as described in 2.2.3. for the amount of free drug.

2.2.11 Environmental Scanning Electron Microscopy Examination

Liposomes and dried lipid films were analysed using an Environmental Scanning Electron Microscope (ESEM) (Philips Electron Optics). The ESEM sample stub was loaded with liposome formulation previously hydrated in PBS and examined under saturated water vapour conditions. Gradual reduction of pressure in the sample chamber resulted in controlled dehydration of the sample environment. Dynamic formation of liposomes was monitored by controlling hydration of dried lipid films. The effect of hydration medium on the stability of drug-free liposomes under controlled dehydration conditions was investigated using PBS and distilled water respectively. The influence of ibuprofen loading on liposome stability was also analysed using controlled dehydration of samples to define a coalescence pressure. A

working temperature of 5°C was maintained in all experiments. To investigate the influence of temperature, MLV made of DMPC:Chol were heated in the sample chamber from a temperature of 5 to 30°C.

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2.2.12 Freeze drying of Liposomes

MLV were prepared as described in 2.2.1. The required concentration of the cryoprotectants was added and SUV made as described in 2.2.2. The sonicated sample was then freeze-dried using a Virtis Advantage freeze-dryer. The samples were frozen at different temperatures ranging from –30 to -70°C. The drying rate was also monitored using different temperatures cycles with variations in the time periods. The freeze-dried samples were hydrated following the DRV method first outlined by Kirby and Gregoriadis, 1985.

2.2.13 Stability studies of freeze dried liposomes

To investigate the stability offered by the addition of cryoprotectants, freeze dried liposomal formulations were tested for their drug retention for a period of 6 weeks. The freeze dried formulations were stored at two temperatures: 4°C and room temperature. The samples were hydrated using the method of Kirby and Gregoriadis, (1985), as outlined above and drug leakage was studied as described in 2.2.3. Size measurements and zeta potential measurements were carried out as described in 2.2.6 and 2.2.7 respectively.

2.2.14 Statistical analysis

Statistical analysis was carried out by analysing the variance (ANOVA) followed by bonferroni comparison test (Glanz, 1997). Significant differences were judged as $P < 0.05$. Student t test was used where mentioned.

Chapter 3

3.0 Ibuprofen- the rational choice

Development of combinatorial chemistry has made a dramatic impact on drug discovery, making it possible to synthesize thousands of compounds per year. However, many of these small molecule (<500 Da) drugs tend to have limited solubility eg <1.5mg/ml in water and biological milieu. Consequently, after administration these compounds can display poor bioavailability often below the therapeutic threshold (Borman, 1998; Kawakami et al., 2002). Therefore, it is vital for the development of new therapeutic agents, that techniques to enhance their solubility and bioavailability are devised.

As mentioned in chapter 1, the biphasic nature of the liposomal systems enables the encapsulation of both water soluble and insoluble drugs into the formulation. A number of studies have been reported investigating the properties of liposomes best suited for maximum solubilsation (Goundalkar and Meizei, 1983; Walde and Ichikawa, 2001; Elmaghraby et al., 1999). However a systematic appreciation of the dictating factors in the formulation of liposomes as solubilising agents is still lacking. In terms of optimum entrapment values of drug candidates into liposomal formulations, it has been reported to be controlled by both the lipid characteristics and the drug properties (Kulkarni et al., 1995) and in order to achieve an optimum encapsulation, various parameters such as liposome size, charge, bilayer rigidity and counter ion pairing need to be considered. The drug loading mechanisms used to prepare liposomes combined with the bilayer composition and the presence of phospholipids with high transition temperatures, all have an effect on drug loading

and the pharmacological performance of the liposomal systems (Gabizon et al., 1998). Therefore the correct selection of the lipids in the development of the liposomal formulations must be employed to ensure the liposome properties (size, charge, *in vivo* distribution, stability and toxicity) are all appropriate (Gupta et al., 1996).

The aim of the initial set of investigations reported was to strategically investigate the effect of liposome bilayer composition in determining bilayer drug loading. The work involved investigation of the various parameters that could control the encapsulation of poorly water soluble drugs and investigate the influence of the structure of such drugs on bilayer loading. To achieve this aim, initial thorough investigations were carried out using ibuprofen as a model drug candidate. Ibuprofen is characterized by a logP value of 3.6, with solubility in water of approximately 50μg/ml. It is a weak acid with a pKa of 5.2. Indeed ibuprofen was a good choice for a problematic drug in terms of solubilisation since drugs with logP in the range of 3-5 have been classed as problematic for liposomal systems due to the fact that they partition easily between the aqueous and the lipophilic environment of the liposomal system thereby being easily lost from the formulation.

3.1 Quantification of Ibuprofen loading

The quantitative assessment for ibuprofen entrapment into liposomal formulations was studied by UV spectrophotometric analysis at 221nm wavelength (Paavola et al., 2001). A UV scan was performed resulting in two peak picks at 221nm and 247nm. 221nm was chosen for our study in line with the work previously reported (Paavola et al., 2001; Teng et al., 2003). The calibration curve was made in 0.01M PBS (pH 7.4) using concentrations in the range of 1 – 30μg/ml resulting in a R^2 value of 0.99 (Appendix 1a). The amount of encapsulated drug into liposomal systems was analysed after centrifugation and separation of the supernatant from the pellet. The centrifugation speed was also carefully optimised to ensure the formation of a stable pellet. The supernatant was assayed for the free drug and the amount of encapsulated drug into liposomes was estimated after deducting the drug concentration in the supernatant from the total amount added initially.

Amount encapsulated (**A**) = total amount added – amount in supernatant

% Encapsulated = (**A** / total amount added initially) * 100 %

The assay for ibuprofen encapsulation efficiency within liposomes was also validated using HPLC (Fig 3.1). The HPLC assay was performed at 221nm wavelength using phosphate buffer: acetonitrile (70:30) as the mobile phase (pH 7.4). The calibration curve was made with concentration ranges of 6- 30μg/ml of the ibuprofen solution. The R^2 was 0.99 (Appendix 1b). The amount of ibuprofen encapsulated into the liposomal bilayer was estimated after centrifugation and separation of the supernatant from the pellet. The pellet was then dissolved in 2ml

of methanol and to assay for the ibuprofen content entrapped in the formulation. The results show that similar entrapment values of ibuprofen were obtained both from the UV assay as well as the HPLC assay (Table 3.1). Further experiments were carried out using the UV assay, which is a simple, reproducible and easy to use quantitative method.

Formulation	**UV assay (% Encapsulation)**	**HPLC assay (% Encapsulation)**
PC:Chol	39.3 ± 0.9	37.7 ± 2.4

Table 3.1: Validation of encapsulation analysis. The encapsulation of ibuprofen with liposomes composed of phosphatidylcholine (PC) and cholesterol (Chol) (16:4μmoles) was measured using UV assay at a wavelength of 221nm and HPLC analysis on a C18 column. The UV assay measured the amount of free drug in the supernatant after centrifugation of the liposome suspension whereas the HPLC assay was a direct measurement of the entrapped drug within the pelleted liposomes. Values denote mean ± S.D. from at least three experiments.

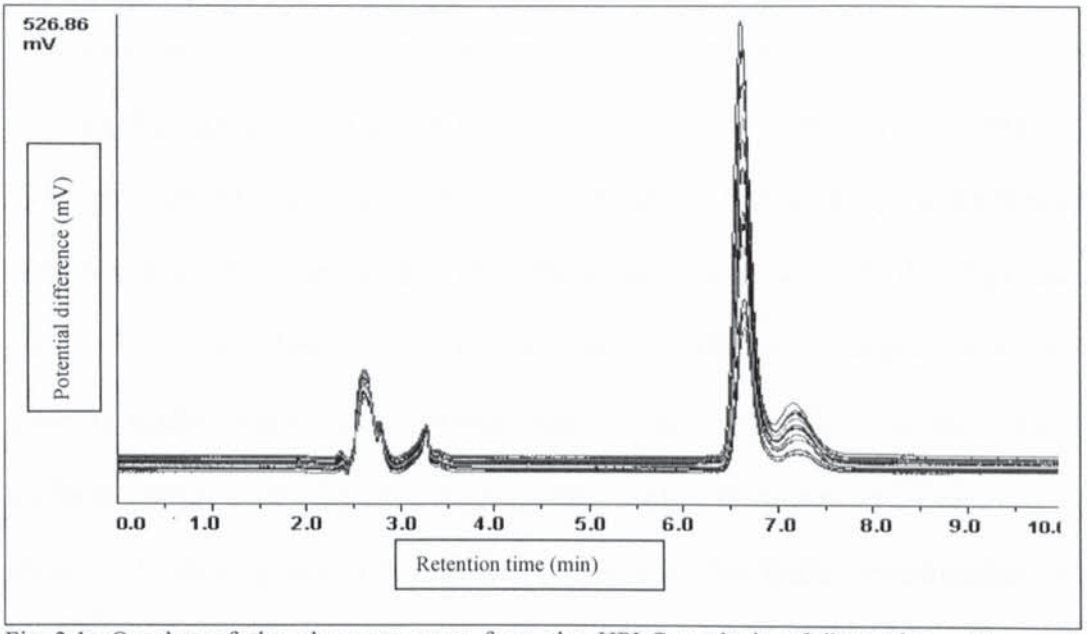


Fig 3.1: Overlay of the chromatograms from the HPLC analysis of ibuprofen. The overlay chromatograms are from the range of concentrations of the ibuprofen solution investigated (15μg/ml – 40 μg/ml). Each concentration was repeated in triplicates and the mean values used for analysis.

3.2 Drug leaching and dilution studies

The effect of repeated leaching from the liposomal formulations to optimise the removal of non-encapsulated ibuprofen was assessed by investigating the loss of any encapsulated drug during subsequent washing. After initial centrifugation, the liposomal pellet was resuspended in 0.01M PBS and subjected to repeated centrifugation processing. The results (Fig 3.2) show that only a small amount of ibuprofen (50 ± 3 μg; 4%) was detected in the supernatant after the second centrifugation with the third wash containing minimal amounts (15 ± 2 μg equivalent to 1.2% of the initial amount of added drug).

This suggests that removal of un-encapsulated ibuprofen from the MLV / ibuprofen suspension mixture can be effectively performed by subjecting the mixture to two centrifugations (total diluent volume of 15ml in each cycle). Repeated washing of liposomes via centrifugation has also been used to measure drug leaching from the vesicles. Leaching studies are an important factor in the industrial development of liposomal formulations. They can give an indication of drug leakage and a measure of bilayer integrity. Gupta et al., (1996) have shown that during the development of the liposomal formulation for a drug ABT-077 developed by Abbott laboratories, leaching studies were used to assess drug retention and bilayer fluidity. Of the fourteen lipid formulations investigated, only formulations consisting of phosphatidylcholine and cholesterol were short listed for further investigation. The criteria for selection included the evaluation of entrapped drug loss from liposomal formulations during centrifugation.

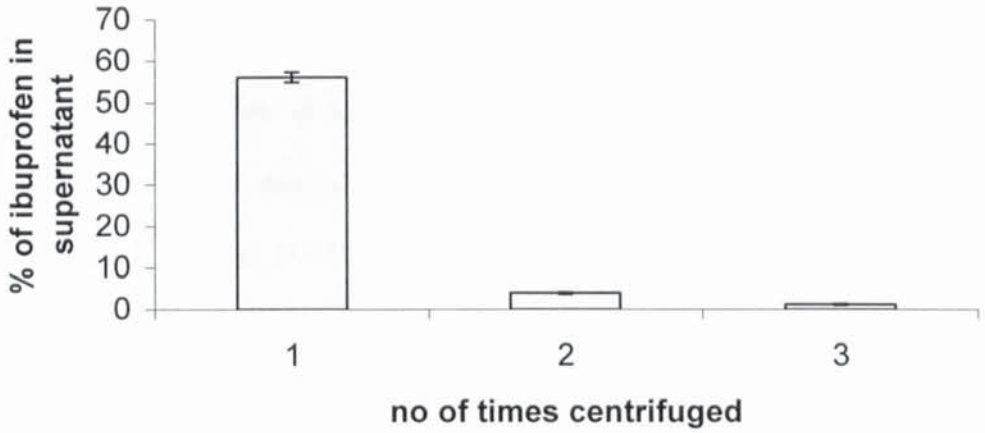


Fig 3.2: The effect of repeated centrifugation on removal of ibuprofen from the liposome/ibuprofen mixture. MLV (PC:Chol; 16:4μmoles) incorporating ibuprofen was prepared in the presence of 1.25mg of ibuprofen as described in section 2.1. The MLV were centrifuged at 23500g for 30min at 4°C. The supernatant was assayed for ibuprofen concentration using UV spectroscopy at 221nm. The results represent mean ± S.D. from at least three experiments.

The effect of liposome concentration during centrifugation was also studied to validate our incorporation studies. The liposomes were prepared as mentioned in 2.1 and were subjected to serial dilutions. The formulation containing 16 μmoles of PC was diluted to half its initial concentration and further dilutions of the same sample resulted in liposomes containing 1μmole of PC. Entrapment values (Fig 3.3) measured after centrifugation of samples containing ibuprofen suggests that the amount of ibuprofen entrapped was not significantly influenced by the dilution of liposomes in PBS. The dilution of liposomal formulations below the saturation solubility of the drug would favour its transfer into the buffer phase (Gupta et al., 1996) and avoid sedimentation during centrifugation of any non-encapsulated ibuprofen which may crystallise out at high concentration. The dilution studies also mimic the sink conditions encountered during an *in vivo* investigation and can give an indication of possible drug retained by the formulation in sink conditions such as those expected *in vivo* (Gupta et al., 1996). With increased dilution of the liposomal suspension, whilst there is no significant difference in incorporation

values, the variations on the mean value clearly increases, suggesting decreased accuracy in the process. This could possibly be attributed to a decrease in the sensitivity of the assay in the highly diluted environment and not the dilution causing a significant release of the encapsulated drug as previously reported (Swarbrick and Boylan, 1994).

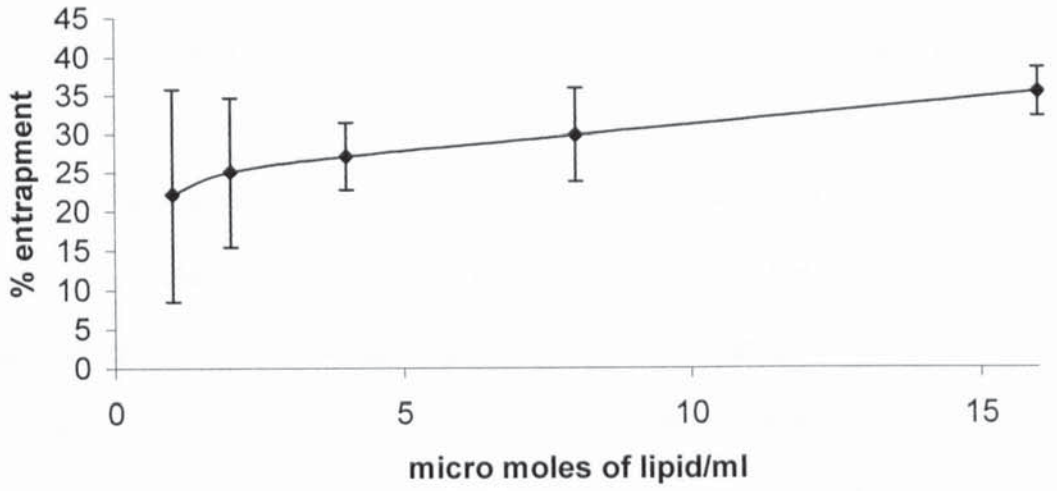


Fig 3.3: The effect of suspension concentration on encapsulation in PC:Chol liposomes (16 μmoles PC and 4 μmoles Cholesterol). MLV incorporating ibuprofen were prepared in the presence of 1.25mg of ibuprofen as described in section 2.2. The MLV suspension was subjected to a series of dilutions ranging from 1μmole of PC to formulations containing 16μmole of PC. The samples were then centrifuged at 23500g for 30min at 4°C. The supernatant was assayed for ibuprofen concentration using UV spectroscopy at 221nm. The results represent mean ± S.D. from at least three experiments.

3.3 Preliminary formulation studies

3.3.1 The effect of liposome composition on ibuprofen entrapment in MLV: Cholesterol Content

The effect of incorporating cholesterol within the lipid composition of PC (16 μmoles) liposome bilayers on ibuprofen entrapment was determined by varying the cholesterol contents from 0-50% mol/mol (0-33% w/w of total lipid; Table 3.2). The presence of cholesterol within PC bilayers was found to have a significant effect on ibuprofen incorporation and increased this incorporation by 9.8% when liposomes contained 4 μmoles of cholesterol compared to PC only liposomes (39.3% vs 29.5% respectively; Table 3.2). Thereafter increases in the cholesterol content to 8 μmoles and 16μmoles reduced drug encapsulation to 23.2% and 17.1% respectively (Table 3.2). The effect of cholesterol content on the size (mean volume diameter) and zeta potential of ibuprofen loaded MLV preparations is shown in Table 3.3. Variation of the cholesterol content of drug free and drug loaded PC liposomes made no significant difference to either the vesicle size or zeta potential (Table 3.3).

Liposome cholesterol content (μmoles)	Ibuprofen entrapment efficiency (%)	Concentration of drug in liposomes (mg ml^{-1})	Ibuprofen drug loading (% w/w)	Concentration of drug in liposomes (% mol/mol)
0	29.5 ± 0.6	0.37 ± 0.01	2.91 ± 0.06	10.1 ± 0.2
4	39.3 ± 0.9*	0.49 ± 0.01	3.33 ± 0.08	10.7 ± 0.2
8	23.2 ± 0.8*	0.29 ± 0.01	1.80 ± 0.06	5.5 ± 0.2
16	17.1 ± 0.2*	0.21 ± 0.01	1.07 ± 0.02	3.1 ± 0.1

Table 3.2: The effect of MLV cholesterol content on encapsulation of ibuprofen in PC:Chol liposomes. MLV were prepared from 16 μmol PC and varying cholesterol content (0-16 μmol) (0-33% w/w ratio). MLV incorporating ibuprofen were prepared in the presence of 1.25mg of ibuprofen as described in section 2.1. Ibuprofen encapsulation efficiency within liposomes was determined as described in section 2.2. * Represents significantly different entrapment values ($P<0.05$). Values denote mean ± S.D. from at least three experiments.

	MLV		MLV incorporating ibuprofen	
Liposome cholesterol content (μmoles)	**Liposome size (μm)**	**Liposome zeta potential (mV)**	**Liposome size (μm)**	**Liposome zeta potential (mV)**
0	4.9 ± 0.5	-5.9 ± 0.4	5.1 ± 0.7	-5.2 ± 0.8
4	4.3 ± 0.5	-6.1 ± 0.1	5.3 ± 0.4	-6.1 ± 0.7
8	4.2 ± 0.4	-6.3 ± 0.8	5.0 ± 0.3	-5.4 ± 0.9
16	4.2 ± 0.5	-5.2 ± 0.9	4.8 ± 0.9	-5.8 ± 0.5

Table 3.3: The effect of MLV cholesterol content and ibuprofen encapsulation PC:Chol liposomes on liposome size and zeta potential. MLV were prepared from 16 μmol PC and varying cholesterol content (0-16 μmol). MLV incorporating ibuprofen were prepared in the presence of 1.25mg of ibuprofen as described in section 2.1. Liposomes size (volume mean diameter) was determined by laser diffraction spectroscopy using a Malvern Mastersizer X at 20°C. The zeta potential of the MLV was measured in 0.001M PBS at 25°C using a Brookhaven Zetasizer. Values denote mean ± S.D. from at least three experiments.

The beneficial role of cholesterol within liposomal drug carriers is well recognised. Early developmental studies of liposomal drug delivery (Gregoriadis and Davis, 1979) demonstrated that inclusion of cholesterol within a liposome formulation to an optimum of 50% mol/mol (1:1 lipid molar ratio) increased the stability and reduced the permeability of liposomal bilayers. At mole fractions between 0.2 and 0.5, depending on the nature of the phospholipids, cholesterol can dissolve within lipid bilayers whereas at higher concentrations cholesterol can form crystal habits (Epand et al., 2003). The inclusion of cholesterol within liposomal bilayers has been shown to result in increased packing densities of phospholipid molecules (Semple et al., 1996). This condensation of the membrane is thought to result from the accommodation of cholesterol in the molecular cavities formed by surfactant monomers assembled into vesicles (Devaraj et al., 2002) as evidenced by surface pressure measurements of monolayer mixtures of surfactants and cholesterol (Rogerson et al., 1987). These studies demonstrated a decrease in effective area per

molecule as the cholesterol content of the monolayer is increased. This space filling action combined with the ability of cholesterol to complex with phospholipids can reduce bilayer permeability to small hydrophilic solutes and ions (Demel et al., 1972; Paphadjopoulos et al., 1973) thereby improving entrapment and retention of hydrophilic drugs (Papahadjopoulos et al., 1973; Senior & Gregoriadis, 1982; Kirby et al., 1980). Biophysical studies (Bernsdorff et al., 1997) of phospholipids-cholesterol bilayers have also shown the addition of 30-50 mol% cholesterol to phosphatidylcholine liposomes can adjust the structural and dynamic properties of membranes and increase the hydrophobicity in the interfacial region of the liposome bilayer, a factor which could influence the incorporation of drugs within the lipid bilayer. The results in Table 3.2 suggest that in the case of the poorly water soluble drug ibuprofen, optimum drug loading occurred in PC liposomes containing 4 μmoles cholesterol content (20% mol/mol). This may be a result of two conflicting factors; on the one hand the increased hydrophobicity (Bernsdorff et al., 1997), increased stability (Gregoriadis and Davis, 1979), decreased permeability (Kirby et al., 1980) of the bilayer with increasing cholesterol content may efficiently trap ibuprofen within the bilayer as the liposomes form. Counteracting this, higher amounts of cholesterol may compete with ibuprofen for packing space within the bilayer therefore excluding the drug as the vesicles assemble and reducing ibuprofen incorporation.

The thermotropic phase behaviour was also characterised using differential scanning calorimetry. Both ibuprofen loaded, and empty MLV were studied. Liposomes made from DMPC only, DSPC only, DMPC:Chol (4:1 molar ratio), DSPC:Chol (4:1 molar ratio) and DSPC:chol:ibuprofen (4:1 molar ratio; 1.25mg

ibuprofen) were analysed. Initial studies involved optimising the heating conditions. Various scan rates of 10°C/min, 7.5°C/min, 5°C/min and 2.5°C/min were attempted and the optimum conditions of 2.5°C/min were identified.

The DSC scans for DSPC liposomes showed a transition peak at an average temperature of 52.3°C (Fig 3.4: Fig 3.5). The addition of 4μmoles of cholesterol to the formulation resulted in the appearance of another peak at a temperature of 42.2°C (Fig 3.6). Further, the addition of ibuprofen to DSPC:Chol formulation resulted in even further lowering of the peak temperature to 26.6°C.

The variations in the transition temperatures of the formulations tested show that the addition of either cholesterol on its own or in combination with the drug has a dramatic influence on the phase behaviour of the liposomes. The reduction in the transition temperature of the pure lipid due to the addition of cholesterol to the mix has previously been attributed to the incorporation of cholesterol molecules into the bilayer matrix (Fang et al., 2003). Further, it has also been previously shown that poorly soluble drugs interact with the liposome bilayer depressing their transitional temperatures. Similar to the effect of cholesterol, this could be due to the "fluidifying" effect caused due to the introduction of the drug in the ordered bilayer of the formulation. The drug molecules act as spacers and disrupt the lipid mosaic resulting in the decrease of gel to liquid phase transition temperature (Castelli et al., 1998). The appearance of smaller peaks before the main transition peak is due to the transformation of the hydrated bilayer from stable lamellar to hexagonal ripple phase. The larger peak represents the main gel to liquid phase transition (Fang et al., 2003). Therefore, our current finding strongly indicates that the added

ibuprofen incorporates itself into the bilayer matrix thereby affecting the transition temperature of the lipids.

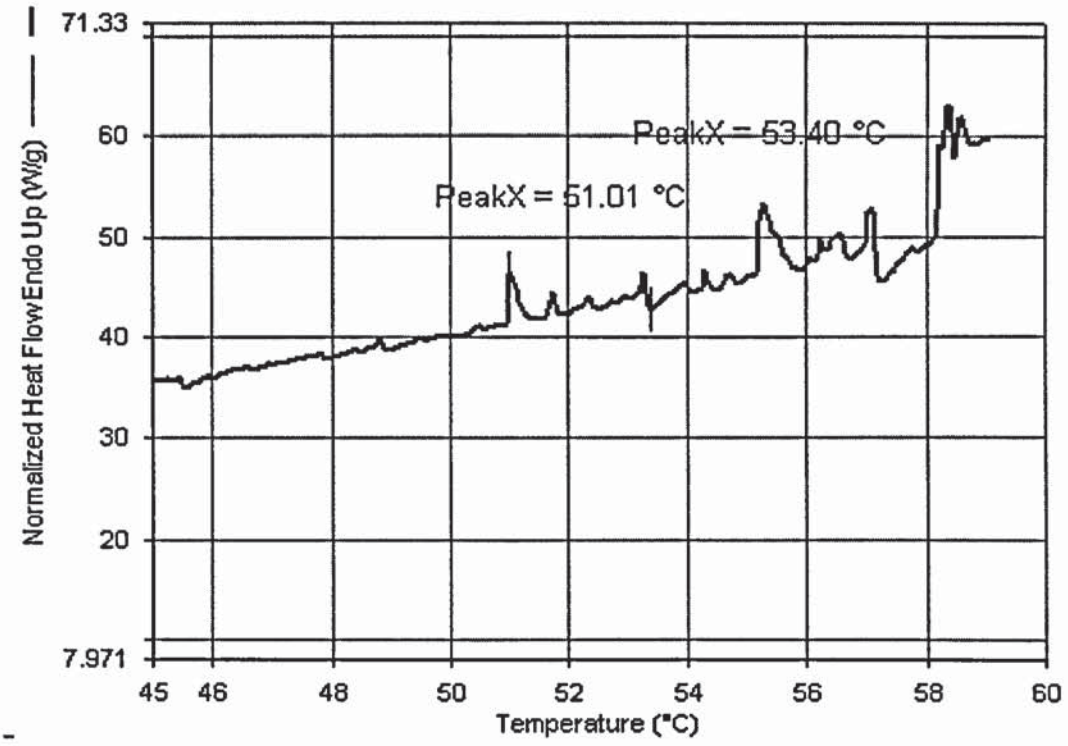


Fig 3.4: The differential scanning traces obtained when liposomes were heated from 45°C to 60°C. The liposomes were made using 16μmoles of DSPC. The scan rate was 2.5°C/min.

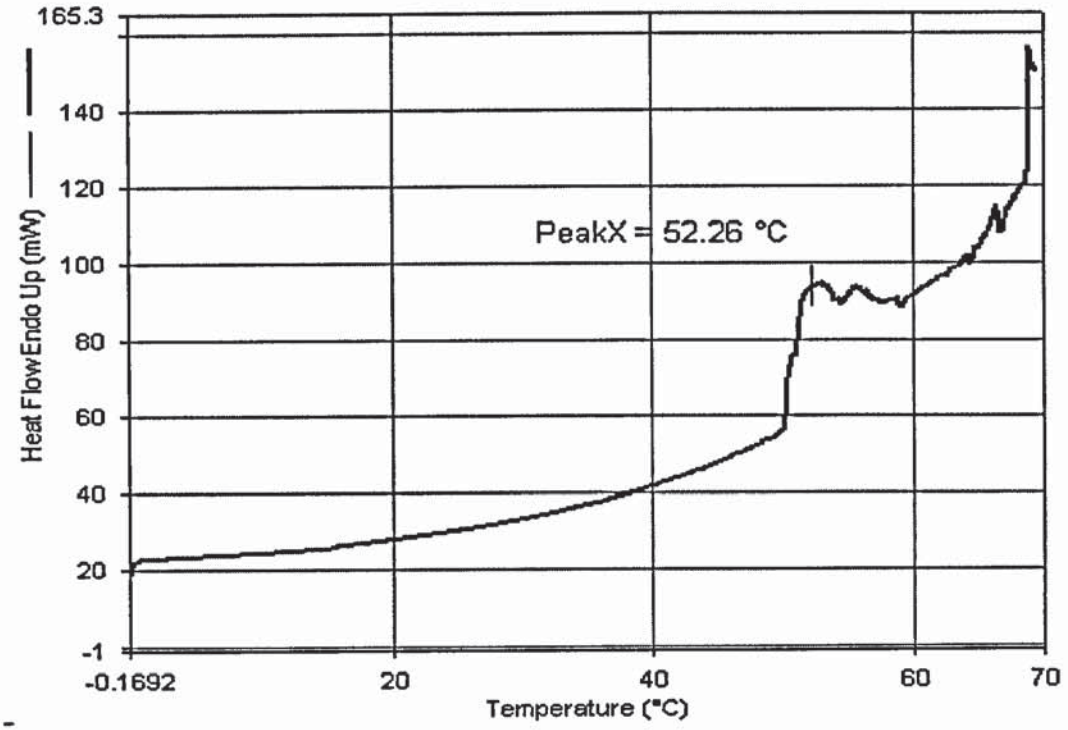


Fig 3.5: The differential scanning traces obtained when liposomes were heated from 0°C to 70°C. The liposomes were made using 16μmoles of DSPC. The scan rate was 2.5°C/min.

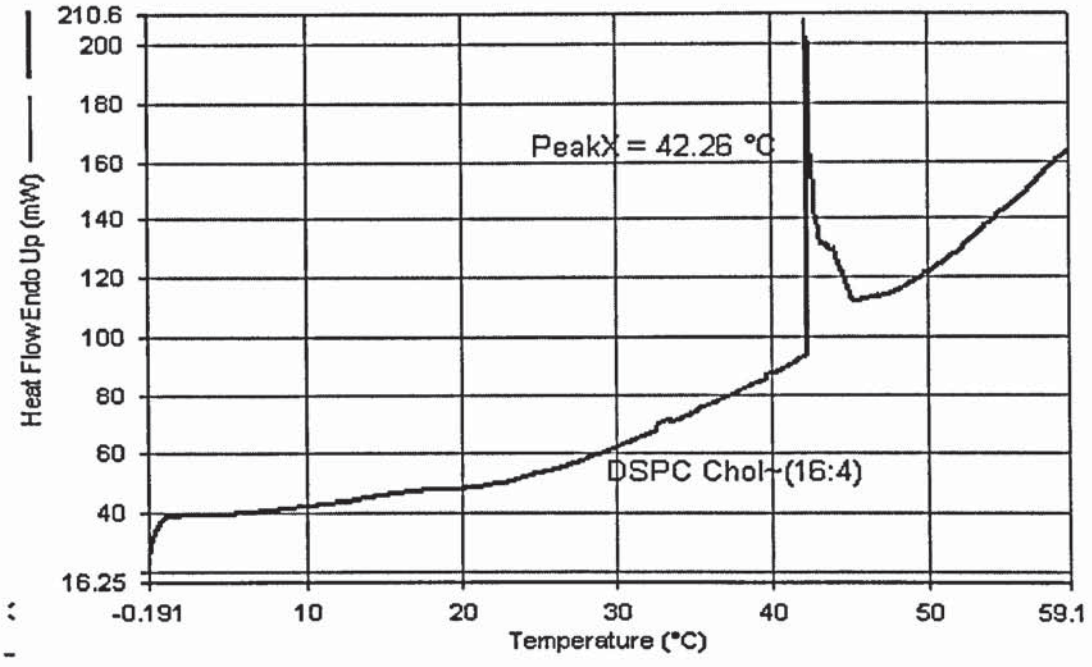


Fig 3.6: The differential scanning thermograms obtained when liposomes were heated from 0°C to 60°C. The liposomes were made using 16μmoles of DSPC and 4μmoles of Cholesterol. The scan rate was 2.5°C/min.

3.3.2 Drug loading

To investigate the influence of drug:lipid ratio during liposome preparation on the encapsulation efficiency, liposomes made from neutral lipids (PC:Chol; 16:4μmoles) and cationic lipids (PC:Chol:SA; 16:4:2μmoles) were tested. The drug loading in neutral and positively charged liposomes was analysed by adding increasing amounts of ibuprofen to the formulation. Fig 3.7 shows the encapsulation efficiency in PC: Chol (16:4μmoles) liposomes with and without 2μmoles of SA. The entrapment statistically ($P<0.05$) increased with the increase in drug concentration with up to 43% (1.75mg of added ibuprofen) in the case of neutral liposomes and up to 44% with 1.5mg ibuprofen with the addition of stearylamine to the above formulation. However, further increase of drug concentration to 2.25mg did not result in the formation of the vesicles (Fig 3.7).

The increase in entrapment with the increase in drug concentration could possibly be attributed to the available space in the bilayer. However the lack of further increase when the concentration of the drug was increased beyond 1.75mg could possibly be attributed to the saturation of the bilayer. Indeed concentration of 2.25mg in the formulation may cause the disruption of the bilayer structure with the liposome bilayer becoming saturated and hindering the formation of vesicle bilayers, which may lead to the formation of microemulsions (Lopez et al., 1998).

Similar to the above studies, maximum drug:lipid loading has been demonstrated when investigating the incorporation of topotecan, an analogue of camptothecin into liposomes composed of DSPC:Chol using an ion gradient method, with Abraham et al (2004) noted that a maximum drug:lipid ratio of 0.2 (wt/wt). When

the concentration of drug exceeded this limit, the drug lipid ratio was either maintained (Abraham et al., 2004) or was noted to reduce below the measured maximum limit as was the case with drug incorporation in liposome formulation (Lopez et al., 1994). Drug loading is also shown to influence the shape of the liposomes with the increased loading of topotecan resulting in the liposomes becoming ellipsoidal in shape compared to the spherical unloaded vesicles (Abraham et al., 2004).

Drug loading also has been shown to influence the release rate *in vitro*. Investigations by Abraham et al (2004) studying the release characteristics in 80% fetal bovine serum at 37°C have shown that drug release from liposomes with a drug lipid ratio of 0.2 was slower when compared to formulations with a loading capacity of 0.1. The decrease in release was attributed to the formation of a more stabilised polymorphic structure of the encapsulated topotecan in the liposomes having a high drug-lipid ratio. Indeed drug loading can also have a major role in the pharmacokinetic distribution of the liposomal systems. Clinical studies have shown that liposomes with high drug loading efficiency and a rigid bilayer demonstrated minimised drug leakage, an important factor in facilitating a desired therapeutic activity (Gabizon et al., 1998).

The progress of liposomal formulations into the clinic should be supported by high entrapment of drug candidates, as well as their retention in the bilayer. For example, formulations with high drug to lipid ratios will tend to reduce the formulation costs as well as any lipid induced toxicity that might occur. Indeed a comparison between the encapsulation efficiency with that of the therapeutic dose

for the drug is one of the key deciding factors for the progress of the formulation (Kulkarni et al., 1995).

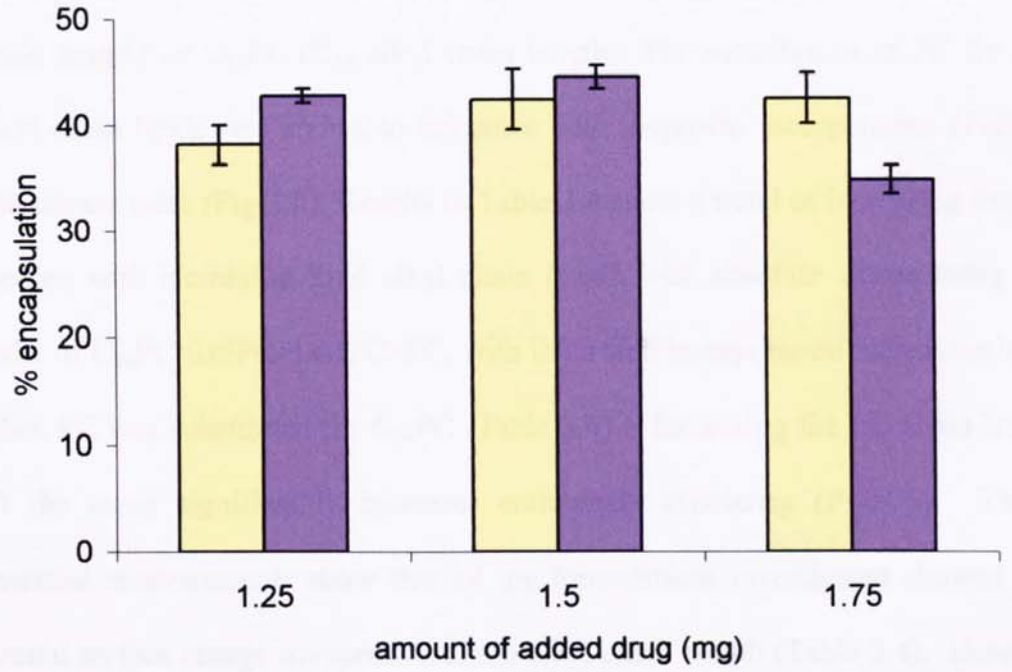


Fig 3.7: The effect of ibuprofen concentration on encapsulation into neutral and positively charged liposomes. MLV were made using PC:Chol (16:4 μmoles; yellow bars) for neutral liposomes and supplemented with 2 μmoles of stearylamine (purple bars) for positively charged liposomes. Ibuprofen was added in increasing amounts varying between 1.25mg to 1.75mg. Encapsulation of ibuprofen was monitored as previously described in 2.2. The results represent mean ± S.D. from at least three experiments.

3.4 The effect of lipid alkyl chain length on liposome encapsulation and release of ibuprofen

To investigate if hydrophobic bilayer volume influenced drug solubilisation, the influence of lipid alkyl chain length on ibuprofen encapsulation was investigated using liposomes prepared from PC, DMPC (C_{14} alkyl chain length), DSPC (C_{18} alkyl chain length) or C_{24}PC (C_{24} alkyl chain length). The substitution of PC for longer alkyl chain lipids was shown to influence both ibuprofen incorporation (Table 3.4) and release rates (Fig 3.8). Results in Table 3.4 show a trend of increasing ibuprofen loading with increasing lipid alkyl chain length with absolute values being in the order of C_{24}PC>DSPC>DMPC>PC, with ibuprofen incorporation increasing by 22% when PC was substituted for C_{24}PC (Table 3.4). Increasing the PC chain length in all the cases significantly increases entrapment efficiency ($P<0.05$). The zeta potential measurements show that all the formulations investigated showed a near neutral surface charge irrespective of the alkyl chain length (Table 3.4). However, a trend of increasing vesicle size was measured with increasing carbon chain length, which could be related to the increased drug loading rather than the formulation

The increase in the entrapment noted in Table 3.4 could possibly be attributed to the increase in the available lipophilic area for the insoluble drug. Previous experiments (Gregoriadis, 1973) have also demonstrated an increase in entrapment of the weakly basic drug Actinomycin D within the lipid phase of the bilayer of liposomes containing dipalmitoyl lecithin (DPPC) compared to liposomes containing egg PC. Studies on solubilisation of Cyclosporin A (Francis et al., 2003), and tetrazepam (Hammad and Muller, 1998) using micelles have similarly shown that solubility is influenced by lipid chain length, with longer alkyl chain surfactants solubilising higher amounts of the added

drug. Presumably, this increased loading capacity could be attributed to the increased hydrophobic area within the longer chain C_{24}PC liposome bilayers, similar to the effects previously demonstrated with micelle formulations (Francis et al., 2003; Hammad and Muller, 1998). Indeed the encapsulation of the antimicrobial agents pefloxacin and ofloxacin into liposomes has similarly been shown to be influenced by the length of the alkyl chain (Puglisi et al., 1995) with longer chain DPPC liposomes displaying higher drug encapsulation when compared to liposomes made from PC. Recent studies encapsulating the anti–tuberculosis drugs rifampicin and isoniazid have also shown that liposomal encapsulation of the insoluble drug rifampicin was influenced by the nature of the alkyl chain length of the lipids (Gursoy et al., 2004). Rifampicin encapsulation was enhanced by approximately 5% when smaller chain egg PC was substituted by longer chain DPPC (Gursoy et al., 2004).

	MLV incorporating Ibuprofen			
Liposome composition	**Ibuprofen entrapment efficiency (%)**	**Concentration of drug in liposomes (% mol/mol)**	**Liposome size (μm)**	**Liposome zeta potential (mV)**
PC:Chol (16:4μmol)	39.3 ± 0.9	10.65 ± 0.2	5.3 ± 0.4	-6.1 ± 0.7
DMPC:Chol (16:4μmol)	42.5 ± 0.8*	11.42 ± 0.2	5.4 ± 0.3	-7.9 ± 1.2
DSPC:Chol (16:4μmol)	43.5 ± 0.7*	11.67 ± 0.2	5.5 ± 0.3	-7.1 ± 1.8
C_{24}PC:Chol (16:4μmol)	61.5 ± 0.9*	15.7 ± 0.2	6.0 ± 0.1	-4.3 ± 1.2

Table 3.4: The effect of lipid alkyl chain length on liposome size, zeta potential and ibuprofen encapsulation. Ibuprofen (1.25mg) was incorporated into MLV of various lipid compositions as shown as described in section 2.1. Ibuprofen encapsulation efficiency within liposomes was determined as previously described in 2.2. Liposomes size (volume mean diameter) was determined by laser diffraction spectroscopy using a Malvern Mastersizer X at 20°C. The zeta potential of the MLV was measured in 0.001M PBS at 25°C using a Brookhaven Zetasizer. * Represents significantly different entrapment values ($P<0.05$). Values denote mean ± S.D. from at least three experiments.

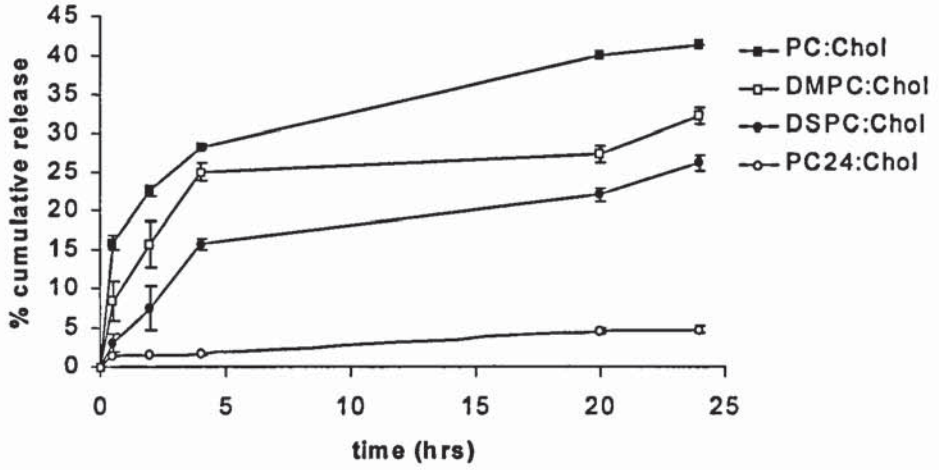


Fig 3.8: The effect of lipid alkyl chain length on the release profile of ibuprofen from liposomes. MLV incorporating ibuprofen composed of: 16μmoles PC, 4μmoles Chol (filled square); 16μmoles DMPC, 4μmoles Chol (open square); 16μmoles DSPC, 4μmoles Chol (filled circle); 16μmoles C_{24}PC, 4μmoles Chol (open circle) were incubated 0.01M PBS at 37°C for up to 24 h. At selected time intervals samples were assayed for ibuprofen release as described in section 2.4 and expressed as % of total incorporated. Results represent mean ± standard deviation, n=3 of percentage cumulative release in PBS at 37°C.

Similarly, the ability of the ibuprofen-loaded MLV to retain their ibuprofen load during incubation in PBS at 37°C correlated to the alkyl chain length of the lipid component of the MLV (Fig 3.8). As shown in Fig 3.8, absolute retention values were again in the order C_{24}PC>DSPC>DMPC>PC. Indeed after only 30 min incubation PC MLV had released 14.5% more of their ibuprofen load compared to C_{24}PC liposomes with the difference increasing to 36% after 24 h incubation (Fig 3.8). Release of steroids from MLV has also been shown to be similarly influenced by lipid alkyl chain length with both hydrocortisone and budesonide being retained longer in DSPC liposomes than in PC MLV (Saarin-Savolainen et al., 1997). Presumably, this again is a result of the increased lipid phase area of the longer lipid alkyl chain liposomes enhancing the stability of the incorporated drug within the bilayer.

Indeed the release profile also coincided with the phase behaviour of the lipids. $C_{24}PC$ is characterised by a high transition temperature of approximately 75°C whereas PC has a low Tc of –5°C with DSPC (55°C) and DMPC (24°C) having intermediate values. The release could be attributed to the physical state of the lipids: the release profiles were determined at 37°C whilst the T_c for $C_{24}PC$ (75°C) DSPC (55°C) is higher than the temperature at which the release studies were carried out and hence the bilayers will be in the ordered gel phase (Saarinen-Savolainen et al, 1997) thereby inhibiting the release of ibuprofen. In contrast, lipids with a T_c less than 37°C will form bilayers of a more fluid phase nature and thus show an enhanced release. Studies by Hu et al., (1995) investigating the encapsulation of the anti cancer agent doxorubicin have shown that the cytotoxic activity of the drug encapsulated into liposomes of different lipid compositions was influenced by the composition of the bilayer. Doxorubicin entrapped in MLV composed of PC exhibited activity compared to that of the free drug whereas DPPC encapsulating doxorubicin showed poor activity. The differences in the activities was attributed to the phase behaviour of the lipids with DPPC liposomes in the gel phase slowing drug release whereas PC liposomes promoted the release essentially due to their fluid nature. Similar results were also reported when rifampicin (water insoluble) and isoniazid (water soluble) encapsulated into lipids of different alkyl chain lengths showed a slow release from organised gel state DPPC MLV when compared to the fluid PC MLV (Gursoy et al., 2004). The egg PC liposomes released around 90% of the entrapped drug whereas the DPPC liposomes released only 50% of the drug (Gursoy et al., 2004).

3.5 The effect of alkyl chain saturation on ibuprofen encapsulation

The effect of incorporating unsaturated alkyl chain lipids within the liposome composition was studied by replacing the PC with other lipids including 1,2-dioleoyl PC (18:1-cis; DOPC) (Fig 3.9), 1,2 dielaidoyl PC (18:1-*trans*; DEPC) (Fig 3.10) and 1,2 dilinoleoyl PC (18:2- 9 *cis*, 12 *cis*; DLPC) (Fig 3.11). DOPC is characterised by a single double bond with a *cis* orientation whereas DEPC also has a single unsaturation but with a *trans* orientation. DLPC has two unsaturated bonds one at 9 carbon and the other at 12 carbon both with cis alignment. All the unsaturated lipids investigated consisted of long (C_{18}) alkyl chains. Ibuprofen incorporation results (Table 3.5) show that the substitution of the unsaturated lipids yields interesting entrapment values with lipids with a C-18 chain length showing similar entrapment of ibuprofen when compared to the saturated DSPC. The substitution of DSPC with DOPC or DEPC resulted in no significant difference in drug loading (Table 3.5) , however substitution with DLPC resulted in a significant ($P<0.05$) reduction in incorporation values (39.5 ± 0.9%; Table 3.5) respectively of the initial amount of ibuprofen (1.25mg) added (Table 3.5). Size analysis of the various MLV formulations revealed no significant difference in the mean volume diameter of the different lipids vesicles (Table 3.5). The surface charge measurements also showed that all the formulations exhibited a near neutral surface charge as expected as all the lipids investigated have a choline head group (Table 3.5).

As discussed in section 3.4, the increase in hydrophobic volume helps in better retention of ibuprofen within the liposomal bilayer. However, the alkyl chain unsaturation depicts a slightly different picture. The encapsulation values in the

single unsaturated lipids (DOPC, DEPC; Fig 3.9 & 3.10 respectively) were not significantly different when compared to the saturated DSPC. The similarity could possibly be attributed to the similar packing efficiency of ibuprofen within the single unsaturated alkyl chains. However, the substitution of DLPC, which contains two unsaturated *cis* bounds on each carbon chain (Fig 3.11) resulted in the lowering of the entrapment by ~4%. Recent work by Komatsu et al., (2001) investigated liposomal formulations made from different acyl chain compositions. Their results indicated that liposomes made from DOPC showed a poor retention of the marker drug (calcein) when compared to saturated phospholipids of DPPC and PC possibly due to the poor bilayer packaging resulting in drug leakage. Studies have also shown that lipid unsaturation influences the permeability of the bilayer. This has been attributed to saturated lipids resisting penetration of water into bilayers whereas bilayers made from unsaturated lipids display enhanced water permeability. This process in turn has been attributed to the looser packing of the unsaturated lipids at the lipid water interface. Similarly, increasing the degree of unsaturation has also been shown to reduce bilayer integrity (Huster et al., 1997; Alamelu and Rao , 1990) with the presence of two unsaturated bonds in the alkyl chain giving encapsulation values lower than DSPC:Chol liposomes. The results reported in Table 3.5 suggest that the presence of one unsaturated double bond per acyl chain (as in the case of DOPC and DEPC made no significant difference to the ability of ibuprofen to pack within the bilayers when compared with DSPC, however further increasing the degree of unsaturation had a detrimental effect on drug loading. This may be a result of the presence of ibuprofen in the bilayer having a stabilising effect and potentially maintaining bilayer integrity. Indeed enhanced stabilisation of liposomes with the inclusion of ibuprofen within MLV

vesicles has been demonstrated using ESEM analysis (Mohammed et al., 2004; Chapter 6).

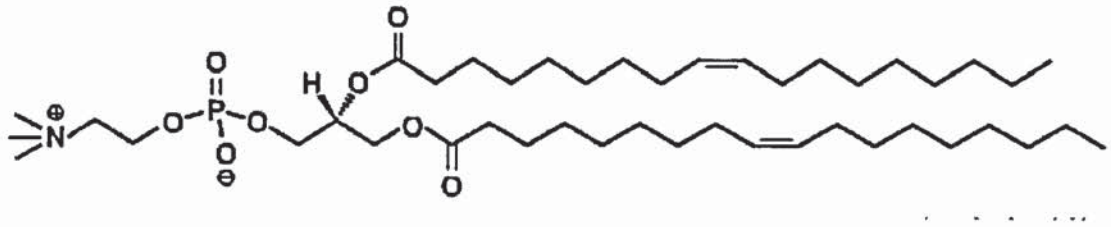


Fig 3.9: Structure of 1,2- dioleoyl PC (18:1-cis; DOPC)

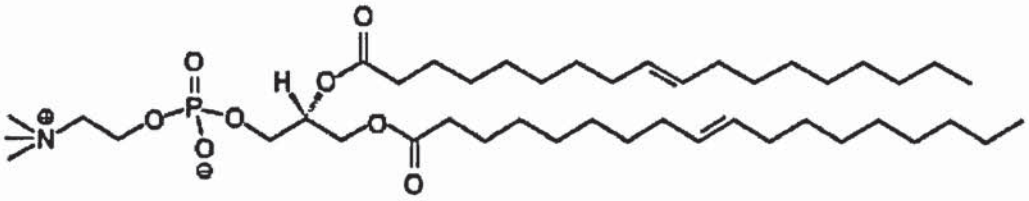


Fig 3.10: Structure of 1,2dielaidoylPC (18:1-*trans*; DEPC)

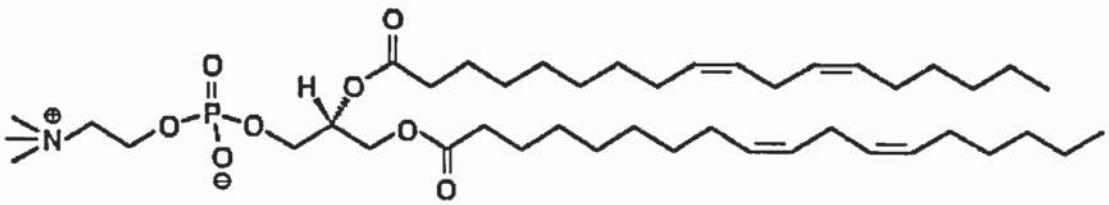


Fig 3.11: Structure of 1,2 dilinoleoyl PC (18:2- 9 *cis*, 12 *cis*; DLPC)

Liposome composition	Ibuprofen entrapment efficiency (%)	Liposome size (μm)	Liposome zeta potential (mV)
DSPC:Chol (16:4μmol)	43.5 ± 0.7	5.5 ± 0.3	-7.1 ± 1.8
DOPC:Chol (16:4μmol)	43.9 ± 0.7	5.5 ± 0.7	-2.9 ± 1.2
DEPC:Chol (16:4μmol)	46.5 ± 2.9	6.1 ± 0.3	-6.1 ± 1.8
DLPC:Chol (16:4μmol)	39.5 ± 0.9*	5.7 ± 0.3	-3.3 ± 1.2

Table 3.5: The effect of lipid alkyl chain length unsaturation on liposome size, zeta potential and ibuprofen encapsulation. Ibuprofen (1.25mg) was incorporated into MLV of various lipid compositions as shown as described in section 2.1. Ibuprofen encapsulation efficiency within liposomes was determined as described in section 2.2. Liposomes size (volume mean diameter) was determined by laser diffraction spectroscopy using a Malvern Mastersizer X at 20°C. The zeta potential of the MLV was measured in 0.001M PBS at 25°C using a Brookhaven Zetasizer. * Represents significantly different entrapment values ($P<0.05$). Values denote mean ± S.D. from at least three experiments.

3.6 Role of charged lipids on ibuprofen encapsulation and release

The inclusion of charged lipids into liposomes has been shown to have significant effect on the physico-chemical attributes of liposomes (e.g. Perrie & Gregoriadis, 2000) The inclusion of charged lipids to liposome formulations in addition to influencing ibuprofen loading (Section 3.2.4) was shown to influence ibuprofen incorporation, MLV size and zeta potential (Tables 3.6 and 3.7). The addition of up to 2 μmoles stearylamine (SA) to the PC:Chol liposome formulation significantly enhanced ($P<0.05$) the incorporation efficiency of ibuprofen by approximately 8% to 47% (Table 3.6) although only a fairly minor change in drug loading was measured. This behaviour may be attributed to the electrostatic attraction between the positively charged head group in SA and the carboxyl group present in dissociated ibuprofen. However, conversely further increases in SA liposome content up to 6μmol resulted in a major reduction in ibuprofen entrapment efficiency and drug loading (27.9 and 25.0% for 4 and 6 μmoles respectively; Table 3.6) to below that of formulations excluding SA. Substitution of SA with 2 μmoles of dicetyl phosphate (DCP), an anionic lipid, also resulted in reduced ibuprofen incorporation efficiency and drug loading relative to PC:Chol liposomes (Table 3.6) suggesting repulsive interactions between the negatively charged lipid and the drug molecule may play a contributing factor in the ibuprofen incorporation within lipid bilayers. The increase in ibuprofen loading on addition of 2 μmoles of SA to the liposome formulation occurred in conjunction with a reversal of surface charge (due to the cationic headgroup of SA) and an increase in vesicle size (based on mean volume distribution) of approximately 0.8μm compared with the equivalent PC:Chol formulation (Table 3.6). In contrast, the incorporation of anionic DCP did

not significantly influence liposome size but did increase the magnitude of the MLV negative charge, again presumably due to the anionic nature of the DCP head-group. These results suggest the increased vesicle size of PC:Chol:SA (16:4:2μmol) may be an outcome of increased drug loading in response to the presence of SA rather than directly due to the presence of SA within the liposomal membrane alone since PC:Chol:SA liposomes formed in the absence of ibuprofen were notably smaller (~1.3μm) than drug loaded PC:Chol:SA MLV (Table 3.6). Characterisation studies of PC:Chol liposomes without SA (Tables 3.2 and 3.3 respectively) also suggest a trend of increased vesicle mean volume size distribution with higher ibuprofen encapsulation.

Previous investigations into the characteristics of charged lipid membranes (Jähnig, et al., 1979) revealed decreased lateral packing of polar heads with increasing surface charge on a lipid bilayer. However, the ordered hydrocarbon chains, due to their attractive van der Waals interaction only partially follow this expansion and in order to minimise a change in packing, the hydrocarbon chains tilt, which decreases bilayer thickness (Fig 3.12) (Jähnig, et al., 1979). This electrostatically induced change in bilayer packaging, combined with the electrostatic interactions between the ibuprofen carboxyl group and the charged head-groups of SA or DCP could both influence the incorporation of ibuprofen within the liposomes. At lower concentrations of SA (1-2 μmoles; 4-9% mol ratio) the opposing charges of the SA and ibuprofen head-groups may electrostatically interact thereby increasing ibuprofen-liposome association (Table 3.6), indeed after incubation for 20 h with a solution containing 1.25mg of ibuprofen, 14.5 ± 0.7% of the ibuprofen remained associated with cationic MLV (PC:Chol:SA; 16:4:2μmol)

after centrifugation compared to only 3.9 ± 0.5% and 2.8 ± 0.5% for PC:Chol (16:4 μmol) and PC:Chol:DCP (16:4:2μmol) MLV respectively. However as the concentration of SA within the liposomal bilayer increases to 4 or 6 μmol there is a marked decrease in ibuprofen encapsulation within the liposomes suggesting that factors besides electrostatic interaction between the opposing charges of ibuprofen and SA are involved. The presence of the charged lipids in the vesicle bilayer also influenced drug release (Fig 3.13). Both the SA and DCP containing liposomes released significantly more ($P<0.001$) incorporated drug (71 ± 2% and 69 ± 3% respectively) over 24 h compared with PC:Chol liposomes (41±0.1%) again suggesting the presence of charged lipids within the liposome bilayer influences the structure of the bilayer and affects both the incorporation and release of ibuprofen from these liposome systems. Changes in the lateral packaging of the liposome bilayers as previously suggested (Jähnig, et al., 1979) may again be responsible for these variations in drug retention within the liposomal bilayers; differences in lipid packaging and interactions may result in increased partitioning of ibuprofen from charged bilayers into the release media compared with PC:Chol liposomes.

Liposome SA content (μmoles)	MLV		MLV incorporating Ibuprofen			
	Liposome size (μm)	Liposome zeta potential (mV)	Ibuprofen entrapment efficiency (%)	Concentration of drug in liposomes (% mol/mol)	Liposome size (μm)	Liposome zeta potential (mV)
0	4.3 ± 0.5	-6.1 ± 0.1	39.3 ± 0.3	10.7 ± 0.2	5.3 ± 0.4	-6.1 ± 0.7
1	4.9 ± 0.2	16.0 ± 5.4	45.4 ± 0.5	11.6 ± 0.1	5.9 ± 0.2	13.4 ± 1.8
2	4.8 ± 0.5	25.7 ± 3.7	47.2 ± 1.9	11.5 ± 0.4	6.1 ± 0.1	19.9 ± 3.3
4	5.1 ± 0.2	39.9 ± 5.3	27.9 ± 0.6	6.6 ± 0.1	5.1 ± 0.5	46.8 ± 3.8
6	5.3 ± 0.1	49.6 ± 1.8	25.0 ± 0.4	5.5 ± 0.1	4.8 ± 0.4	53.8 ± 4.9

Table 3.6: The effect of MLV cationic lipid content in PC:Chol liposomes on liposome size, zeta potential and ibuprofen encapsulation. MLV were prepared from 16 μmol PC, 4μmol CHOL and varying SA content (0-6μmol). MLV incorporating ibuprofen were prepared in the presence of 1.25mg of ibuprofen as described in section 2.1. Ibuprofen encapsulation efficiency within liposomes was determined as described in section 2.2. Liposomes size (volume mean diameter) was determined by laser diffraction spectroscopy using a Malvern Mastersizer X at 20°C. The zeta potential of the MLV was measured in 0.001M PBS at 25°C using a Brookhaven Zetasizer. Values denote mean ± S.D. from at least three experiments.

Liposome formulation	MLV		MLV incorporating Ibuprofen			
	Liposome size (μm)	Liposome zeta potential (mV)	Ibuprofen entrapment efficiency (%)	Concentration of drug in liposomes (% mol/mol)	Liposome size (μm)	Liposome zeta potential (mV)
PC:Chol (16:4 μmoles)	4.3 ± 0.5	-6.1 ± 0.1	39.3 ± 0.9	10.7 ± 0.2	5.3 ± 0.4	-6.1 ± 0.7
PC:Chol:SA (16:4:2 μmoles)	4.8 ± 0.5	25.7 ± 3.7	47.2 ± 1.9*	11.5 ± 0.4	6.1 ± 0.1	19.9 ± 3.3
PC:Chol:DCP (16:4:2 μmoles)	4.9 ± 0.3	-28.2 ± 3.3	34.3 ± 0.3*	8.6 ± 0.1	5.1 ± 0.2	-24.5 ± 1.3

Table 3.7: The effect of charged lipids on MLV size, zeta potential and ibuprofen incorporation MLV were prepared from 16 μmol PC, 4μmol Chol and 2μmol of either SA (cationic MLV) or DCP (anionic DRV). Liposomes size (volume mean diameter) was determined by laser diffraction spectroscopy using a Malvern Mastersizer X at 20°C. The zeta potential of the MLV was measured in 0.001M PBS at 25°C using a Brookhaven Zetasizer. * Represents significantly different entrapment values ($P<0.05$). Values denote mean ± S.D. from at least three experiments.

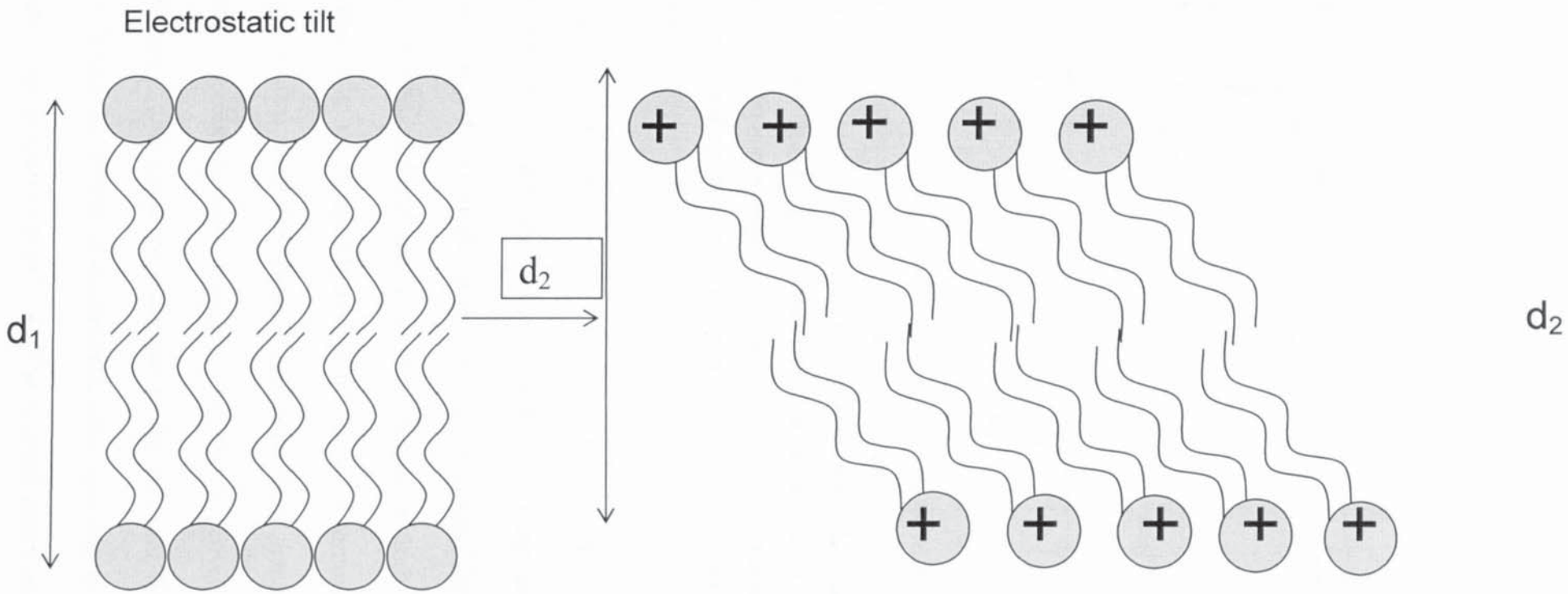


Fig 3.12: Schematic representation of the electrostatically induced tilt of a lipid bilayer in the presence of charged surfactant head-groups. Electrostatic repulsion between charged-head groups decreases the lateral packaging of the lipid polar heads. However, the ordered hydrocarbon chains, due to their attractive van der Waals interaction only partially follow this expansion and in order to minimise a change in packing, the hydrocarbon chains tilt, which decreases bilayer thickness.

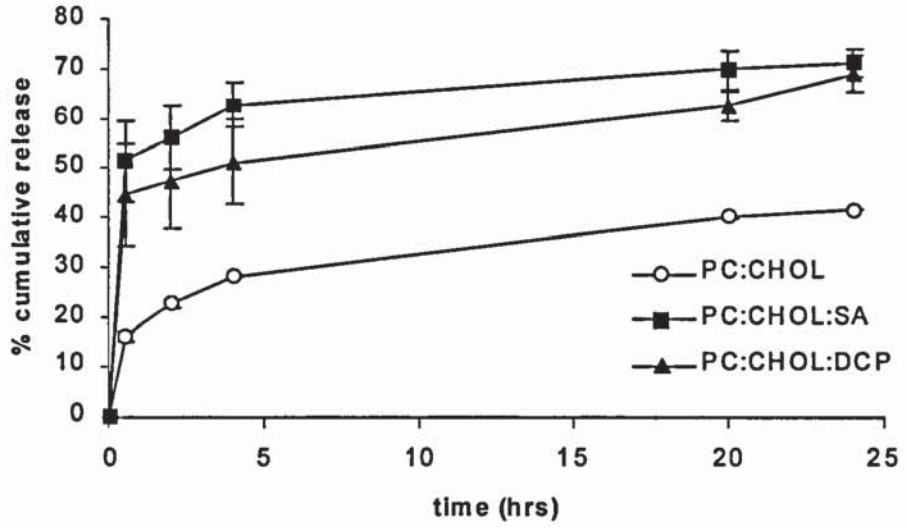


Fig 3.13: The effect of incorporation of charged lipids on the release profile of ibuprofen from MLV. Multilamellar vesicles incorporating ibuprofen composed of: 16μmoles PC, 4μmoles Chol (open circle); 16μmoles PC, 4μmoles Chol, 2μmoles SA (filled triangle); PC, 4μmoles Chol, 2μmoles DCP (filled square) were incubated 0.01M PBS at 37°C for up to 24 h. At selected time intervals samples were assayed for ibuprofen release as described in section 2.4 and expressed as % of total incorporated. Results represent mean ± standard deviation, n=3 of percentage cumulative release in PBS at 37°C.

The partitioning behaviour of ibuprofen in solution into neutral and charged liposomes was investigated using the addition of stearylamine (positively charged liposomes) and dicetyl phosphate (negatively charged liposomes) as appropriate. The aim was to determine if any free ibuprofen from solution in PBS would associate with the charged bilayer of the liposome formulation. The results show (Fig 3.14) that ibuprofen partitions/associates to a greater extent into positively charged liposomes when compared to the negatively charged and neutral liposomes. This could be attributed to the fact that ibuprofen associates with stearylamine by means of electrostatic attraction due to the oppositely charged nature of the molecules at pH 7.4. The minimal partitioning/association to neutral and negatively charged liposomes indicates that a small degree of electrostatic association is involved.

Recent studies by Boija et al., 2004 have also shown that partitioning of free drug (indomethacin, lidocaine) into neutral liposomal bilayer was minimal and the presence of cholesterol also reduces the partitioning behaviour by improving the bilayer packaging. The investigations also revealed that the presence of negatively charged detergents increased the partitioning of positively charged drug molecules across the bilayer.

The solubility of drug molecules carrying a charge can also be influenced by the charge on the micellar solubilising systems. For example, carbendazim (logP 1.7) a positively charged molecule at pH 2 was poorly entrapped into non ionic Tween80 and 20, cationic myristoyl canitine, however higher entrapment values were reported when the charged surfactants were replaced with oppositely charged sodium lauryl sulphate (Ni et al., 2002).

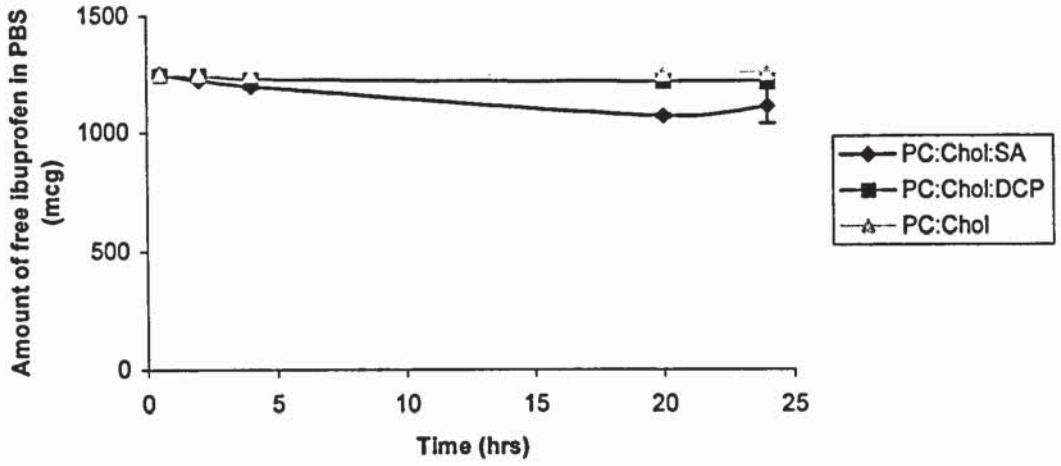


Fig. 3.14. The results show the partitioning behaviour of ibuprofen (1.25mg) at pH 7.4 in PBS into neutral, positively charged lipososomes (PC:Chol:SA) and negatively charged liposomes (PC:Chol:DCP). Free ibuprofen (1.25mg) was added to the media and quantifying the free drug left in the media was used to assess the amount of partitioned drug. The results represent mean partition ± S.D, n=3.

3.7 The influence of hydrogen ion concentration (pH) of the hydration medium on encapsulation

To investigate the effect of the hydrogen ion concentration on the entrapment of ibuprofen, PC:Chol (16:4μmoles) MLV were tested for drug incorporation at pH ranges above and below the pKa (5.2) of ibuprofen. The results (Fig 3.15) show that over the pH range tested (3.9-7.4) the encapsulation of ibuprofen was optimum at pH 4.7 (56.2 ± 2.9%). Investigation of the size of the liposomes (Table 3.8) showed this increase in drug loading correlated with an increase in vesicle mean volume diameter (7.4±0.5μm) unlike liposomes prepared in the absence of ibuprofen which had measured mean volume diameters between 3.8 and 4.8μm irrespective of the hydration media pH (Table 3.8). Zeta potential analysis (Table 3.8) revealed that the surface of unloaded liposomes became increasingly cationic as the pH of the hydration media decreased whilst ibuprofen loaded liposomes retained a slightly anionic zeta potential (-4.6 to -5.8 mV) independent of the hydration media pH.

The results in Figure 3.15 show incorporation of ibuprofen within liposomal bilayer increases as the pH decreases from 7.4 to 4.7. This could be attributed to the decrease in pH promoting the conversion of ibuprofen, which has a pKa of 5.2, from the weakly acid structure to the molecular, lipophilic form which will favour bilayer incorporation. However, decreasing the pH of the media further from 4.7 to 3.9 results in a substantial reduction in the ibuprofen loading within liposomes suggesting that the effect of the hydration media pH on drug ionisation is not the only factor controlling drug bilayer incorporation and it may also significantly influence drug loading by altering the characteristics of the liposome bilayer.

In terms of the size and zeta potential characteristics of the liposomes studied, it is interesting to note that the increased vesicle size of ibuprofen loaded MLV at pH 4.7 (Table 3.8) can be seen as an outcome of increased drug loading rather than a controlling factor since the vesicle size of unloaded liposomes was not influenced by hydration media pH. Conversely, the hydration media pH is shown to influence the zeta potential of unloaded liposomes but not ibuprofen loaded liposomes (Table 3.8). Within the neutral pH range the anisotropic phosphatidylcholine within the liposomal bilayer is arranged such that the negatively charged phosphate groups are on the outer surface of the bilayer followed by positively charged ammonium groups. This charge distribution of the lipid molecule can hinder the movement of charged molecules into the lipid bilayer (Avdeef et al., 1998). As the pH is reduced, the negatively charged phosphate groups of PC becomes protonated due to the excess hydrogen ions in the hydration medium resulting in an increase in cationic surface charge. This is clearly demonstrated in table 3.8 with the zeta potential of unloaded liposomes increasing with decreasing pH. This change in charge distribution has been suggested (Gregoriadis, 1993) to cause the reduction in the hydrophilicity of the lipid head group, thereby eventually reducing the partitioning barrier for lipophilic molecules and again promoting ibuprofen bilayer loading at reduced pH. Indeed, at pH 4.7 significant amounts (13%) of the drug entered the unloaded liposome bilayer after incubation for 24 h. In comparison, partitioning of ibuprofen into the liposome bilayer was not observed at pH 7.4 over the same time period (Fig 3.16). The increase in cationic surface charge of unloaded liposomes could also result in electrostatic interaction and surface adsorption of any dissociated weakly acidic ibuprofen present to the cationic

surface of the liposome. Indeed the reduced zeta potential of the liposomes incorporating ibuprofen compared to unloaded liposomes suggests a degree of electrostatic interaction could be occurring. However none of these factors explain the decreased ibuprofen MLV loading at pH 3.9 compared to 4.7 (Fig 3.16) suggesting that more complex factor may also be involved again perhaps relating to a bilayer re arrangement as suggested by Jahnig (1979).

Indeed recently, research (Moussaoui, et al., 2002) has shown that liposomal bilayers undergo complex structural rearrangements in the pH range of 4. This structural re-arrangement may result in a reduced loading of ibuprofen within the lipophilic region of the bilayer. Therefore it may be that the enhanced ibuprofen loading at pH 4.7 is a balanced combination of enhanced lipophilicity of molecular ibuprofen combined with complex electrostatic and structural changes within the bilayer which can promote drug bilayer loading and/or association.

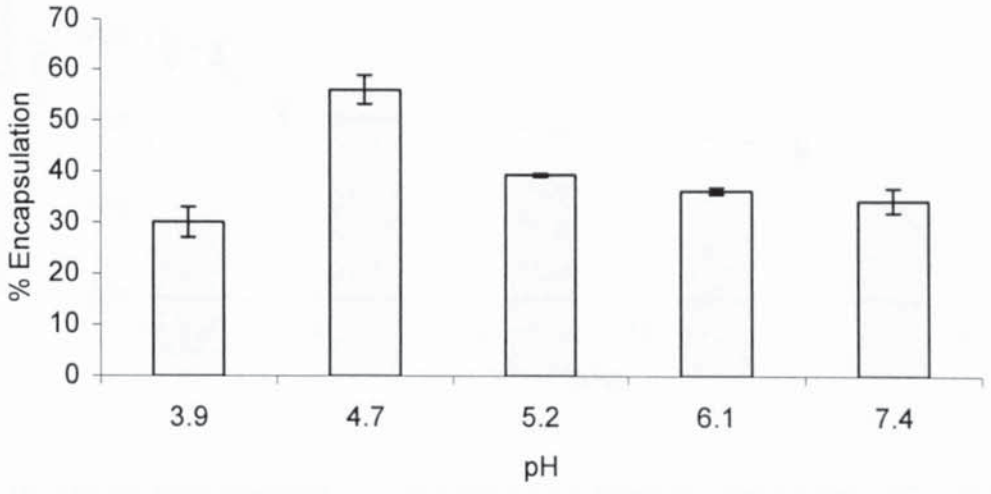


Fig.3.15. The effect of hydration media pH on ibuprofen encapsulated into the liposomal bilayers. Ibuprofen (1.25mg) was incorporated within MLV composed of PC:Chol (16:4μmoles) prepared by hydration of a dry lipid film using media with a range of pH between 3.9 and 7.4. The pH of the hydration medium was adjusted with 0.01M HCl. The results represent mean ibuprofen encapsulation±S.D,n=3.

Hydration media pH	Zeta potential (mV)		Vesicle size (μm)	
	MLV	Ibuprofen loaded MLV	MLV	Ibuprofen loaded MLV
3.9	12.3 ± 0.8	-4.7 ± 1.2	3.8 ± 0.8	5.2 ± 0.6
4.7	9.1 ± 0.4	-5.3 ± 0.8	4.5 ± 0.3	7.4 ± 0.5
5.2	1.6 ± 0.7	-4.6 1 ± .1	4.1 ± 0.5	6.1 ± 0.3
6.1	-1.5 ± 0.9	-5.8 ± 0.8	4.8 ± 0.2	5.4 ± 0.6
7.4	-5.9 ± 0.4	-4.9 ± 0.7	4.3 ± 0.6	5.0 ± 0.4

Table 3.8: The effect of hydration media pH on MLV size and zeta potential Liposomes were prepared in the presence or absence of ibuprofen (1.25mg) and in hydration media with a pH between 3.9 and 7.4. Liposomes size (mean volume diameter) was determined by laser diffraction spectroscopy using a Malvern Mastersizer X at 20°C. The zeta potential of the MLV was measured in 0.001M PBS at 25°C using a Brookhaven.

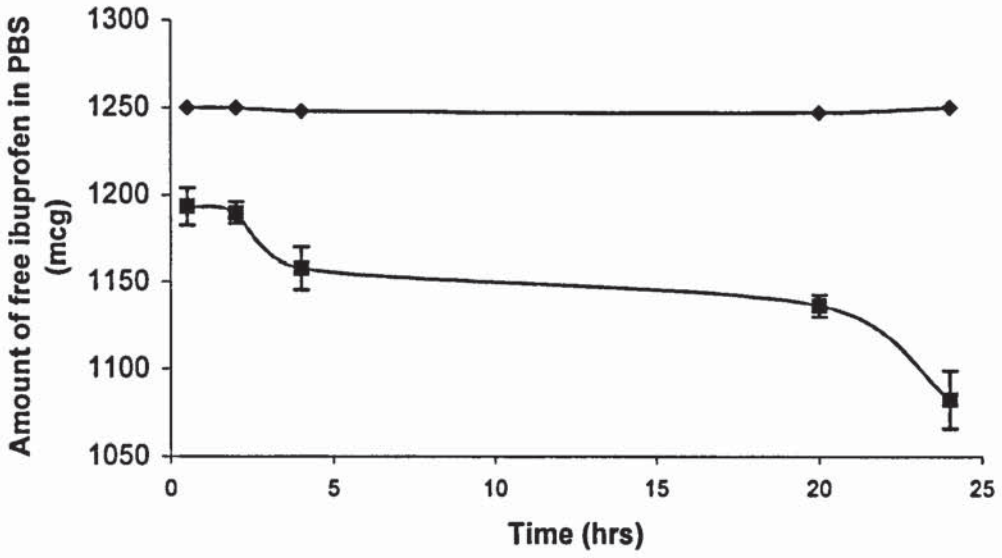


Fig.3.16. The partitioning behaviour of free ibuprofen into empty liposomes made of PC and Chol at pH 4.7 and pH 7.4 in PBS at 37°C. Free ibuprofen was added to empty MLVs made from 16:4 of PC:Chol. Samples were taken at specified intervals, centrifuged and the amount of ibuprofen assayed on a UV spectrophotometer at 221nm wavelength. The results are mean values ± S.D, n=3.

3.8 The effect of temperature on ibuprofen release from liposomes

Previous studies, looking at the release profiles of water soluble drugs (eg isoniazid: Gursoy et al., 2004) have shown that formulation of liposomes using high-transition lipids such as DSPC (Tc = 55°C) displayed enhanced retention of drugs within their aqueous environment compared to lower transition temperature lipids (such as PC; Tc < 0°C). Lipid molecular arrangements within bilayers can be characterised into two different patterns of arrangements, the choice of which is dictated by the lipids transition temperature. Lipids can either arrange into an ordered gel structure, if incubated at a temperature below their transition temperature, or alternatively at higher temperatures they form fluid structures. This change in arrangement is known to have a dramatic effect on the release profiles of aqueous soluble drugs (Gursoy et al., 2004) therefore the influence of liposome composition on the release of bilayer-loaded liposomes was investigated to determine if similar profiles occurred. Results in Fig 3.17 demonstrate the release characteristics between liposome formulations when the drug is present in the hydrophobic bilayer area rather than the aqueous environment: only minimal drug release (~10% over 24 h; Fig 3.17) was measured from liposomes composed of DSPC or DMPC and cholesterol at 15°C compared to PC:Chol liposomes which release almost half of the total loaded ibuprofen (~40% over 24; Fig 3.17) over the same time period. Similar to previous reports using aqueous soluble drugs, this could be related to both DSPC and DMPC lipid bilayers being in the ordered gel state at 15°C whereas PC bilayers, due to their low Tc, would adopt the fluid state at the same temperature. The increased thermal motion of the lipid alkyl chains when in the fluid state, is reported (Swarbrick and Boylan, 1994) to cause an

enhanced motion at the junction of the acyl with the head-group. This leads to repulsion between the adjacent acyl chains eventually disrupting the ordered arrangement of the lipids within the bilayer which could result in an increased release of bilayer loaded drug as shown in Fig 3.17. Indeed, addition of PC to DMPC liposomes (PC:DMPC:Chol; 8:8:4 μmoles) resulted in a dramatic increase in ibuprofen release rates to similar levels as those measured with PC:Chol liposomes alone (35% after 24h; Fig 3.17) suggesting that inclusion of 40% of low Tc PC within DMPC liposomes is able to disrupt the lipid bilayer and potentially force it into the fluid, and hence more leaky, molecular arrangement.

However, despite the high transition temperature of DSPC, increase of the temperature of the release medium to 37°C increased release rates of ibuprofen from DSPC and DMPC to 26 and 32% respectively (Fig3.17) despite the transition temperature of DSPC being well above this temperature. This may be attributed to the presence of cholesterol and/or ibuprofen within the bilayer, which may influence the physical state of the lipids. Cholesterol has a planar steroid ring structure, which is responsible for its rigid molecular structure (Wang and Quinn, 2002). Due to this, the interactions with the phospholipids molecules in a bilayer are specific to individual lipids and these interactions can give rise to various phases in the bilayer (Wang and Quinn, 2002). The phase structures of the bilayer membranes containing cholesterol has been classified into three states: liquid disordered which occurs at low cholesterol levels, liquid ordered which occurs at high cholesterol content (above 20mole%) and solid ordered arrangement which is present at low cholesterol content below the transition temperature. The presence of cholesterol in concentrations greater than 33mol% in the phospholipid bilayer

prevents its transition from ordered gel to liquid crystal phase (Tirosh et al., 1997). Studies on the influence of cholesterol concentration on the phase behaviour of the lipids has shown that low concentrations of cholesterol (5mole%) reduced the transition temperature from ordered gel to liquid crystalline to 21°C and to 10°C in the presence of 20mole% of cholesterol (Wang and Quinn, 2002). However among the formulations that we investigated, the cholesterol concentration was 20mole% which could potentially induce the formation of liquid disordered structures in the liposome bilayer causing a lowering of the transition temperature (~10°C) as was reported in section 3.3.1.

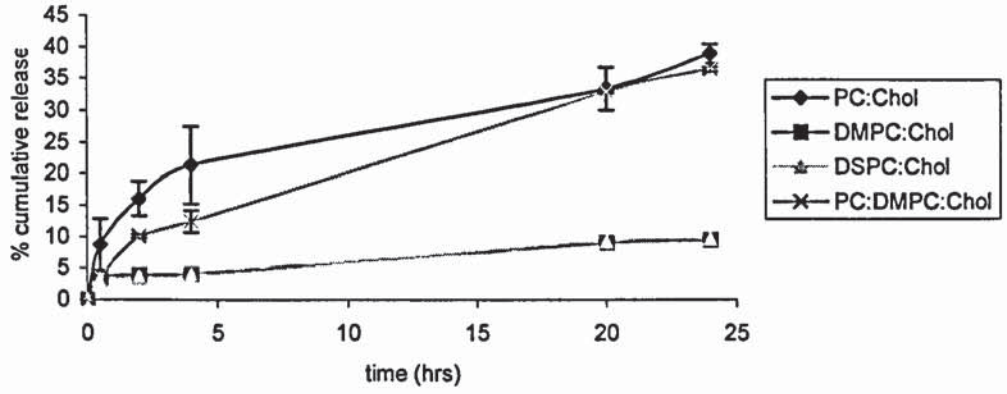


Fig 3.17. The release profile of ibuprofen from liposomes made from different lipid:Chol (16:4μmoles). MLV were made using lipid:Chol of 16:4. PC:DMPC:Chol was made using 8:8:4 μmoles of the respective lipids. The results represent percentage cumulative release (mean ± SD; n=3) in PBS at 15°C.

3.9 The influence of addition of surfactants on ibuprofen encapsulation

The influence of non-ionic surfactants on neutral liposomal formulations was initially investigated by the addition of Tween 80. Previous investigations involving solubilisation of estradiol have shown to be effected by the addition of neutral surfactant Tween 20 and Tween 80 (Maghraby et al., 2000). However in our present studies the addition of 4μmoles of Tween 80 to a neutral liposome formulation consisting of 16 μmoles of PC and 4 μmoles of Chol with 1.25mg of added ibuprofen resulted in lowering the encapsulation to 32 ± 0.2% (Table 3.9). Measurements of mean volume diameter also did not show any significant increase in size nor was a change in zeta potential measured. The release studies of the formulation incorporating Tween 80 showed an initial burst release with around 45% of the encapsulated drug being released in the first half hour, and after 24hr a total of approximately 62% of the entrapped drug was released compared to the PC:Chol liposomes without Tween 80 which released 9% and 39% at similar time points (Fig 3.18). The burst release followed by a sustained release could potentially be due to the formation of deformable liposomes due to the addition of surfactant molecules as was reported by El Maghraby et al., (1999). The presence of a neutral single chain surfactant molecule has been reported to possibly destabilise the rigidity and packing behaviour of the neutral two-chain phospholipid.

	PC:Chol	PC:Chol:Tween 80
% Encapsulation	39.3 ± 0.9	32.0 ± 0.2*
Size (μm)	5.3 ± 0.4	4.5 ± 0.5
Zeta Potential (mV)	-6.1 ± 0.7	-3.7 ± 3.0

Table 3.9: The effect of addition of tween 80 on liposome size, zeta potential and ibuprofen encapsulation. Ibuprofen (1.25mg) was incorporated into MLV of PC:Chol with the addition of 4μmoles of tween 80 as shown as described in section 2.1. Ibuprofen encapsulation efficiency within liposomes was determined as described in section 2.2. Liposomes size (volume mean diameter) was determined by laser diffraction spectroscopy using a Malvern Mastersizer X at 20°C. The zeta potential of the MLV was measured in 0.001M PBS at 25°C using a Brookhaven Zetasizer. * Represents significantly different entrapment values ($P<0.05$). Values denote mean ± S.D. from at least three experiments.

The above result prompted us to investigate in more detail the role of additional surfactant to neutral liposomal formulation. α-Tocopherol (Fig 3.19) was used to study the role of single chain surfactant as this offers the advantage of being anti-oxidant thus protecting phospholipids from chemical degradation. The properties of α-tocopherol partly resemble cholesterol in that it is insoluble and a non-swelling amphiphile. The neutral liposomal formulations were tested using different concentration ranges of tocopherol (Table 3.10). The results show that the concentration of α-tocopherol added to the formulation has a wider role in influencing both the encapsulation of ibuprofen as well as possibly affecting the bilayer fluidity. The addition of up to 1 μmoles of tocopherol to PC:Chol formulation did not significantly alter the entrapment. However an increase in concentration from 1.5 μmoles had a significant effect on the amount of ibuprofen entrapment ($P< 0.001$) with further increase in the tocopherol concentration to 3 μmoles had no additional benefits with percentage entrapment still around 43 – 45%. The increase of tocopherol concentration beyond 3μmoles up to 4μmoles

lowered ibuprofen entrapment to below that measured for PC:Chol without tocopherol incorporated. The inclusion of up to 4μmoles of tocopherol did not have a significant effect on size or zeta potential.

The variations in encapsulation could possibly be explained by the surfactant nature of tocopherol. The entrapment of poorly soluble drugs into micelles in the presence of tocopherol is influenced by the log P of the drug and the fatty acid chain length (Nielson et al., 2001). The addition of α tocopherol to medium chain bile salts improved the encapsulation of insoluble drugs than with long chain bile salts and it has been shown that the solubilisation of griseofulvin (logP 2.2) was significantly improved in the presence of tocopherol whereas the solubility of felodipine (logP 4.8) was lowered in the presence of tocopherol (Nielson et al., 2001). The results indicate that the solubilising capacity of tocopherol is dependent on the logP of the drug. However our results also show that the influence of tocopherol is also concentration dependent. Lower concentrations (7-13% molar concentration) have a positive influence on drug solubilisation whereas increase of concentration could have a negative impact. The higher concentrations could possibly be having an effect on bilayer packaging thus lowering the entrapment of the added drug similar to the effect noted with cholesterol in section 3.3.1. Thus a correct choice of tocopherol concentration is essential and may require to be optimised for a given drug as may be the case with cholesterol when aiming to prepare liposome based solubilising agents.

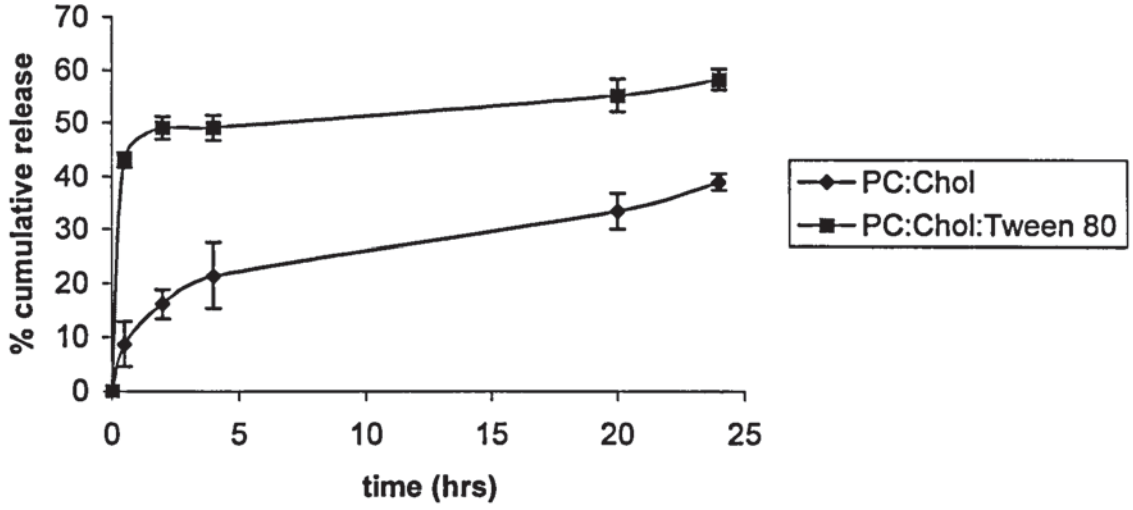


Fig 3.18: The effect of surfactant on the release profile of ibuprofen from liposomes at pH 7.4. Multilamellar vesicles incorporating ibuprofen composed of: 16μmoles PC, 4μmoles Chol and 2μmoles of tween 80 were incubated 0.01M PBS at 37°C for up to 24 h. At selected time intervals samples were assayed for ibuprofen release as described in section 2.4 and expressed as % of total incorporated. Results represent mean ± standard deviation, n=3 of percentage cumulative release in PBS at 37°C.

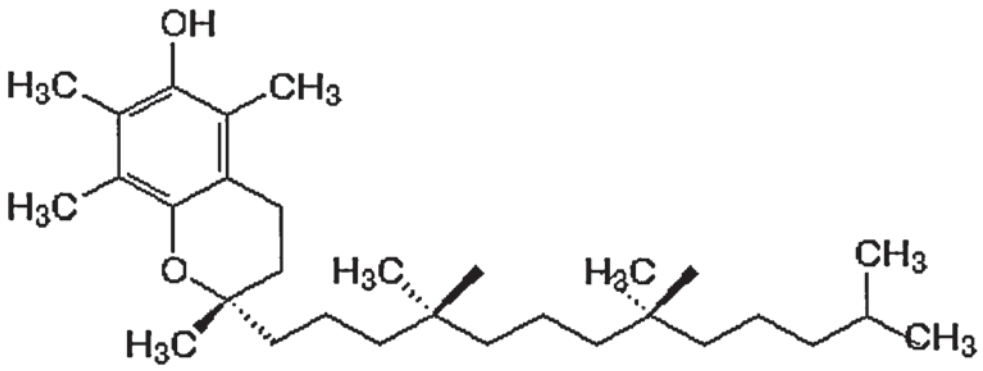


Fig 3.19 Structure of alpha tocopherol

Liposome formulation	% Encapsulation	Conc of drug in liposomes (%mole/mole)	Mean volume diameter (μm)	Zeta potential (mV)
PC:Chol (16:4μmoles)	39.3 ± 0.9	10.7 ± 0.2	5.3 ± 0.4	-6.1 ± 0.7
PC:Chol:Toco (16:4:0.5μmoles)	38.2 ± 1.2	9.9 ± 0.5	5.4 ± 0.8	-2.1 ± 1.5
PC:Chol:Toco (16:4:1.0μmoles)	37.9 ± 0.7	9.7 ± 0.3	4.9 ± 0.8	1.5 ± 1.0
PC:Chol:Toco (16:4:1.5μmoles)	43.6 ± 0.6*	11.3 ± 0.3	5.9 ± 0.5	-2.4 ± 1.3
PC:Chol:Toco (16:4:2.0μmoles)	42.8 ± 1.5*	11.1 ± 0.6	5.6 ± 0.3	-1.8 ± 0.5
PC:Chol:Toco (16:4:2.5μmoles)	43.4 ± 2.1*	11.3 ± 0.6	5.9± 0.9	-4.2 ± 0.6
PC:Chol:Toco (16:4:3.0μmoles)	45.7 ± 0.6*	12.2 ± 0.4	5.9 ± 0.4	- 3.7 ± 1.2
PC:Chol:Toco (16:4:3.5μmoles)	37.4 ± 1.2*	9.8 ± 0.3	5.6 ± 0.3	-3.2 ± 1.0
PC:Chol:Toco (16:4:4.0μmoles)	35.4 ± 1.1*	9.2 ± 0.4	5.8 ± 0.3	- 2.8 ± 2.0

Table 3.10: The effect of tocopherol concentration on the encapsulation of ibuprofen. The MLV were prepared using various amounts of tocopherol (0.5-4.0μmoles) keeping the ratio of PC:Chol (16:4) constant. Liposomes size (mean volume diameter) was determined by laser diffraction spectroscopy using a Malvern Mastersizer X at 20°C. The zeta potential of the MLV was measured in 0.001M PBS at 25°C using a Brookhaven. * Represents significantly different entrapment values ($P<0.05$). Values denote mean ± S.D. from at least three experiments.

3.10 Conclusions

Incorporation of the poorly-water soluble drug ibuprofen, into liposomes was shown to be influenced by the MLV cholesterol and α-tocopherol content, both the lipid alkyl chain properties and the presence of charged lipid head-groups within the MLV formulation. Optimum drug loading was shown to be promoted by using MLV formulations containing 20% (total lipid) cholesterol; or potentially 13% tocopherol; 9% stearylamine and the inclusion of long akyl chain lipids such as dilignoceroyl phosphatidylcholine (C_{24}PC), however to enhance retention of the incorporated drug the charged lipid should be omitted from the formulation. Enhanced ibuprofen incorporation in cationic liposomes was shown to coincide with an increased vesicle mean volume diameter; however the presence of ibuprofen did not significantly influence the zeta potential of any of the liposome formulations tested.

Encapsulation of poorly water soluble acidic drugs in liposomal formulations can also be controlled by changing the ionised state of the drug and the bilayer by modification of the hydration media pH. Higher encapsulation can be achieved by optimising the charge barrier between the drug and the lipid head group thereby facilitating incorporation of the drug molecule into the liposome bilayer.

The presence of the surfactants in the bilayer also controls the encapsulation of the insoluble drugs. The optimum concentration of the surfactant promotes drug retention.

Chapter 4

4.0 Liposomal solution to problematic drugs

Poor water solubility of the drug molecules has been an area of extensive research due to the low therapeutic availability exhibited often due to the low dissolution profile. The two possible approaches to overcome the drawback involve either the chemical modification of the drug itself or employing a suitable carrier system (Leigh et al., 2001).

As already demonstrated in chapter 3, liposomes due to the versatility of the delivery system can act as carriers for lipophilic hydrophilic drugs such as ibuprofen, however the solubility and partitioning behaviour of a drug molecule will govern its location in the liposome structure (Gulati et al., 1998). Highly hydrophilic drugs (logP<1.7) are located exclusively in the aqueous compartment of the liposomes, eg: arabinosylcytosine (Tokunaga et al., 1988). Highly lipophilic drugs with $logP_{oct}$ >5 are entrapped in the lipid bilayer almost completely, eg: cyclosporin (Vadiei et al., 1989). Drugs with intermediate partition coefficients with logP between 1.7 and 4.0 pose a problem because they partition easily between the lipid and aqueous phases and are lost very easily from liposomes (Gulati et al., 1998). Despite the enormous amount of work, which has investigated and documented the application of liposomes as carrier systems for the delivery of hydrophilic drugs, their application as solubilising agents for the lipophilic drug molecules has received limited attention. Although previous research has shown that poor water solubility of the drug molecules (cyclosporin A) could be improved by forming lipid complexes, introducing single chain lipids (Leigh et al., 2001), introducing acyl chain lengths on the drug molecule (Mayhew et al., 1996) a general layout to illustrate the suitability of

different lipid characteristics with the physico chemical properties of the drug molecules is lacking. Liposome encapsulation efficiency of lipophilic drugs has been known to be influenced by both the characteristics of the drug and the liposomal membrane (Walde and Ichikawa, 2001; Balasubramanian and Straubinger, 1994).

Following on from our initial investigations using ibuprofen, the aim of the work was to study an array of drug candidates with varying physico-chemical properties and to investigate liposomal solubilising systems. Drugs from the acidic group included NSAID (non steroidal anti inflammatory drugs) such as flurbiprofen, sulindac and indomethacin were investigated to provide a range of molecular weight/size. To investigate the effect of drug charge, the basic drug lignocaine and the neutral drug progesterone was included. The drug properties investigated included molecular weight, log P, nature of the functional group and molecular size. The lipid properties investigated included the nature of the lipid head group, hydrophobic volume space, transition temperature and influence of single chain surfactants.

4.1 Quantification of drug loading

The quantitative assessment for drug entrapment for all the different drugs investigated was studied by UV spectrophotometric analysis. A UV scan was performed after blanking the spectrophotometer with 0.01M PBS on Vision software (version 3.4). The resulting peak pick was chosen for our study (Table 4.1). The calibration curve was made in 0.01M PBS (pH 7.4) using different concentration ranges and a R^2 value of 0.99. The amount of encapsulated drug into liposomal systems was analysed after centrifugation (Beckman J20) and separation of the supernatant from the pellet. The centrifugation speed was also carefully optimised to ensure the formation of a stable pellet (Table 4.1). The supernatant was assayed for the free drug and the amount of encapsulated drug into liposomes was estimated after deducting the drug concentration in the supernatant from the total amount added initially as described in section 3.1.

The assay for Indomethacin was also validated using ^{14}C labelled indomethacin. Liposomes were made incorporating the non-labelled indomethacin along with known activities of ^{14}C Indomethacin. The MLV were passed through a column prepacked with sephadex G-50 to separate the free drug from the encapsulated drug. 1.0ml aliquots were collected from the column, vortexed with 5ml of scintillation fluid and analysed on a beta counter (Fig 4.1). The results show that the encapsulation values obtained with the radioactive material were similar to that reported using the UV spectrophotometric assay (Table 4.2). Further experiments were carried out using the UV assay, which is a simple, reproducible and easy to use quantitative method.

Drug	Wave length (nm)	Concentration range	R^2 value	Centrifugation speed (rpm)/time (min)	Reference
Flurbiprofen	284	1-18 μg/ml	0.99	15000/35	Appendix1c
Indomethacin	320	0-35 μg/ml	0.99	13500/40	Appendix1e
Sulindac	324	0-25 μg/ml	0.99	14000/25	Appendix1d
Lignocaine	262	50-400 μg/ml	0.99	15000/30	Appendix1f
Progesterone	248	0-10 μg/ml	0.99	14500/30	Appendix1g

Table 4.1 Summary of the calibration curves. The calibration curves were made by dissolving the drug in PBS (pH 7.4) and an initial UV scan was done to determine the wavelength. The R^2 value for all the curves was 0.99. The centrifugation speed and duration were optimised for each drug. Values denote mean ± S.D. from at least three experiments.

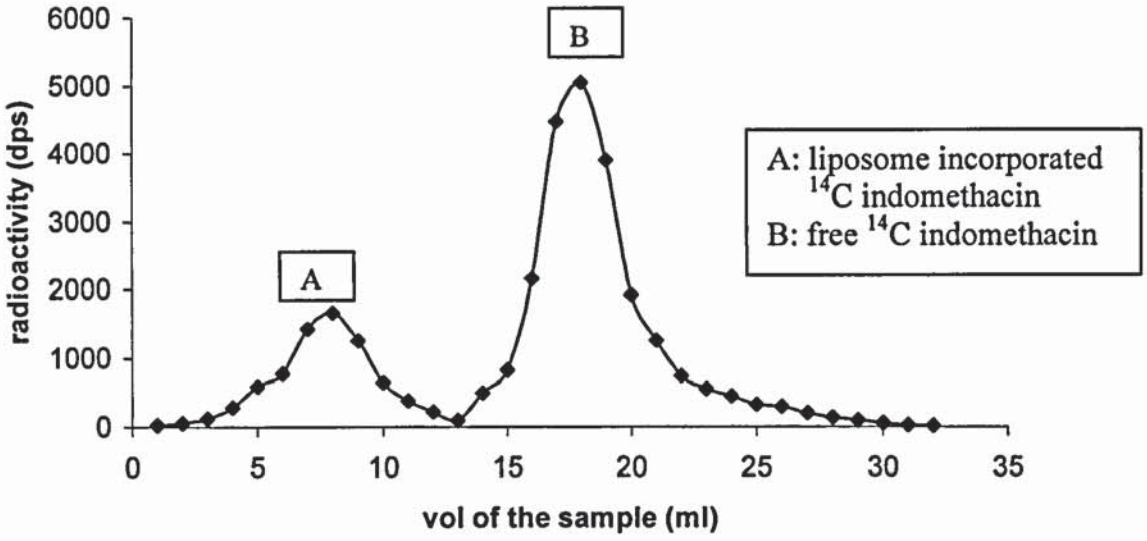


Fig 4.1 Encapsulation studies using radio labeled 14C indomethacin. The radio labeled indomethacin was encapsulated in liposomes composed of phosphatidylcholine (PC) and cholesterol (Chol) (16:4μmoles). The liposomes were separated from the free drug by column separation. Samples (1ml) were collected and analysed on a beta scintillation counter. Values denote mean ± S.D. from at least three experiments.

Formulation	UV assay (% Encapsulation)	Radioactive assay (% Encapsulation)
PC:Chol (16:4μmoles)	32.21 ± 2.6	31.3 ± 2.4

Table 4.2: Validation of encapsulation analysis. The encapsulation of indomethacin with liposomes composed of phosphatidylcholine (PC) and cholesterol (Chol) (16:4μmoles) was measured using UV assay at a wavelength of 320nm and radio labeled ^{14}C. The UV assay measured the amount of free drug in the supernatant after centrifugation of the liposome suspension whereas the radio assay was a direct measurement of the entrapped drug within the liposomes. Values denote mean ± S.D. from at least three experiments.

4.2 Drug loading of poorly soluble drugs in liposomal systems

To further investigate the influence of drug/lipid ratio in vesicle solubilisation systems, drug loading of a range of poorly soluble drugs (sulindac, progesterone, indomethacin, flurbiprofen and lignocaine; Table 4.3) were tested similar to the studies performed with ibuprofen in section 3.3.2. The results show that the mole/mole drug loading efficiency of all the drugs investigated increases with the increase in the concentration of the drug (Fig 4.2).

Drug	Mol wt	Solubility (μg/ml)	pKa	LogP	Nature of the functional group
Flurbiprofen	244.3	45	4.7	4.1	acidic
Indomethacin	357.8	80	4.5	3.2	acidic
Sulindac	356.4	20	4.7	3.4	acidic
Lignocaine	234.3	85	7.9	3.4	basic
Progesterone	314.5	40	-	3.9	neutral

Table 4.3 Summary of the drug characteristics. The various properties of the drug candidates investigated included molecular weight, size, logP and charge on the functional group.

However the results also show that the trend in increase in the drug/lipid ratio is also dictated by other properties of the drug candidates. Sulindac and flurbiprofen exhibit a higher loading into the bilayer essentially due to the strong surface-active nature and high logP (Table 4.3) respectively of the drug candidates (Pignatello et al., 2002). Sulindac and progesterone are also characterised by very poor water solubility of the order of 20μg/ml and 10μg/ml respectively, the lowest when compared to all the other drugs investigated. Thus a strong driving force to enter the lipid bilayer may be

expected for both the drug molecules despite their relatively lower log P. However the drug loading studies indicate that the surface active nature of the drug candidate has a greater influence on drug loading when compared to the low solubility profiles of the drug molecules. The surface activity of the drug molecules might enable the drug candidate to penetrate deeper into the alkyl chain length of the lipid molecule and provide improved retention in the bilayer. (Pignatello et al., 2002)

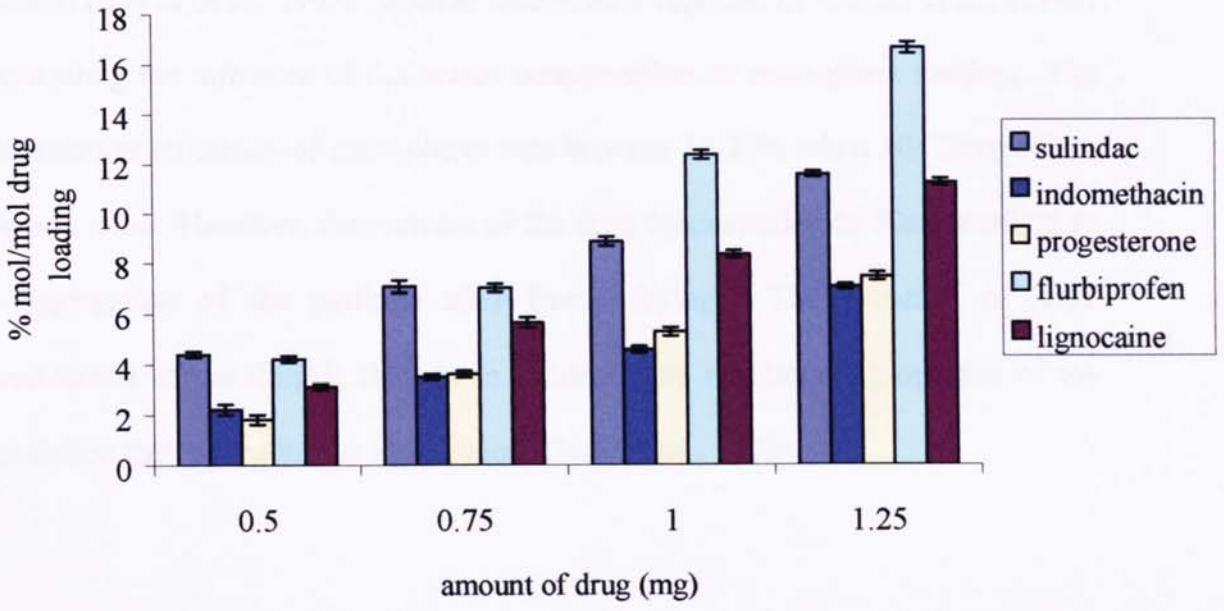


Fig 4.2: The effect of drug concentration on encapsulation into neutral liposomes. MLV were made using PC:Chol (16:4 μmoles). The drug was added in increasing amounts varying between 0.5mg to 1.25mg. Encapsulation of the drug was monitored as previously described in 2.2. The results represent mean ± S.D. from at least three experiments.

The drug/ lipid ratio has been an area of interest for some time as it affects the stability of the formulation and dictates the *in vivo* performance of the formulation (Gabizon et al., 1998). For example, research by Elorza et al., (1997) investigating the influence of the amphiphilic bile salts on the stability of the liposomal formulations composed of DPPC MLV have shown that the increase of the surfactant concentration increases its partitioning into the bilayer. The above results also show that with the increase of the amount of the added drug, the loading efficiency also is

enhanced. However when a saturation phase of the bilayer is reached, it begins to solubilise the bilayer resulting in the partial formation of the mixed micelles. Further increase of the surfactant ratio causes the complete disruption of the liposomal bilayer leading to the formation of mixed micelles. Thus the effective molar ratio (the amount present in the bilayer) of the amphiphile was thought to have a wider impact on liposomal stability than the total molar concentration (total amount present in the medium) (Elorza et al., 1997). Similar results were reported by Gorner et al., (1999) investigating the influence of lignocaine concentration on nanosphere loading. The encapsulation efficiency of nanospheres was between 18-20% when 10/ 25mg of the drug was used. However, the increase of the drug concentration to 50mg resulted in the aggregation of the particles after freeze drying. The presence of large concentration of the drug is thought to influence the mechanical properties of the formulation thereby leading to aggregation (Gorner etal., 1999).

4.3 Influence of drug properties on liposomal loading

To investigate the influence of drug properties including molecular weight, size, log P and solubility a range of drugs (sulindac, flurbiprofen, indomethacin, lignocaine, and progesterone) were tested. The phyisco-chemical properties of these drugs and their entrapment in liposomes composed of PC and Cholesterol (16:4 μmole) is shown in Table 4.4 and their effect on drug loading is shown in Table 4.4 and Fig 4.3.

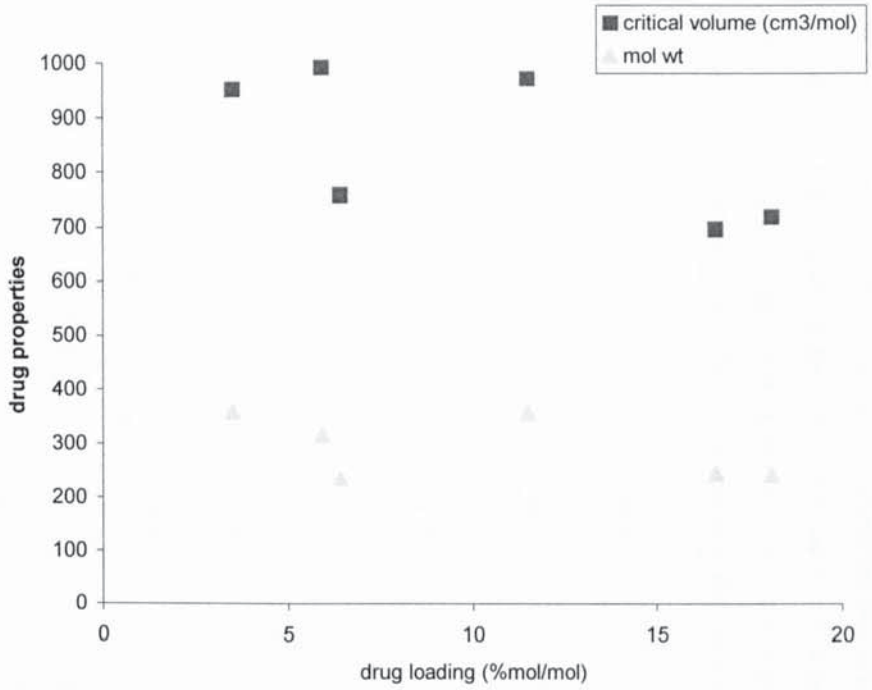


Fig 4.3 The relationship between mol/mol drug loading and the drug properties such as molecular weight and critical volume. Molecular weight was taken from the manufacturers data sheet and the critical volume calculated using CS Chem3D Pro 5.0; CambridgeSoft, Ma

Drug	Mol wt	Solubility in H_2O (μg/ml)	log P	% Entrapment	% mol /mol	Size (μm)	Zeta potential (mV)
Flurbiprofen	244.3	45	4.1	52.0 ± 0.9	16.6 ± 0.3	6.3 ± 0.8	-6.0 ± 1.3
Indomethacin	357.8	80	3.2	32.2 ± 2.5	3.5 ± 0.5	6.7 ± 0.4	-6.3 ± 1.6
Sulindac	356.4	20	3.4	52.4 ± 0.5	11.5 ± 0.10	6.6 ± 0.1	-5.0 ± 1.3
Mefenamic acid	241.3	10	5.3	56.0 ± 2.5	18.12 ± 1.8	6.4 ± 0.9	-3.8 ± 2.6
Lignocaine	234.3	80	3.4	33.6 ± 0.5	6.4 ± 0.10	4.9 ± 1.2	-3.4 ± 2.0
Progesterone	314.5	40	3.9	29.9 ± 5.3	5.9 ± 0.16	6.5 ± 0.6	-3.1 ± 4.9

Table 4.4 The effect of drug properties on liposome size, zeta potential and encapsulation. Flurbiprofen, indomethacin, sulindac, lignocaine and progesterone (1.25mg) were incorporated into MLV composed of phosphatidylcholine (PC) and cholesterol (Chol) (16:4μmoles) as described in section 2.1. Drug encapsulation efficiency within liposomes was determined as previously described in 2.2. Liposomes size (volume mean diameter) was determined by laser diffraction spectroscopy using a Malvern Mastersizer X at 20°C. The zeta potential of the MLV was measured in 0.001M PBS at 25°C using a Brookhaven Zetasizer. Values denote mean ± S.D. from at least three experiments.

4.3.1 Influence of molecular weight, drug properties and size

The results (Table 4.4) show that molecular weight may play a role in influencing drug encapsulation in the bilayer but is not the single factor affecting liposomal drug loading. A decrease in encapsulation was observed in the neutral liposomes with an increase in molecular weight and size of the drug molecule for flurbiprofen, mefenamic acid, progesterone and indomethacin. Mefenamic acid (Mol wt 241) showed an entrapment of 56 ± 2.5% followed by flurbiprofen (Mol wt: 244) with an entrapment of 52.0 ± 0.9% when compared to 32.2 ± 2.5% encapsulation with indomethacin (Mol wt: 358) and progesterone (Mol wt: 314) exhibiting intermediate encapsulation of 29.9 ± 5.3%. However, sulindac (Mol wt: 356) and lignocaine (Mol wt: 234) did not follow the pattern (Fig 4.3). Statistical variance analysis showed that the entrapment values were significantly different for all the compounds except for mefenamic acid and flurbiprofen that have similar molecular weights ($P<0.05$). Similarly, the effect of the molecular critical volume shows no direct correlation with drug loading, however there is a trend of increasing drug loading with decreased critical volume with again sulindac and lignocaine most notably deviating from this trend (Fig 4.3). Clearly, from this data none of these factors individually can be taken as indicative factors controlling drug loading. However, the general decrease in the encapsulation efficiency with the increase in the molecular weight/critical volume of the drugs could possibly be attributed to the reduced efficiency in packing the larger molecules into the bilayer. The arrangement of the drug molecules in the colloidal systems is a vital parameter influencing drug loading. Liposomal encapsulation of the pro drugs of 5-flouro-2' deoxyuridine (FudR) showed that the amount of prodrug incorporated was influenced by the molecular weight. When 5'-O-palmitoyl-FUdR

and 3',5'-O-dipalmitoyl-FUdR were incorporated into liposomes made of egg PC a maximum of 200 micrograms and 90 micrograms of the prodrug was encapsulated per mg egg phosphatidylcholine as lipid matrix (Supersaxo et al., 1988).

The results show that drug incorporation into the liposomal bilayer is also influenced by other physical characteristics of the drug molecules including the binding constant (K_B: the affinity of the drug candidate to the lipophilic environment), aqueous solubility and surfactant properties apart from the molecular weight. Sulindac is characterized by very poor water solubility of the order of 20μg/ml whereas flurbiprofen and indomethacin have solubilities of approximately 40μg/ml and 80μg/ml respectively. Previous studies investigating the association of indomethacin with zwitterionic detergent hexadecylphosphocholine, which is structurally similar to phospholipids, have demonstrated that ionised indomethacin is characterized by poor binding affinity to the lipophilic part of the detergent molecule (Castro et al., 2001), clearly a factor that would influence drug loading in liposomes. Similarly, NSAIDs have previously been reported to have a very predominant lipophilicity due to their low aqueous solubility and dissolution rate especially in their acidic form (Fini et al., 1984, 1988) and NSAIDs like ibuprofen, indomethacin and sulindac have also been shown to have surface active properties (Fini et al., 1995). The surface active property of the NSAIDs were studied by varying the drug concentration and the ionic strength of the medium (Fini et al., 1995). The results yielded separate categories of compounds. Firstly compounds like indomethacin which although surface active form aggregates at very low drug concentration in pure water and solvents with low ionic strength. Alternatively, sulindac molecules would only associate with each other in the presence of higher drug concentration and ionic strength when compared

to indomethacin (Fini et al., 1995). However, these drugs associate very strongly with the lipophilic environment due to the fact that hydrophilicity imparted to these molecules by the ionized carboxylic acid is superseded by the hydrophobicity of the rest of the molecule and the molecules insert themselves into the lipophilic environment (Fini et al., 1995). Therefore the higher entrapment of sulindac compared to indomethacin into the liposomal bilayer could be associated with this surface active property which could better enable sulindac to pack into the lipid bilayer. In contrast indomethacin although surface active, forms aggregates at low drug concentrations, leading to self association between molecules, the reduced need (in terms of achieving a state of minimum free energy) to solubilise within the liposome and a poor binding affinity for the lipophilic environment, resulting in decreased encapsulation into the liposomal bilayers.

Studies on flurbiprofen entrapment into polymers have shown that it exists as isolated molecules within the polymer network and penetrates deeper into the polymer network with improved retention in the polymer matrix (Pignatello et al., 2002). Flurbiprofen is characterized by the presence of two aromatic rings in its structure whereas lignocaine has a long alkyl chain consisting of five carbon atoms attached to a single aromatic benzene ring. The presence of aromatic rings in the structure of the molecules has a greater influence on the drug properties in that the aromatic ring imparts the molecule rigidity and lowers the association between the molecules thus preventing any aggregation (Fini et al., 1995). It may be possible that the structural arrangement of atoms in flurbiprofen lessens association between the molecules and the ability to penetrate deeper into the lipophilic environment enables it to better pack

in the bilayer matrix although slightly higher in molecular weight when compared to lignocaine.

4.3.2 The effect of log P of the drug

To investigate if the logP of the drug candidates could be taken as an indicative factor in predicting encapsulation/drug loading in liposomes the results were also considered from this aspect (Table 4.3). The results show that the encapsulation of the poorly water-soluble drugs generally increases with the increase in the log P of the drug (Fig 4.3) except for sulindac and progesterone (Table 4.4). Statistical analysis showed that the encapsulation values were significantly different from each other ($P<0.05$).

The increase in entrapment with the increase in the log P (the ability to partition in favour of the lipophilic environment when compared to the aqueous phase) of the drug candidate could possibly be attributed to the increase in lipophilicity of the drug molecule. Investigation into the entrapment of anthracyclines with similar structures into liposomes made from DSPC has shown that idarubicin entrapment was higher when compared to doxorubicin (Dos Santos et al., 2004). This increase in entrapment was attributed to the higher partition coefficient of idarubicin aiding in its transbilayer movement resulting in increased drug loading (Dos Santos et al., 2004). Similar results were also reported by Sasaki et al., (1986) suggesting that drug incorporation was related to the lipophilicity and partition coefficient of the drug candidates and it was proposed that drugs with higher log P were retained more effectively than molecules with intermediate logP suggesting the ability of the molecules to better

partition into the lipophilic environment. However, studies by Bradbury et al., (2002) demonstrated that molecular packaging and not Log P was a controlling factor in liposome bilayer loading of a series of benzoates. *In vivo*, investigations into the amount of dose absorbed by the subcutaneous administration of mixed micelles consisting of low molecular weight drugs showed that the log P of the drugs was directly proportional to the amount of the drug found in the lymphatic system. The results suggested that the drug lipophilicity is the primary pre requisite for improved therapeutic activity (Supersaxo et al., 1991).

However again, among the series of the drug candidates investigated not all those studied followed this trend of increasing Log P increasing drug loading, in particular sulindac and progesterone did not follow the trend. The deviation of sulindac from the trend and its ability to be better retained in the liposomal bilayer could, as mentioned possibly be due to the surface active nature of the molecule which enables it to penetrate deeper into the lipophilic bilayer. Similarly the low drug loading of progesterone could possibly be attributed to its molecular structure. Progesterone is a steroidal moiety characterized by a bulky structure. The inability of progesterone to accommodate into the liposomal bilayer due to its bulky structure could possibly be responsible for its low encapsulation despite its higher log P value.

4.3.3. Vesicle Characteristics (size and zeta potential)

The mean volume diameter of the vesicles may be influenced by several factors such as molecular weight, drug loading, and log P of the drug candidate. However the results (Table 4.4) show that it would be difficult to implicate any of these factors here as all the drugs investigated had a similar vesicle size in the range of 5-6.5μm.

The surface charge measurements (Table 4.4) show that all the formulations exhibited a near neutral surface charge indicating that the drug tested in these studies had no significant influence on the charge of the vesicle bilayer.

4.3.4 Release studies

The aqueous solubility of drug candidates is in the order of sulindac< flurbiprofen/progesterone< lignocaine/indomethacin (Table 4.4). Measuring the release rates of the drugs into PBS at physiological temperature also demonstrates a similar trend with sulindac releasing ~22% of the total amount of the encapsulated drug whereas -indomethacin and lignocaine loaded vesicles released ~35% in the same time period of 24 hr with flurbiprofen and progesterone exhibiting intermediate release (Fig 4.4). This correlation suggests that the higher solubility of the drug molecule in the polar environment may to some extent promote diffusion from the bilayer enabling a higher release.

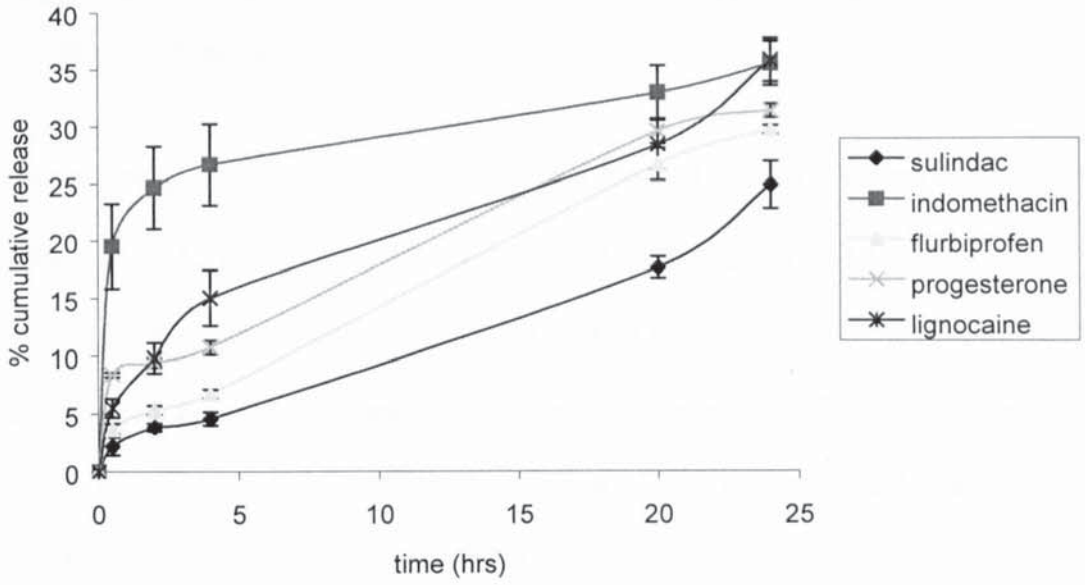


Fig 4.4: The effect of drug properties on the release profile from neutral liposomes. MLV incorporating the different drugs composed of: 16μmoles PC, 4μmoles Chol were incubated 0.01M PBS at 37°C for up to 24 h. At selected time intervals samples were assayed for ibuprofen release as described in section 2.4 and expressed as % of total incorporated. Results represent mean ± standard deviation, n=3 of percentage cumulative release in PBS at 37°C.

4.4 The influence of charged bilayers on drug incorporation and release

4.4.1. Drug Incorporation

To investigate the influence of the addition of a charged lipid to a neutral formulation, stearylamine (SA) (2μmoles; cationic lipid) and dicetyl phosphate (DCP) (2μmoles; anionic lipid) were added individually to PC:Chol (16:4μmoles) MLV. The influence of the charged lipids was tested with an array of drugs of different characteristics: sulindac, flurbiprofen, indomethacin (anionic at pH 7.4), lignocaine (cationic at pH 7.4) and progesterone (no charge at pH 7.4).

The addition of the charged lipid to the neutral formulation had a significant effect on entrapment, size and zeta potential of the ionisable drugs (Table 4.5): the inclusion of the positively charged lipid SA within the vesicle composition increased the entrapment efficiency of all the anionic drugs investigated in line with the observations for ibuprofen (section 3.6). Sulindac had an increase of approximately 8.1% entrapment when compared to the neutral formulation whereas indomethacin and flurbiprofen incorporation increased by 14.8% and 20.1% respectively (Table 4.5). The enhancement in drug entrapment was significantly higher for all the drugs investigated ($P<0.05$) when compared to 'neutral' formulations. The analysis of mean volume diameter showed all the MLV investigated were in the size range of 4.5-7μm (Table 4.5). The positive charge on the surface of the liposomes reflects the presence of the cationic lipid head groups in the bilayer (Table 4.5).

The addition of the anionic lipid DCP lowered the entrapment of all the anionic drugs again in agreement with the findings for ibuprofen (section 3.6). The entrapment of sulindac decreased by 17.4% when compared to the neutral formulation whereas

indomethacin and flurbiprofen encapsulation decreased by 13.1% and 10.4% respectively (Table 4.5). The size analysis showed that the mean volume diameter was in the range of 5.5-7μm (Table 4.5). The zeta potential measurements show a reversal of charge to negative due to the presence of an anionic head group in the DCP.

The addition of the charged lipid to the neutral formulations encapsulating lignocaine (basic in nature) had an opposite effect to those demonstrated with the acid NSAID: inclusion of SA decreased the entrapment to 21% when compared to 34% in the case of neutral lipids. However the addition of DCP significantly ($P < 0.05$) enhanced the entrapment of lignocaine to 42% followed by an increase in size of the MLV. The zeta potential measurements showed a high positive surface with the addition of SA and a high negative surface charge when DCP was included. In contrast, progesterone bilayer loading did not show any significant differences ($P>0.05$) in entrapment with the addition of the charged lipids to the neutral PC:Chol formulation.

The results show that the presence of the charged lipids in the bilayer and the nature of the functional group have a major influence on drug entrapment characteristics. The anionic drugs investigated are characterised by the presence of a negatively charged carboxyl group at pH 7.4. Lignocaine (pKa 7.9) also exists in an ionic form (cationic) at pH 7.4. However progesterone lacks any ionisable groups and therefore exists in the unionised molecular form at pH 7.4. The increase in encapsulation with the ionisable drugs could be attributed to the interaction between the oppositely charged lipid head group and the ionized functional group of the drug candidate (Barratt et al., 1994). The decrease in encapsulation relative to PC:Chol liposomes

measured when similarly charged drugs and bilayers were combined suggests that repulsive interactions between these similarly moieties inhibits incorporation within lipid bilayers. Alternatively, the presence of opposite charges between the lipid head group and the incorporated drug is demonstrated in Table 4.5 and has also been demonstrated by Pignatello et al., (2002). Indeed recent investigations by Manosroi et al., (2004) published at a similar time to these reported studies (Mohammed et al., 2004) have shown that the presence of the charged lipids influences drug entrapment, stability and skin permeation of amphotericin B encapsulated into liposomes. In this study, neutral, cationic and anionic liposomes were tested for the entrapment of insoluble amphotericin B for delivery through the skin. The results showed that the entrapment was highest in the presence of the cationic lipid stearylamine (90%) when compared to the neutral and the anionic MLV. As previously noted, this increased encapsulation could be due to the electrostatic interactions between the opposite charges in combination with changes in the lateral packing of the liposome bilayers due to the presence of charged lipids in the liposome bilayer (Jahnig et al., 1979).

Drug	PC:Chol (16:4) MLV			PC:Chol:SA (16:4:2) MLV			PC:Chol:DCP (16:4:2) MLV		
	Entrapment (%)	Size (μm)	Zeta potential (mV)	Entrapment (%)	Size (μm)	Zeta potential (mV)	Entrapment (%)	Size (μm)	Zeta potential (mV)
Sulindac	52.4 ± 0.5*	6.6 ± 0.1	-5.0 ± 1.3	60.5 ± 0.1*	6.8 ± 0.1	31.1 ± 3.0	35.0 ± 1.2*	7.0 ± 0.4	-20.7 ± 3.6
Flurbiprofen	52.0 ± 0.9*	6.3 ± 0.8	-6.0 ± 1.3	72.1 ± 0.1*	6.6 ± 0.5	21.1 ± 3.0	41.6 ± 1.4*	6.1 ± 0.3	-18.2 ± 1.5
Progesterone	29.9 ± 5.3*	6.5 ± 0.6	-3.1 ± 4.9	28.4 ± 5.8*	5.8 ± 0.3	14.2 ± 4.5	26.5 ± 8.1*	6.4 ± 0.5	-9.6 ± 2.1
Indomethacin	32.2 ± 2.5*	6.7 ± 0.4	-6.3 ± 1.6	47.1 ± 5.0*	6.0 ± 0.3	21.2 ± 1.3	19.0 ± 2.5*	6.9 ± 0.1	-18.9 ± 4.3
Lignocaine	33.6 ± 0.5	4.9 ± 1.2	-3.4 ± 2.0	21.0 ± 4.0	4.5 ± 1.2	33.2 ± 8.5	42.0 ± 2.5	5.4 ± 0.5	-22.7 ± 4.2

Table 4.5: The effect of charged lipids on PC:Chol liposomes on liposome size, zeta potential and drug encapsulation. MLV were prepared from 16 μmol PC, 4μmol Chol and 2 μmol of either SA (cationic lipid) or DCP (anionic lipid) was added. MLV incorporating the different drugs were prepared as described in section 2.1. Drug encapsulation efficiency within liposomes was determined as described in section 2.2. Liposomes size (volume mean diameter) was determined by laser diffraction spectroscopy using a Malvern Mastersizer X at 20°C. The zeta potential of the MLV was measured in 0.001M PBS at 25°C using a Brookhaven Zetasizer. * Represents significantly different entrapment values ($P<0.05$). Values denote mean ± S.D. from at least three experiments.

4.4.2. Drug Release

The addition of the charged lipids increased the release rate of all the drugs studied as was previously observed in the case of ibuprofen (section 3.6). The addition of 2μmoles of either the cationic stearylamine (Fig 4.5) or anionic dicetyl phosphate (Fig 4.6) increased the release by ~30% after 24hr when compared to the neutral vesicles. The presence of the charged lipids resulted in the release of higher amounts of the encapsulated drug at time points as early as 2hr with the charged liposomes releasing ~10% of the drug when compared to ~5% in the case of neutral formulations (Fig 4.5 vs Fig 4.4 respectively).

The increase in the release could possibly be due to the presence of a charged bilayer surface (zeta potential; Table 4.5), which can influence the packaging behaviour of the lipids in the bilayer. As noted in section 3.6 the presence of a charged bilayer causes changes in the lateral packaging of the bilayer lipids (Jahnig et al., 1979) resulting in the variations in drug retention when compared to the neutral PC:Chol formulations (Fig 4.3). Indeed recent investigations by Manosroi et al., (2004) demonstrated that when amphotericin B was incorporated in vesicles containing stearylamine or dicetyl phosphate, it improved the release of the encapsulated drug with stearylamine liposomes showing ~10 fold increase when compared to the other formulations. The presence of charged moieties (stearylamine, dicetylphosphate) in the liposomal bilayer would also increase the water permeability due to its ionic nature resulting in the increased release of the encapsulated drug when compared to the neutral liposomal formulations.

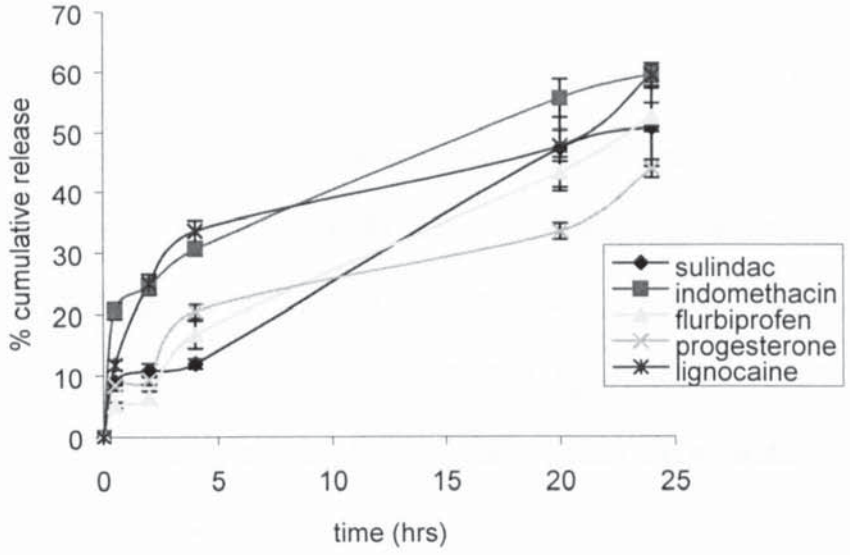


Fig 4.5: The effect of incorporation of charged lipids on the release profile of the different drugs from MLV. Multilamellar vesicles composed of: 16μmoles PC, 4μmoles Chol, 2μmoles SA were incubated 0.01M PBS at 37°C for up to 24 h. At selected time intervals samples were assayed for ibuprofen release as described in section 2.4 and expressed as % of total incorporated. Results represent mean ± standard deviation, n=3 of percentage cumulative release in PBS at 37°C.

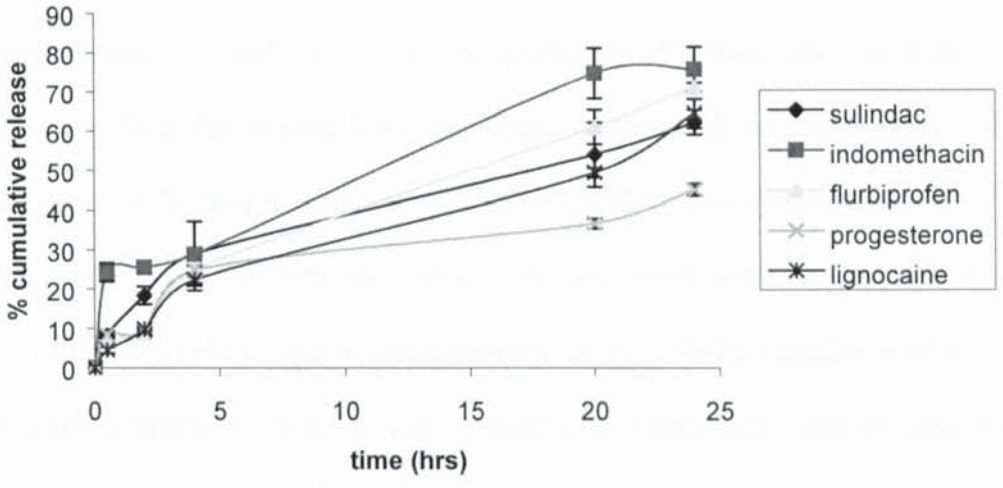


Fig 4.6: The effect of incorporation of charged lipids on the release profile from MLV. Multilamellar vesicles incorporating the different drugs composed of: PC, 4μmoles Chol, 2μmoles DCP were incubated 0.01M PBS at 37°C for up to 24 h. At selected time intervals samples were assayed for ibuprofen release as described in section 2.4 and expressed as % of total incorporated. Results represent mean ± standard deviation, n=3 of percentage cumulative release in PBS at 37°C.

4.4.3 Partitioning studies of free drug

Partitioning studies were carried out to investigate the association of free drug with the liposomal bilayer. The studies investigated the partitioning/association of free drug with MLV made from neutral PC:Chol (16:4μmoles), cationic PC:Chol:SA (16:4:2μmoles) and anionic PC:Chol:DCP (16:4:2μmoles) lipids. The drugs studied included flurbiprofen (negatively charged at pH 7.4), lignocaine (positively charged at pH 7.4) and progesterone (neutral at pH 7.4). The working temperature was 37°C and the buffer was PBS (pH 7.4).

Interestingly the results suggest that the association of the free drug with the bilayer is dictated both by the nature of the functional group of the drug candidate and the lipid composition of the liposomes. Previous studies with ibuprofen showed only minor association of the drug with the vesicles after incubation, and this was again shown with lignocaine. However in contrast, 90μg and 48 μg respectively of flurbiprofen and progesterone associated into the neutral liposomes after incubation for 24hr (Fig 4.7). However when the neutral liposomes were substituted for the cationic MLV the association of flurbiprofen increased to 110μg with no association measured for either progesterone or lignocaine (Fig 4.8). The incubation with the anionic MLV with lignocaine resulted in 37μg association after 24 hr with the negatively charged MLV. However minimal association was measured for flurbiprofen and progesterone. The results suggest that two factors play a prominent role in drug partitioning. The association of flurbiprofen and progesterone with the neutral MLV suggests that log P of the drug candidate controls the partitioning of the free drug into the bilayer in the case of neutral liposomes. Both flurbiprofen (logP 4.1) and progesterone (log P 3.9)

are characterised by high log P when compared to the other drugs investigated including ibuprofen (log P 3.6). Although it would be expected that flurbiprofen does not partition into the neutral bilayer, as was seen in the case of ibuprofen (section 3.7), it could be possible that the differences in the log P of the two drug molecules enables better partitioning of flurbiprofen into the bilayer. Drug loading studies (section 4.2) also point to the fact that flurbiprofen better partitions into the bilayer and is retained in the hydrophobic bilayer. The addition of the charged lipids to the neutral liposomes has shown that the nature of the functional group of the drug candidate also plays a vital role in drug partition. The addition of the cationic stearylamine improved the association of the anionic flurbiprofen possibly due to the electrostatic attraction between the oppositely charged lipids. Similar behaviour between lignocaine and the anionic lipid might be responsible for the partition/association of lignocaine with anionic MLV. Progesterone however did not partition/associate into the charged lipids possibly due to lack of any electrostatic association with the charged lipids.

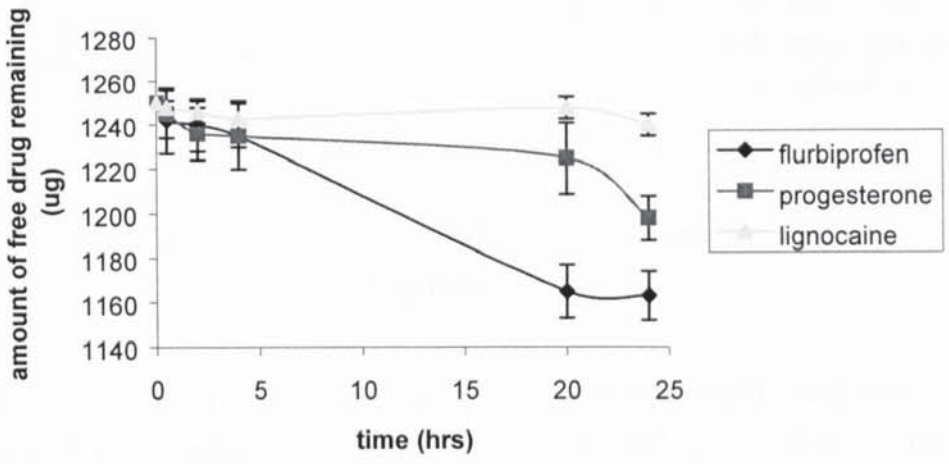


Fig. 4.7: The results show the partitioning behaviour of free drug (1.25mg) at pH 7.4 in PBS into neutral MLV composed of 16μmoles PC, 4μmoles Chol. Free drug (1.25mg) was added to the media and quantifying the free drug left in the media was used to assess the amount of partitioned drug. The results represent mean partition ± S.D, n=3.

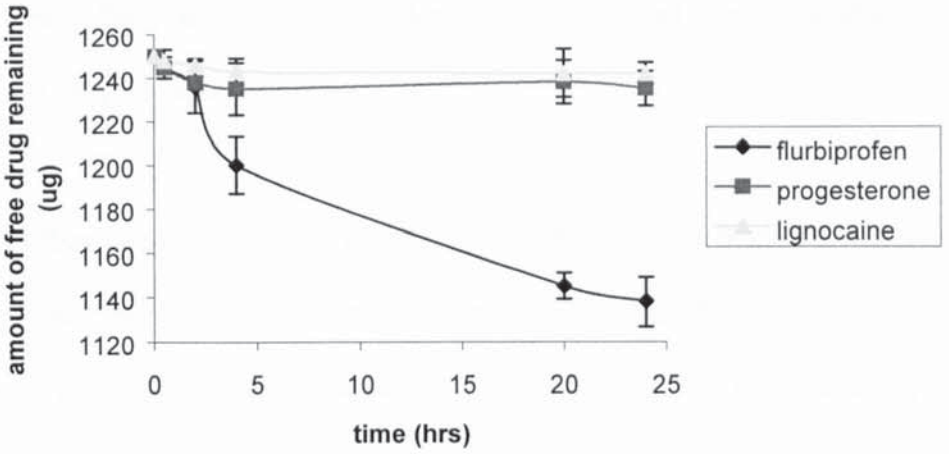


Fig. 4.8: The results show the partitioning behaviour of free drug (1.25mg) at pH 7.4 in PBS into cationic MLV composed of 16μmoles PC, 4μmoles Chol and 2μmoles of SA. Free drug (1.25mg) was added to the media and quantifying the free drug left in the media was used to assess the amount of partitioned drug. The results represent mean partition ± S.D, n=3.

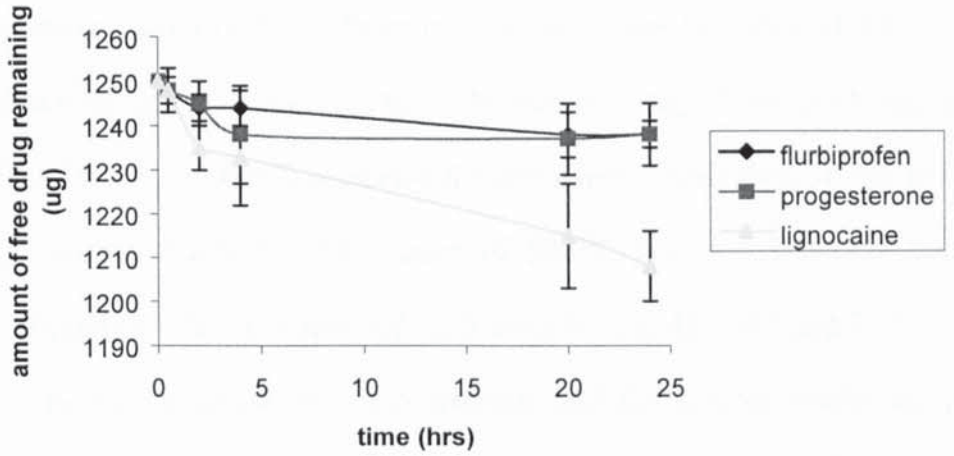


Fig. 4.9: The results show the partitioning behaviour of free drug (1.25mg) at pH 7.4 in PBS into anionic MLV composed of 16μmoles PC, 4μmoles Chol and 2μmoles of DCP. Free drug (1.25mg) was added to the media and quantifying the free drug left in the media was used to assess the amount of partitioned drug. The results represent mean partition ± S.D, n=3.

4.5 The influence of Alkyl chain length on liposomal drug solubilisation

4.5.1. Drug Loading

To further investigate our hypothesis that increasing the bilayer hydrophobic volume enhanced bilayer drug loading (section 3.4) the effect of varying the alkyl chain length of the lipids used within the liposome composition on encapsulation and release of three different drugs (sulindac, progresterone, indomethacin, and flurbiprofen) was studied. These four drugs were employed as they allow the effect of both charge and log P to be considered. In this study liposomes were prepared from Egg PC, DMPC (C14 alkyl chain length), or DSPC (C18 alkyl chain length) and combined with cholesterol at a 4:1 molar ratio.

The incorporation of these drugs into the liposomes is shown in Table 4.6 and the results confirm our initial findings: substitution of egg PC for the longer chain lipids (either DMPC or DSPC) increased the entrapment of all three of the poorly soluble drugs tested. Indeed the application of DMPC (Table 4.6) within the liposomes significantly ($P<0.05$) increased drug loading by 3.7, 43.5, 8.7 and 27.2% respectively for sulindac, progresterone, indomethacin, and flurbiprofen whilst the presence of DSPC significantly increased ($P<0.05$) the loading of these four drugs even further to 6.2, 49.2, 23.1 and 41.0% (Table 4.7). Whilst there was no significant difference in mean vesicle size or zeta potential for the different formulation, the data suggested a trend of increasing vesicle size with increased drug loading, as had previously been demonstrated in Section 3.4

As previously discussed (section 3.4), the increase in encapsulation in the longer chain lipids could possibly be attributed to the increased lipophilic bilayer area within the vesicle structure which would result from the longer alkyl lipid chains. Studies on entrapment of actinomycin D into long chain lipids based liposomes (Gregoriadis, 1973), and encapsulation of Cyclosporin A and other drugs into micelles (Francis et al., 2003; Neilsen et al., 2001) have shown that drug incorporation into these systems is promoted by lipid chain length with the longer alkyl chains both solubilising and retaining higher amounts of the drug in question. However, our results also demonstrate that the physico-chemical characteristics of the drug influence the extent to which enhanced solublisation is promoted. For example, flurbiprofen (Log P 4.1) encapsulation was enhanced to up to 93% in DSPC (C_{18}) liposomes however indomethacin (logP 3.2) encapsulation was not enhanced to a similar extent and only increased to 55% in the same liposome formulation. In contrast, both progresterone and sulindac which have log P values around 4 displayed higher drug incorporation values in DSPC liposomes compared to indomethacin suggesting that log P alone cannot be taken as indicative factor of drug solubilisation in liposomes. Other factors previously mentioned (section 4.3), may play a contributing role include bilayer packaging of the molecules within the bilayer and lipophilic interactions within the bilayer. For example, it has already been noted that indomethacin displays poor binding within the lipophilic environment (Fini et al., 1995)

4.5.2 Drug Release

The release profiles of indomethacin, sulindac, progesterone and flurbiprofen from both DMPC and DSPC liposomes was studied (Fig 4.10 & 4.11). Comparison between the two lipid formulations confirm previous findings with our ibuprofen studies that the nature of the lipid within the liposome composition influenced the rate of drug release, with the shorter chain/lower transition temperature lipid DMPC-based liposomes releasing more of their incorporated drug compared with their DSPC counter parts. However the time at which the differences on release became apparent, was dependent on the drug incorporated. For examples, after 1 hr ~4% of flurbiprofen and sulindac was released from both the DSPC and DMPC formulation. In contrast, at the same time point approximately 6% of indomethacin was released from the DSPC liposome formulation compared with 12% from the DMPC counterpart formulation suggesting that both the structural characteristics of the liposome bilayer and the drug characteristics are dictating the release rates.

Comparison between the release rates of the drug from similar formulation, reveal that with both the DMPC and DSPC liposome formulations, the cumulative drug release displayed a trend of indomethacin > progesterone > sulindac/flurbiprofen. In the case of the DSPC formulations, these differences between the drug formulations did not become significant ($P<0.05$) until the 20 hr time point, with initially around 3-5% of total incorporated drug being released over 4 hr with no significant difference between the four drugs (Fig 4.11). In contrast, drug release from the DMPC formulations already display the above trend as early as 2 hr with over 11.9% of the incorporated indomethacin being released at this time compared to 6.2, 4.2 and 3.7%

of progesterone, flurbiprofen and sulindac being released respectively (Fig 4.10). From this trend there is no clear indicative correlation between the drug's log P or molecular weight. Similar to the results presented in Fig 4.4, where the release profile of the four drugs were ranked indomethacin > progesterone > flurbiprofen > sulindac from PC-based liposomes there is a potential influence of drug solubility influencing drug release to an extent with the most soluble of the four drugs, indomethacin, displaying the highest release and sulindac releasing the lowest amount of incorporated drug in all formulations tested. However this trend is not displayed when cationic or anionic liposome formulations were investigated (Fig 4.5 and 4.6) suggesting that changes in the bilayer arrangements, as a result of incorporating charged surfactants may also contribute to the release rates of drugs from vesicle membranes making an indicative guide to such release profiles difficult at this stage to establish, and suggesting that any new formulations developed would need to be independently investigated.

Drug	%Encapsulation	Conc of drug in liposomes %mol/mol	Size (μm)	Zeta potential (mV)
Indomethacin	40.9 ±1.9	5.0 ± 0.4	7.4 ± 1.0	-1.2 ± 2.8
Sulindac	56.1 ±1.4	12.3 ± 0.3	6.5 ± 0.4	-2.9 ± 6.4
Flurbiprofen	79.2 ±1.2	25.3 ± 0.3	6.7 ± 0.4	-6.1 ± 3.0
Progesterone	73.4 ± 2.5	10.8 ± 0.4	7.3 ± 0.7	-3.4 ± 2.8

Table 4.6: The effect of lipid alkyl chain length on liposome size, zeta potential and encapsulation. Drug (1.25mg) was incorporated into MLV of DMPC:Chol (16:4μmoles) as described in section 2.1. The encapsulation efficiency within liposomes was determined as previously described in 2.2. Liposomes size (volume mean diameter) was determined by laser diffraction spectroscopy using a Malvern Mastersizer X at 20°C. The zeta potential of the MLV was measured in 0.001M PBS at 25°C using a Brookhaven Zetasizer. Values denote mean ± S.D. from at least three experiments.

Drug	%Encapsulation	Conc of drug in liposomes %mol/mol	Size (μm)	Zeta potential (mV)
Indomethacin	55.3 ± 1.3	6.7 ±0.2	7.5 ± 0.4	-2.3 ± 0.8
Sulindac	58.6 ± 0.5	12.8 ± 0.1	6.7 ± 0.2	2.4 ± 3.0
Flurbiprofen	93.1 ± 0.5	29.7 ± 0.2	6.8 ± 0.6	-3.4 ± 0.5
Progesterone	79.1 ± 1.5	11.2 ± 0.4	7.6± 0.1	-2.1 ±1.0

Table 4.7: The effect of lipid alkyl chain length on liposome size, zeta potential and drug encapsulation. Drug (1.25mg) was incorporated into MLV of DSPC:Chol (16:4μmoles) as described in section 2.1. The encapsulation efficiency within liposomes was determined as previously described in 2.2. Liposomes size (volume mean diameter) was determined by laser diffraction spectroscopy using a Malvern Mastersizer X at 20°C. The zeta potential of the MLV was measured in 0.001M PBS at 25°C using a Brookhaven Zetasizer. Values denote mean ± S.D. from at least three experiments.

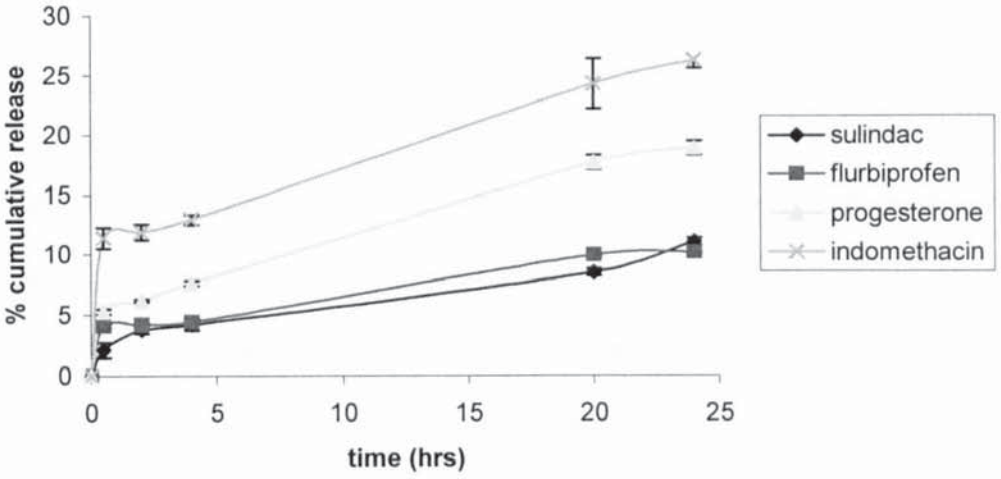


Fig 4.10: The effect of lipid alkyl chain length on the release profile of the different drugs from liposomes. MLV composed of: 16μmoles DMPC, 4μmoles Chol were incubated in 0.01M PBS at 37°C for up to 24 h. At selected time intervals samples were assayed for ibuprofen release as described in section 2.4 and expressed as % of total incorporated. Results represent mean ± standard deviation, n=3 of percentage cumulative release in PBS at 37°C.

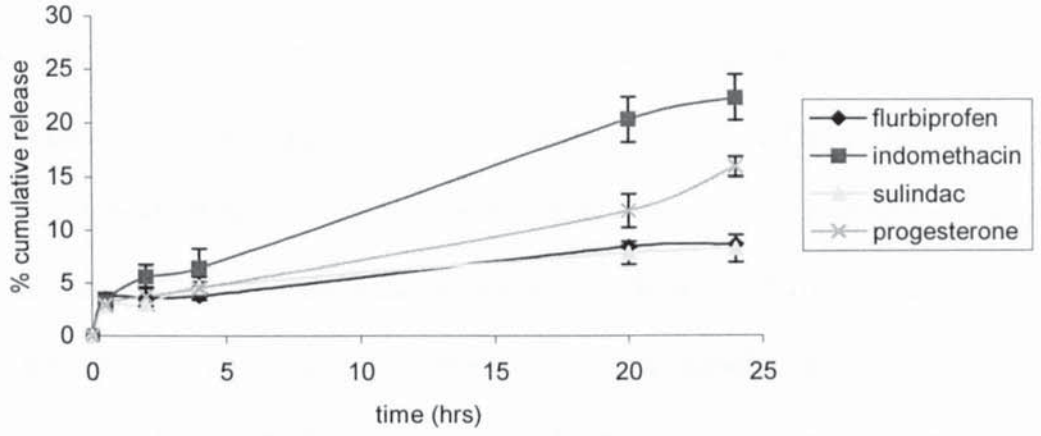


Fig 4.11: The effect of lipid alkyl chain length on the release profile of the different drugs from liposomes. MLV composed of: 16μmoles DSPC, 4μmoles Chol were incubated in 0.01M PBS at 37°C for up to 24 h. At selected time intervals samples were assayed for ibuprofen release as described in section 2.4 and expressed as % of total incorporated. Results represent mean ± standard deviation, n=3 of percentage cumulative release in PBS at 37°C.

4.6 The effect of alpha tocopherol

To further investigate the role of alpha tocopherol in improving the solubilisation of the insoluble drugs, drug candidates with different logP and drug characteristics were studied. The concentration of the alpha tocopherol investigated was 1, 3 and 4μmoles with PC:Chol (16:4μmoles) as differences in encapsulation were noted with ibuprofen (section 3.8) with the above concentration ranges.

The influence of the addition of α-tocopherol was investigated with flurbiprofen (log P 4.1), Sulindac (log P 3.4) and progesterone (log P 3.9) to determine if the log P of the drug candidate was related to improved solubilisation in the presence of α-tocopherol. The results show that the addition of α-tocopherol influences drug encapsulation and size and is possibly related to the log P of the drug candidate (Table 4.8). Inclusion of 1, 3 and 4 μmoles of α-tocopherol in the neutral PC:Chol formulation incorporating sulindac did not have any effect. However when flurbiprofen and progesterone were investigated, the results show that the addition of the surfactant does affect drug incorporation. The addition of 1μmole of α-tocopherol did not have any significant influence on drug incorporation when compared to the neutral formulations both in the case of flurbiprofen and progesterone ($P>0.05$). However increase of concentration to 3 and 4 μmoles increased the entrapment of the drug. Similar results were also obtained with ibuprofen as discussed in section 3.8. The mean volume diameter of the MLV was in the range of 5-6μm with no significant effect on the zeta potential of the formulation.

The properties of α-tocopherol partly resemble cholesterol in being insoluble and a non-swelling amphiphile. It may be possible that thorough investigation is needed to optimise the concentration of α-tocopherol in a liposome formulation to improve solubility. The concentration of cholesterol also needs to be optimised as variations in entrapment have been reported with the change of cholesterol concentration (Mohammed et al., 2004). Cholesterol due to its amphiphilic nature plays a dual role of influencing bilayer permeability and rigidity. An optimum concentration of cholesterol is therefore needed to prevent low entrapment of drugs due to higher fluidity and low permeability of the bilayer (Dos Santos et al., 2004). Nacka et al., (2001) have shown that the effect of tocopherol is dependent on its concentration and similar behaviour was also reported in section 3.8 when ibuprofen entrapment was assessed with different concentrations of the surfactant. However previous research has also pointed out that drug solubilisation using micelles with the addition of α-tocopherol was also dependent on the log P of the drug candidates (Nielsen et al., 2001). The addition of tocopherol to formulations encapsulating griseofulvin (log P 2.2) significantly improved the entrapment whereas drugs with higher log P Lu28-179 (log P 8), felodipine (log P 4.8) saw a decrease in encapsulation with the addition of α-tocopherol. Our results suggest that the addition of α-tocopherol to liposomal systems can benefit encapsulation in some cases but this is dependent on the log P of the drug candidate.

Drug	PC:Chol (16:4) MLV with 1μmole of tocopherol			PC:Chol (16:4) MLV with 3μmole of tocopherol			PC:Chol (16:4) MLV with 4μmole of tocopherol		
	Entrapment (%)	Size (μm)	Zeta potential (mV)	Entrapment (%)	Size (μm)	Zeta potential (mV)	Entrapment (%)	Size (μm)	Zeta potential (mV)
Sulindac	51.4 ± 0.9	5.2 ± 0.8	-2.6 ± 2	49.2 ± 2.8	5.6 ± 0.9	-1.4 ± 2	51.5 ±1.3	5.1 ± 0.5	-3.8 ± 3
Flurbiprofen	51.8 ± 1.8*	5.8 ± 1	-1.8 ± 4.3	55.6 ± 0.8*	6.3 ± 0.3	-3.2 ± 4	57.8 ± 0.7*	6.3 ± 0.6	-2.4 ± 3
Progesterone	31.5 ± 3.2*	5.1± 0.8	-3.3 ± 2.5	34.8 ± 1.2*	5.5 ± 0.5	-2.2 ± 0.5	36.4 ± 0.5*	5.7 ± 0.3	-3.5 ± 0.5

Table 4.8: The effect of tocopherol concentration on drug encapsulation, MLV size and zeta potential. The MLVs were prepared using various amounts of tocopherol (1-, 3 and 4μmoles) keeping the ratio of PC:Chol (16:4) constant. Liposomes size (mean volume diameter) was determined by laser diffraction spectroscopy using a Malvern Mastersizer X at 20°C. The zeta potential of the MLV was measured in 0.001M PBS at 25°C using a Brookhaven. * Represents significantly different entrapment values ($P<0.05$).

4.7 Conclusions

The results demonstrate that liposomes could be effectively employed in enhancing the solubility of the poorly water soluble drugs. However properties of the drug molecules must be considered collectively when designing liposome-based solubilisation systems. The presence of the oppositely charged lipids within the bilayer has shown to improve drug loading with similar results being obtained with the substitution of the longer alkyl chain lipids. The liposomal solubilisation can also be possibly exploited to control the release characteristics *in vitro* with the charged lipids showing a relatively faster release when compared to the slow release in the case of longer chain lipids. The addition of alpha tocopherol to the bilayer may carry an additional advantage of improvement in solubilsation provided the concentration is optimised as demonstrated by the results (section 4.6).

Chapter 5

5.0 Freeze-drying of liposomes

Liposomes have been successfully employed for the encapsulation of various classes of synthetic drugs (actinomycin; Gregoriadis et al., 1973: oestradiol; Elmaghraby et al., 1999: ibuprofen; Mohammed et al., 2004) and biologicals (e.g. DNA; Kroll et al., 1997: Perrie et al., 2000: protein; Parmar et al., 1999). However the success of a formulation not only depends on its ability to encapsulate and deliver the active moiety, but is also judged by its ability to retain its constituents during storage and hence liposomes are often characterized by drug leakage, vesicle fusion or bilayer disintegration during storage. In particular the arrangement of phospholipids within the bilayer of small vesicles is characterized by loosely packed headgroups and tightly packed alkyl chains in the outer layer with the opposite arrangement in the inner layer of the bilayer. This leads to a thermodynamically unstable state, which leads to aggregation and/or fusion of the vesicles to counteract this instability (Komatsu et al., 2001).

To circumvent this problem, freeze-drying has been used as an effective approach to render the liposomes stable without compromising their physical state or encapsulation capacity. However, freeze-drying of liposome systems without appropriate stabilizers will again lead to fusion of vesicles- a factor exploited by the dehydration-rehydration method (Kirby and Gregoriadis, 1984). To promote stability during the freeze-drying process most commonly studied cryoprotectants include saccharides (sucrose, trehalose, lactose) and their derivatives (Bendas et al., 1996).

A detailed review by Crowe et al., (1988) outlines the mechanism of stabilization offered by the saccharides. The leakage of the encapsulated material in the liposomes during freeze-drying was attributed to two parameters: phase separation of lipids around their gel-fluid transition temperature and fusion of the membranes of the liposomes. Thus materials that could possibly exhibit the formation of stable boundaries between the liposomes could prevent aggregation of the vesicles. The mechanism of protection offered by the cryoprotectants has been attributed to different factors. Crowe et al., (1988) have proposed that the cryoprotectants function by replacing the bound water of the lipid head group. They suggested that the polar region of the lipid head group interacts with the cryoprotectant thus replacing the water around the bilayer (Water replacement hypothesis). Alternatively, Koster et al (1994) proposed the formation of a vitrious layer (glass formation around the bilayer) of the cryoprotectant around the bilayer, which depresses the transition temperature of the phospholipids thus preventing any drug leakage during gel to fluid phase transformations. Hydrogen bond formation between the cryoprotectant molecules and/or also the lipid head group was also proposed as a possible means for cryoprotection (Crowe et al., 1987).

In light of this, various saccharides (glucose, sucrose, trehalose etc) have been investigated thoroughly as stabilizing solutes (Crowe et al., 1994; Suzuki et al., 1996; Miyajima, 1997; Wolkers et al., 2004). However these detailed investigations, the fluctuations in the degree of effectiveness have not been thoroughly evaluated (Crowe et al., 1994) and is currently not understood, therefore cannot be fully exploited.

The aim of the work was to assess alternative cryoprotectants, in particular amino acids, as stabilizing moieties during the freeze-drying of SUV. Previous investigations involving amino acids as cryoprotectants included work by Crowe et al., 1994 that investigated proline and Osterberg, 1993 (Histidine). This current work emphasizes on basic, polar amino acids, which include arginine (Fig 5.1), lysine (Fig 5.2) and histidine (Fig 5.3) structures. The freeze-drying efficiency of these amino acids was compared to that offered by trehalose, the most commonly used cryoprotectant sugar.

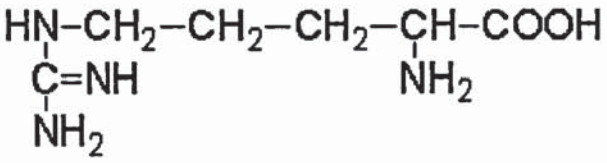


Fig 5.1 Structure of the amino acid arginine

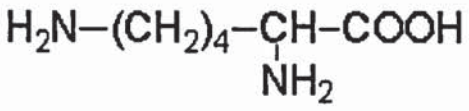


Fig 5.2 Structure of the amino acid lysine

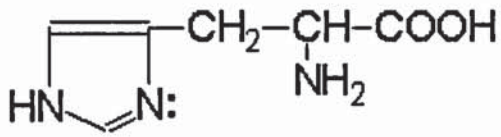


Fig 5.3 Structure of the amino acid histidine

5.1 Amino acids as cryoprotectants: influence of cryoprotectant concentration

To investigate the efficiency of amino acids as cryoprotectants, PC:Chol (16:4μmoles) SUV were subjected to lyophilisation in the presence of various concentrations of the cryoprotectants ranging from 2 moles to 10 moles per mole of the lipid. All formulations were pre frozen at –70°C for 20 min followed by drying which was carried out in two stages: –50°C for 10 h and at –30°C for 24 h.

Size analysis prior to freeze-drying revealed fairly uniform SUV below 120nm (Table 5.1). As expected, despite the addition and increase in concentration of the amino acids from 2 moles per mole of lipid to 10 moles per mole of lipid to the SUV suspension, the vesicles size was not affected, demonstrating that the presence of amino acids in the suspension does not influence SUV size prior to freeze-drying. However rehydration of the formulations after freeze-drying revealed that the concentration of the cryoprotectant influences the size significantly (Table 5.2). In the absence of amino acids, SUV fusion resulted in rehydrated vesicles in the size range of ~ 3 μm, over 30 times bigger than the original lyophilized SUV. Alternatively all the cryoprotectants tested inhibited to some degree this fusion with the molar ratio of 4/1 being optimal for all the amino acids and the carbohydrate trehalose. Interestingly, SUV freeze dried in the presence of both lysine and histidine began to demonstrate a significantly higher size (t test; $P<0.001$) when the concentration was increased above 8 moles per mole of the lipid. Cryo-stabilized SUV containing trehalose however did not show such behavior with vesicle size remaining low even at a concentration of 10 moles per mole of lipid.

The process of freeze drying involves three stages: prefreezing (initial freezing of the liposmal suspension) primary drying (water loss from the formulation by sublimation without the intermediate liquid stage) secondary drying (removal of any adsorbed surface bound water to obtain a free flowing dry powder). Previous investigations by Suzuki et al., (1996) studying the effect of concentration of glucose oligomers on freeze-drying have reported that the stabilization offered by these cryoprotectants is influenced by both the concentration and molecular size of the cryoprotectant with DPPC liposomes lyophilized in the presence of maltotriose aggregating when either 7 mM or 15mM was used. However an intermediate concentration of 10mM was found to be optimal for stabilizing such vesicles. Similarly when PC and DOPC vesicles were freeze-dried with maltodextrins, which has a large number of glucose residues it resulted in the fusion of the liposomes and a measured increase in vesicle size (Suzuki et al., 1996). The protection offered was significantly high when small molar ratios of glucose derivatives were used. An increase in glucose residues has been suggested to promote the hydrophobic interactions between the saccharides and the liposomal membrane thereby leading to fusion or aggregation of the liposomes (Miyajima 1997). Investigations by Partida et al., (1992) have also shown that the cryoprotection offered by proline and glycine-betaine was concentration dependent with 54mM proline and 53mM glycine-betaine protecting the freeze dried ram spermatozoa. Conversely, higher concentrations of the amino acids lowered the sperm motility.

Histidine is characterized by the presence of a penta cyclic ring in its structure whereas arginine has three NH_2 groups (branched structure) when compared to two NH_2 groups in lysine. All the amino acids investigated are charged molecules capable of forming hydrogen bonds with the phosphate of the lipid head group, which is an

important feature, exhibited by most of the saccharides (Crowe et al., 1994). During freeze-drying it may be possible that the amino acids interact electrostatically with the phosphate head group in the lipid molecule thereby forming a barrier between the individual liposomes. These layers of amino acids could possibly be acting as cushions preventing any damage during freeze-drying (Kundu et al., 2001). Previous investigations involving the use of amino acids as cryoprotectants for the stabilization of sarcoplasmic reticulum during freeze thawing have shown that the protection offered by the amino acids is inversely proportional to their concentration (Lalonde et al., 1991). The increase in concentration of the cryoprotectants alters the hydrophobic environment around the vesicles with higher concentrations promoting fusion (Miyajima 1997). A molar ratio of 4 per mole of lipid was optimum for all the amino acids investigated. The increase of molar concentration to 8 moles of the amino acid may influence the hydrophobic environment to a higher degree in the case of straight chain lysine and cyclic histidine when compared to the branched structure of arginine thus promoting fusion as was reported for saccharides (Crowe et al., 1994). Our results (Table 5.2) reveal a biphasic nature of cryoprotection offered by the amino acids as was reported by Kundu et al., (2001). In the studies, the freeze-drying ability of proline, glutamine, histidine, arginine, lysine, alanine and glycine was investigated for the cryoprotection of goat sperm cells. The initial increase in the concentration of the cryoprotectants (upto 120mM) stabilized the motility of the sperm cell on rehydration but further increases resulted in the loss of the motility of the sperm suggesting cell damage upon rehydration.

Investigations on the protection offered by the amino acids were also extended to ibuprofen encapsulated SUV. The size analysis before freeze-drying showed SUV

with size range below 200nm irrespective of the presence of cryoprotectant. Upon freeze-drying and rehydration, the ibuprofen loaded SUV showed a similar size pattern (Table 5.3) as that observed with the unloaded SUV. The increase in size of the formulations corresponded with the increase in the molar ratio of the amino acids. Thus the presence of the encapsulated drug in the bilayer did not influence the cryoprotection offered by the amino acids suggesting that ibuprofen does not interact with the cushioning effect of amino acids and probably is associated with the lipid alkyl chain length (Moahmmed et al., 2004).

Cryoprotectant	Size (nm) of SUV with varying concentration of cryoprotectant (mol of cryoprotectant/ mol of lipid)				
	2/1	4/1	6/1	8/1	10/1
Control	83 ± 21	95 ± 4	123 ± 15	89 ± 21	134 ± 6
Trehalose	121 ± 12	79 ± 12	98 ± 12	93 ± 9	109 ± 21
Lysine	89 ± 3	95 ± 14	137 ± 12	93 ± 8	121 ± 9
Histidine	127 ± 13	147 ± 9	126 ± 20	118 ± 13	127 ± 23
Arginine	156 ± 25	167 ± 8	109 ± 16	126 ± 17	99 ± 12

Table 5.1 The effect of cryoprotectant concentration on PC:Chol liposome size before freeze-drying. SUV were prepared from 16 μmol PC and 4 μmol of cholesterol. SUV containing the cryoprotectants were prepared as described in section 2.2.12. Liposomes size (volume mean diameter) was determined by laser diffraction spectroscopy using a Brookhaven Zetaplus at 25°C. Values denote mean ± S.D. from at least three experiments.

Cryoprotectant	Size in nm of SUV with varying concentration of cryoprotectant (mol of cryoprotectant/ mol of lipid)				
	2/1	4/1	6/1	8/1	10/1
Control	2857 ± 235	3988 ± 210	2867 ± 99	2988 ± 522	3121 ± 640
Trehalose	147 ± 12	145 ± 9	130 ± 15	148 ± 12	475 ± 21
Lysine	298 ± 9	106 ± 7	393 ± 23	1517 ± 80	1567 ± 19
Histidine	450 ± 4	200 ± 12	483 ± 23	834 ± 23	1523 ± 15
Arginine	303 ± 21	180 ± 18	523 ± 18	246 ± 13	1862 ± 50

Table 5.2 The effect of cryoprotectant concentration on PC:Chol liposomes size after freeze-drying. SUV were prepared from 16 μmol PC and 4 μmol of cholesterol. SUV containing the cryoprotectants were prepared as described in section 2.2.12. Liposomes size (volume mean diameter) was determined by laser diffraction spectroscopy using a Brookhaven Zetaplus at 25°C. Values denote mean ± S.D. from at least three experiments.

Cryoprotectant	Size in nm of SUV with varying concentration of cryoprotectant (% mol of cryoprotectant/ mol of lipid)				
	2/1	4/1	6/1	8/1	10/1
Control	3878 ± 213	4090 ± 250	4435 ± 129	3884 ± 235	3986 ± 515
Trehalose	443 ± 17	235 ± 12	399 ± 23	398 ± 21	654 ± 19
Lysine	437 ± 13	189 ± 5	486 ± 25	1356 ± 91	774 ± 38
Histidine	538 ± 25	176 ± 13	644 ± 9	754 ± 18	1653 ± 24
Arginine	389 ± 15	184 ± 4	672 ± 18	653 ± 12	1752 ± 32

Table 5.3 The effect of cryoprotectant concentration on PC:Chol liposomes incorporating ibuprofen on liposome size after freeze-drying. SUV were prepared from 16 μmol PC and 4 μmol of cholesterol with 1.25mg of added ibuprofen. SUV containing the cryoprotectants were prepared as described in section 2.2.12. Liposomes size (volume mean diameter) was determined by laser diffraction spectroscopy using a Brookhaven Zetaplus at 25°C. Values denote mean ± S.D. from at least three experiments.

5.2 Influence of pre-freezing temperature on the performance of the cryoprotectants

To further assess the influence of temperature on pre-freezing unloaded formulations (4/1) were frozen at -70°C, -50°C and –30°C. The primary drying was carried out at –50°C, -40°C and –20°C respectively for 10h (Table 5.4). The secondary drying was done at –30°C, -25°C and –10°C for 24h respectively. The results (Table 5.4) show that the pre-freezing temperature has an influence on the liposome characteristics. The formulations frozen at –70°C and –50°C were capable of efficiently offering protection during freeze-drying with rehydrated liposome size remaining below 200 nm with cryoprotectants. In contrast rehydrated SUV pre-frozen at -30°C in the presence of cryoprotectant were significantly larger with vesicle sizes similar to liposomes freeze-dried in the absence of cryo-protectants (Table 5.4). This data demonstrates that all SUV pre-frozen at -30°C were subject to fusion.

Studies by Crowe et al., (1994) have shown that the cryoprotection offered by the various solutes is influenced by various factors including the transition temperature of the lipids, Tg of the solutes and molar ratio of the solutes. Trehalose (Tg=-30°C) and dextran (Tg=-10°C) were tested using liposomes made from DPPC. When the samples were frozen below the Tg for over 4hr, they retained most of the encapsulated material. However when the frozen samples were warmed to temperatures above the Tg of the cryoprotectant for less than 4hr, it resulted in complete leakage of the entrapped material. This was attributed to the increased mobility of the solutes at temperatures higher than the Tg which eventually cause the encapsulated material to leak. Thus the formation of a glassy phase (vitrification)

during freezing of the sample is also one of the requirements for efficient cryoprotection (Crowe et al., 1994). Miyajima, (1997) have reported that either the formation of the glassy solid or the visco elastic liquid in the interstices of the SUV prevents them from aggregation or fusion. The poor size reproducibility of the various cryoprotectants can possibly be attributed to the high freezing temperature. Prefreezing temperatures of –70°C and –50°C followed by primary drying temperatures of –50°C and -40°C respectively for 10h are below the Tg for all the solutes employed. However pre-freezing at –30°C followed by drying at –20°C did not protect the formulation possibly due to lack of formation of the glassy state thereby promoting fusion of the liposomes.

Investigating for the influence of duration of primary drying (Table 5.5) showed that there were no significant differences in the sizes of SUV (t test; $P< 0.001$) when the drying was carried out at -40°C for 6, 10 or 14hr (Table 5.5). The results are in agreement with those reported by Crowe et al., 1994 emphasizing that liposomes dried for more than 4hr are stable. The primary drying is characterized by sublimation, which involves the gradual loss of the moisture when a difference in vapour pressure is maintained between the ice interface and condensor (Mellor, 1978). The temperature of the sublimation phase determines the moisture content of the formulation. Temperatures below the transition temperature for the cryoprotectants favour the loss of the moisture to as low as 0.05mg water per mg of dry weight of the formulation. The surface adsorbed moisture is however lost during the secondary phase of drying which involves conditioning the sample surface with minimal interaction with the moisture (Crowe et al., 1994).

Formulations	-70°C freezing temperature		-50°C freezing temperature		-30°C freezing temperature	
	Size (nm) before freezing	Size (nm) after freeze-drying	Size (nm) before freeze-drying	Size (nm) after freeze-drying	Size (nm) before freezing	Size (nm) after freeze-drying
Control	105 ± 21	3654 ± 401	89 ± 6	4563 ± 341	94 ± 32	4554 ± 348
Trehalose	98 ± 14	156 ± 23	118 ± 21	167 ± 12	129 ± 14	2434 ± 281
Lysine	112 ± 9	199 ± 21	165 ± 14	145 ± 14	89 ± 9	3889 ± 92
Histidine	103 ± 8	142 ± 12	99 ± 14	187 ± 6	122 ± 15	4750 ± 373
Arginine	89 ± 25	145 ± 9	97 ± 13	146 ± 21	128 ± 13	4554 ± 274

Table 5.4 The effect of pre-freezing temperature on PC:Chol liposome size after freeze-drying. SUV were prepared from 16 μmol PC and 4 μmol of cholesterol. SUV containing the cryoprotectants were prepared as described in section 2.2.12. The formulations were frozen at –70°C, -50°C and –30°C. Liposomes size (volume mean diameter) was determined by laser diffraction spectroscopy using a Brookhaven Zetaplus at 25°C. Values denote mean ± S.D. from at least three experiments.

Formulations	6hr primary drying		10hr primary drying		14hr primary drying	
	Size (nm) before drying	Size (nm) after freeze-drying	Size (nm) before drying	Size (nm) after freeze-drying	Size (nm) before drying	Size (nm) after freeze-drying
Control	105 ± 12	3654 ± 222	89 ± 12	4563 ± 304	94 ± 15	4554 ± 382
Trehalose	98 ± 21	125 ± 12	128 ± 6	167 ± 21	119 ± 16	143 ± 32
Lysine	102 ± 12	145 ± 16	125 ± 12	143 ± 12	89 ± 13	128 ± 18
Histidine	113 ± 18	142 ± 24	99 ± 23	164 ± 18	122 ± 20	176 ± 16
Arginine	89 ± 19	145 ± 9	97 ± 13	165 ± 14	99 ± 13	118 ± 22

Table 5.5 The effect of primary drying duration on PC:Chol liposome size. SUV were prepared from 16 µmol PC and 4 µmol of cholesterol. SUV containing the cryoprotectants were prepared as described in section 2.2.12. The formulations were frozen at -50°C but were dried for 6, 10, 14hr at –40°C. Liposomes size (volume mean diameter) was determined by laser diffraction spectroscopy using a Brookhaven Zetaplus at 25°C. Values denote mean ± S.D. from at least three experiments.

5.3 Stability studies

Stability studies were carried out with SUV made from PC:Chol (16:4μmoles) incorporating 1.25mg ibuprofen (37.6% encapsulation) with 4 moles of the cryoprotectants per mole of the lipid. The concentration of the cryoprotectant was fixed at 4moles as previous studies demonstrated that (Table 5.4) fusion of the SUV was dependent on cryoprotectant concentration (Suzuki et al., 1996). The samples were tested for 42 days for drug leakage and size.

The results show that formulations made with lysine displayed improved stability and could be compared to those made from trehalose (Fig 5.4). Although the formulations consisting of histidine and arginine retained around 90% of the encapsulated drug, the size analysis reveal that fusion of vesicles had started to occur (Table 5.6). The enhanced stability offered by lysine with minimal leakage (2.5%) over 42 days shows that lysine protects the formulation in the freeze dried form more effectively. The size analysis of the formulations (Table 5.6) revealed that the drug leakage corresponds with the fusion of the liposomes with arginine and histidine stabilized vesicles increasing in size by ~105% and 77% respectively over 42 days storage compared with lysine or trehalose stabilized vesicles which increased by only ~25% over the same time period.

Previous research (Crowe et al., 1994; Suzuki et al., 1996) has reported that leakage of the encapsulated material from the freeze dried formulation is accompanied by liposome fusion. Suzuki et al., (1996) investigating the role of saccharides as cryoprotectants have shown that that maximum retention offered for liposomes made

from egg PC was only 55% in the presence of more than three residues of glusose. This was attributed to the weaker interactions between the lipid head group and the glucose units surrounding the liposomes (Suzuki et al., 1996). Studies by Miyajima (1997) involving the analysis of various types of saccharides as cryo protectants have shown that drug leakage occurs to the extent of 90% and is dependent on the nature of the cryo protectant and the phospholipid. Wolkers et al., 2004 have also shown that the leakage of the encapsulated material from the freeze dried formulation is also influenced by other factors including the pH. The increase in pH promotes the negative charge in the ions (phosphate in this case) surrounding the phospholipid thereby increasing the competition for interacting with the glucose molecules. The increased competition thus favours poor binding of the glucose molecule with the phospholipid head group eventually leading to leakage of the encapsulated material.

5.4 Conclusions

Our results suggest that lysine may offer cryoprotective effects to liposomes similar to that of trehalose. The higher leakage observed with histidine and arginine could possibly be attributed to the weaker binding of the amino acid to the phospholipid and the gradual fusion of the vesicles over a prolonged period of time thus promoting higher rates of leakage.

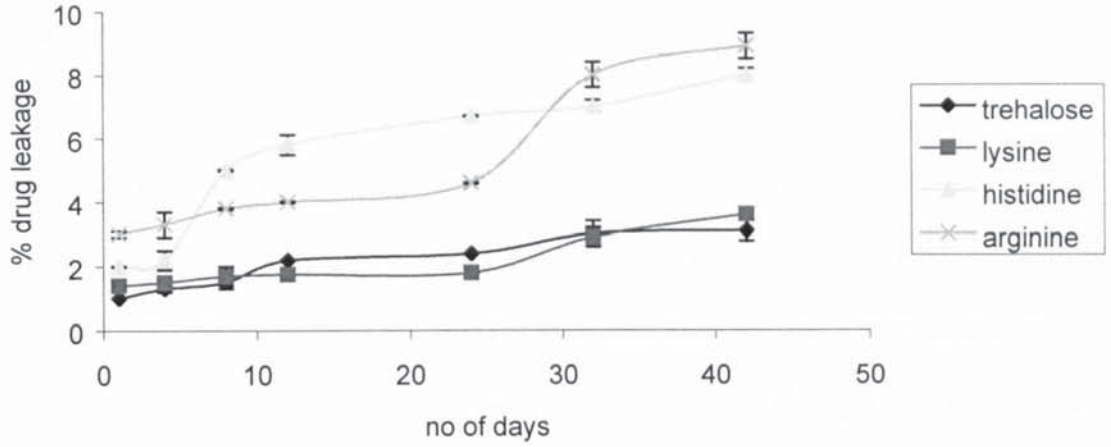


Fig 5.4 The influence of addition of cryoprotectants on stability. SUV were prepared from 16 μmol PC and 4 μmol of cholesterol with 4 mol of cryoprotectants added per mole of the lipid. At selected time intervals of 1, 4, 8, 12, 24, 32, 42 days samples were rehydrated and assayed for ibuprofen leakage as described in section 2.4 and expressed as % of total released. Results represent mean ± standard deviation, n=3 of percentage cumulative release in PBS at 37°C.

	Size (nm) analysis						
Formulation	**Day 1**	**Day 4**	**Day 8**	**Day12**	**Day 24**	**Day 32**	**Day 42**
Trehalose	199 ±21	198 ± 28	231 ± 16	234 ± 22	237 ± 17	248 ± 21	249 ± 35
Lysine	189 ±15	193 ± 3	198 ± 22	221± 2	231± 30	233 ± 23	239 ± 21
Histidine	176 ± 21	194 ± 32	243 ± 15	288 ± 22	289 ± 15	301 ± 30	312 ± 12
Arginine	163 ± 14	187 ± 3	210 ± 26	283 ± 38	298 ± 12	323 ± 21	334 ± 20

Table 5.6 The influence of cryoprotectant liposome stability. SUV were prepared from 16 μmol PC and 4 μmol of cholesterol. SUV containing the cryoprotectants were prepared as described in section 2.2.12. The formulations were freeze dried and stored in a fridge. Liposomes size (volume mean diameter) was determined by laser diffraction spectroscopy using a Brookhaven Zetaplus at 25°C. Values denote mean ± S.D. from at least three experiments.

Chapter 6

6.0 Environmental Scanning Electron Microscopy: a novel wet mode analysis technique for liposomes

Microscopy has been extensively employed for the study of formulation morphology and surface modifications of systems under differing conditions and such techniques have provided enormous data on surface characteristics of the formulations and on variables such as size, phase behaviour and structural changes of the systems (Mohammed et al., 2004; Arunothayanun et al., 1999). In addition data from micrographs have supported other physico-chemical studies of pharmaceutical systems for example; viscosity, flow behaviour and the dye retention capacity of the niosomes where it has been has been substantiated with the micrographs of the formulation which demonstrated the enhanced flow and dye retention of niosomes composed of (hexadecyl diglycerol ether) $C_{16}G_2$: Chol: (polyoxyethylene 24 cholesteryl ether) SolulanC_{24} (49:49:2) was a result of the spherical nature of the vesicles compared to the poor flow characteristics of $C_{16}G_2$: SolulanC_{24} (91:9) niosomes which were shown to form polyhedral structures (Arunothayanun et al., 1999).

To study liposome morphology, various techniques including scanning electron microscopic studies (SEM; eg Lopez et al., 2001) and freeze fracture analysis (Egelhaaf et al., 1996) have previously been employed. However, techniques such as conventional SEM analysis require samples to be dried or the water fixed before imaging with the disadvantage that images are often poorly representative (Donald, 1998). Freeze fracture is also known to result in changes in the morphology of

liposomes due to the mechanical stresses encountered during specimen preparation (Egelhaaf et al., 1996).

The advent of atomic force microscopy (AFM) has provided further avenues for sample analysis in the wet mode. This technique involves interpretation of interactions between a sub microscopic probe and the sample surface in both contact as well as non-contact modes. Unfortunately, the contact mode involves the probe touching the sample surface and can thereby causes a physical damage to the sample or damage to the probe in cases of hard sample surfaces. Alternatively, non-contact mode is less sensitive due to lack of any contact with the sample surface.

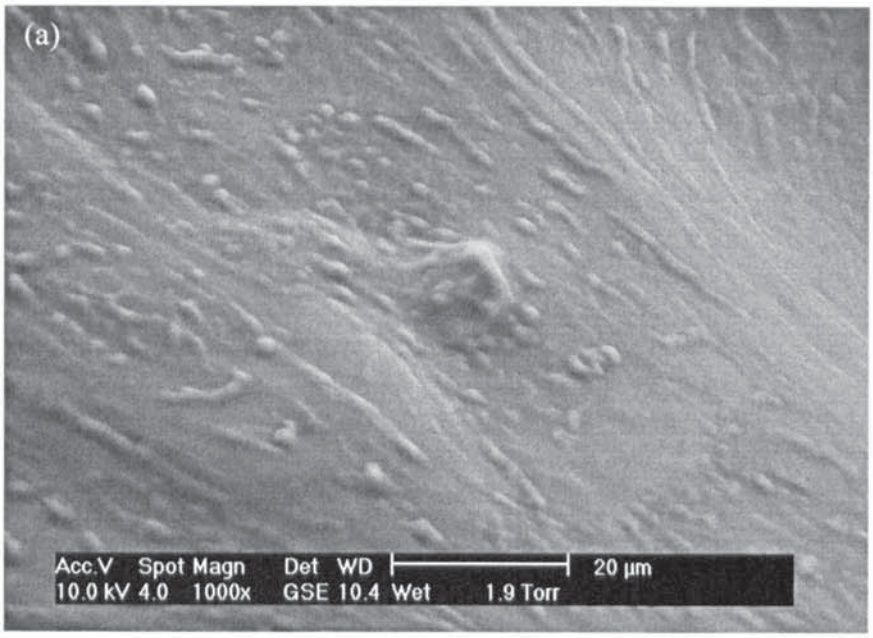
Environmental scanning electron microscopy (ESEM) can overcome such problems with its ability to image wet systems without prior sample preparation. Further, ESEM allows variation of the sample environment through a range of pressures, temperatures and gas compositions. Indeed, ESEM has already proved valuable for analysis of hydrated samples such as thermo-responsive microspheres (D'Emanuele and Dinarvand, 1995), polymeric surfactant micelles (Cao and Li, 2002), dendrimers (Sui et al., 2000) and colloidal latex dispersions (Donald, 2000). In our study, we have used ESEM to dynamically follow the changes in structure of lipid films and liposome suspensions as water condenses on to or evaporates from the sample. In particular, changes in liposome morphology were studied using ESEM in real time to investigate the resistance of liposomes to coalescence during dehydration thereby providing an alternative assay of liposome formulation and stability.

6.1 ESEM assessment of liposome stability

As an alternative assay of liposome formulation and stability, and to dynamically follow the changes in structure of lipid films and liposome suspensions as water condenses on to or evaporates from the sample, samples were observed using ESEM under various state of hydration or dehydration. Liposome formulation from dry lipids had been explained previously in terms of a "budding off" mechanism with vesicles forming from organised lipid lamellar arrays due to increased stress associated with phospholipid hydration (Swarbrick and Boylan, 1994). At the molecular level, liposome assembly can be explained by the structural changes accompanying phospholipids hydration. Early investigations, using X-ray structural data (Small,1967), demonstrated that the surface area per phosphatidylcholine molecule gradually increased as the weight fraction of water increased, due to more water being incorporated into the phosphorylcholine head group region. These observations suggested 9 molecules of water were associated with each lipid molecule at a lecithin: water weight fraction of 0.85:0.15 (Small, 1967). Further increases in the weight fraction of water (above 16%) in the sample resulted in the appearance of a "free water layer" separating the lipid bilayers leading to the formation of myelin figures (multilamellar cylinders) and anisotropic droplets (Small, 1967). To visualise the formation of liposomes from hydration of a dry lipid film, ESEM was used to investigate the morphology of a dry lipid film containing PC and Chol (4:1 molar ratio) subjected to controlled hydration in the ESEM sample chamber under an operating pressure maintained at ~2.0 torr. Following sufficient hydration of the lipid film, cylinders and spherical structures were clearly observed forming from the film (Fig 6.1a & Fig 6.1b) similar in nature to those described by Small, (1967).

To investigate the stability of preformed MLV during dehydration, liposomes prepared as described (Section 2.1) were also studied. Initially, drug-free MLV (PC:Chol; 4:1 molar ratio) suspended in PBS (pH 7.4) were investigated. At a pressure of 4.0 torr, MLV appeared as spherical vesicles of various sizes, in accordance with that of the mean volume size measured using laser diffraction, (4.3 ± 0.5μm; Table 3.3) superimposed on a background moisture film (Fig 6.2a). Gradual reduction of the ESEM operating pressure to 3.4 torr resulted in crystallisation of salts from the PBS suspension medium, forming large cuboid-shaped crystals (Fig 6.2b). At 2.9 torr pressure (Fig 6.2c), the liposomes were observed to be coalescing due to evaporation of the surrounding aqueous environment and salt crystals were also visible, interspersed between the lipid globules. Further reduction of pressure to 1.9 torr (Fig 6.2d) resulted in the liposomes losing their spherical shape as flattening and spreading occurred to form lipid patches. Partially spread, hemispherical structures are also visible.

Similarly, ibuprofen loaded liposomes were investigated; PC:Chol liposomes incorporating ibuprofen were prepared and washed via centrifugation and suspended in PBS. Reduction of the ESEM operating pressure to 2.8 torr again resulted in salt crystallisation from the PBS medium (Fig 6.3a); however, unlike the drug-free liposomes in PBS which showed signs of coalescence at 2.9 torr (Fig 6.2c), drug loaded liposomes were maintained at 2.0 torr (Fig 6.3b &6.3c). Closer examination of the liposome morphology in Fig 6.3c, shows small grains or specks on the liposome surface which may be attributed to either small PBS crystals or adsorbed ibuprofen. Attempts to remove these small grains by washing the drug loaded liposomes twice more, via centrifugation, were unsuccessful.



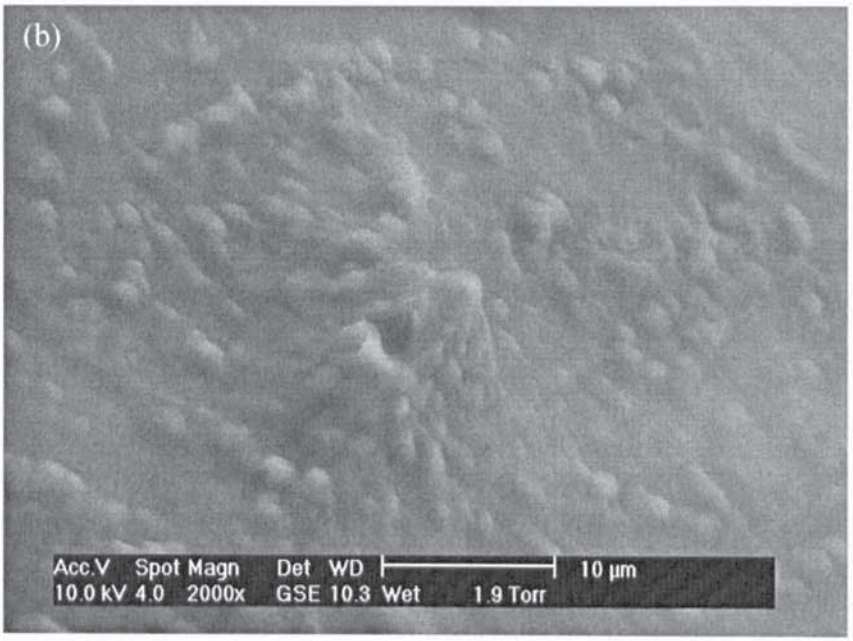
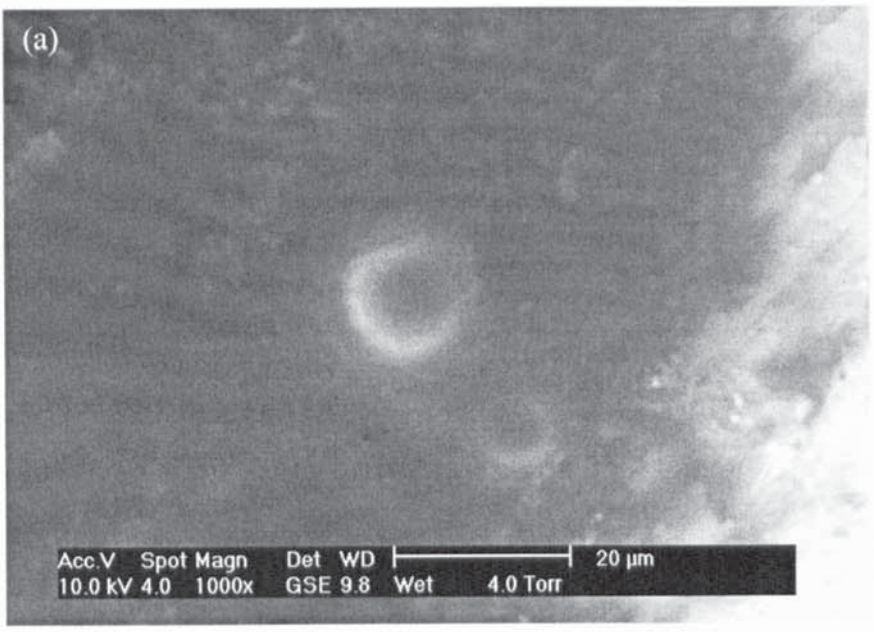


Fig 6.1: Environmental scanning-electron micrograph of a dry lipid film containing a mixture of PC and cholesterol (4:1 molar ratio) and subjected to controlled hydration in the ESEM sample chamber under an operating pressure maintained at 1.9 torr. a) 1000X magnification b) 2000X magnification.

Further reduction of the operating pressure to 1.9 torr revealed clear images of stable spherical liposomes despite the continued evaporation of the surrounding aqueous medium (Fig 6.3d) demonstrating that the liposomes with incorporated ibuprofen tolerated reduced operating pressure more effectively than drug-free liposomes which tended to flatten at a pressure of 2.9 torr (Fig 6.2c). Indeed ibuprofen-loaded liposomes were able to withstand pressure reductions to 1.4 torr without losing their spherical form (Fig 6.3e).

The ESEM analysis clearly demonstrates that ibuprofen incorporation enhances the stability of PC:Chol liposomes with drug-loaded liposomes showing enhanced resistance to coalescence during dehydration compared to drug-free liposomes. This behaviour suggests that the incorporation of ibuprofen within the liposomal structure may enhance vesicle stability due to the presence of amphiphilic ibuprofen interdigitating between the lipid chains of the bilayer and enhancing the rigidity of the PC:Chol (16:4 μmole) bilayer. Indeed, studies (Fatouros and Antimisiaris, 2002) investigating hydrophilic drug retention have demonstrated the ability of membrane incorporated amphiphilic drugs such as prednisolone, diazepam and griseofulvin to enhance membrane integrity and significantly stabilise liposomes. Liposomes composed of PC and any of the three amphiphilic drugs tested (prednisolone, diazepam and griseofulvin) where shown to retain significantly more of the vesicle-entrapped hydrophilic compound carboxyfluroscein after incubation for up to 24 h in the presence of buffer or serum proteins (Fatouros and Antimisiaris, 2002). Further, recent studies by Maghraby et al., (2004), investigating the role of penetration enhancers using DSC, have shown that the presence of molecules between the lipid alkyl chains have an influence on the arrangement of the lipid molecules in the bilayer. The presence of

cholesterol and other penetration enhancers has been shown to affect the fluidity of the bilayer in a concentration dependent manner. Investigations by Maghraby et al., (2004) involved deciphering the penetration effect of the surfactants: tween 80, span 80 and sodium cholate on the membrane properties of DPPC liposomes. It was found that the orientation of the molecules in the bilayer and the HLB (hydrophilic lipophilic balance) value of the enhancers controlled the bilayer fluidity. The arrangement of lipids in the bilayer is characterised by the presence of empty spaces below the bulky head group which are responsible for the origin of the pre-transition state thereby affecting the arrangement of the lipid molecules. It was found that at higher concentrations of sodium cholate (above 8.2 mol%) the pre transition peak of DPPC was abolished thereby suggesting that the liposomes would transform from gel to fluid state without undergoing changes at the pre-transition stage. This has been attributed to the arrangement of sodium cholate which orients itself alongside the lipid alkyl chain with the carboxylic head group interacting with the head group of DPPC thus possibly rendering the formulation a stable arrangement. Similarly, it could be possible that the ibuprofen orients itself along side with the lipid molecule and occupies the empty space below the head group of the lipid thereby stabilising the formulation.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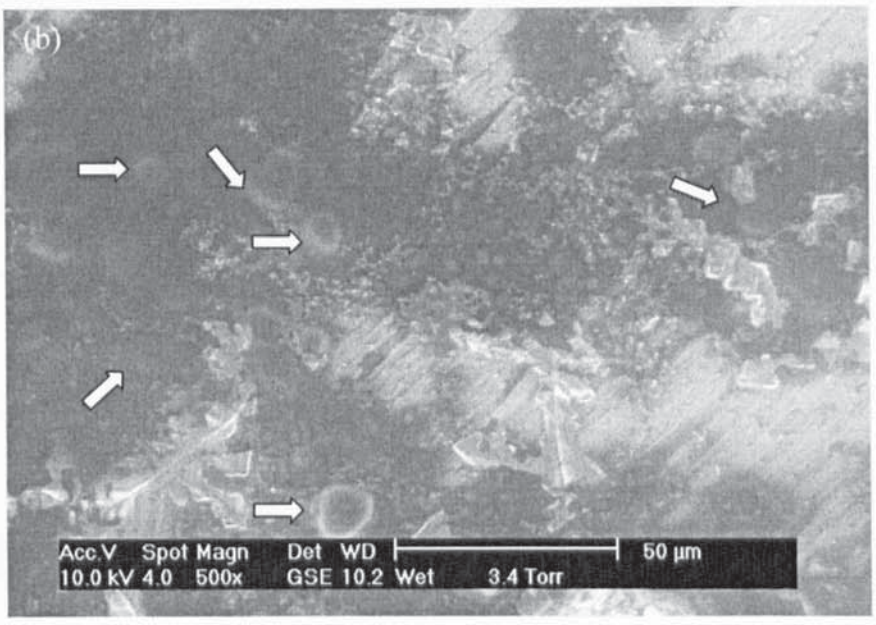
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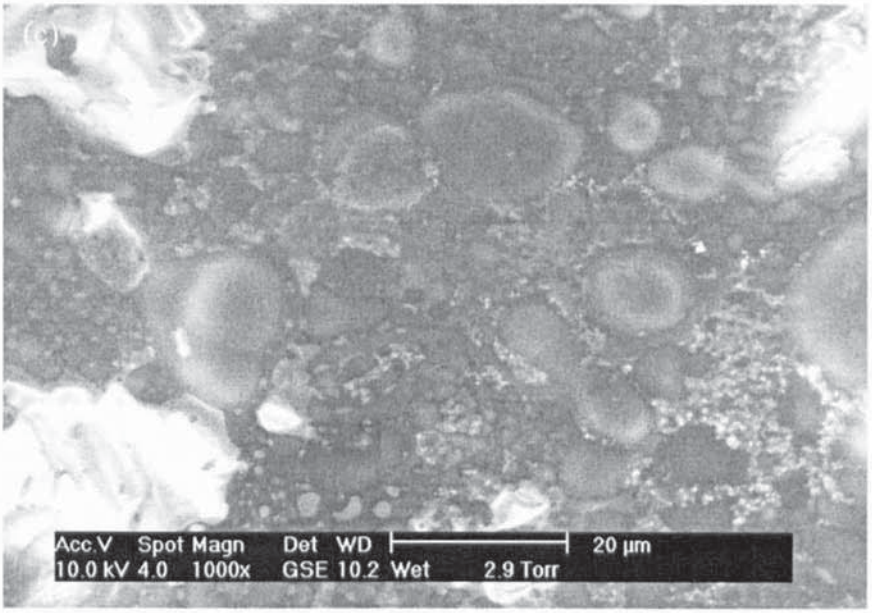


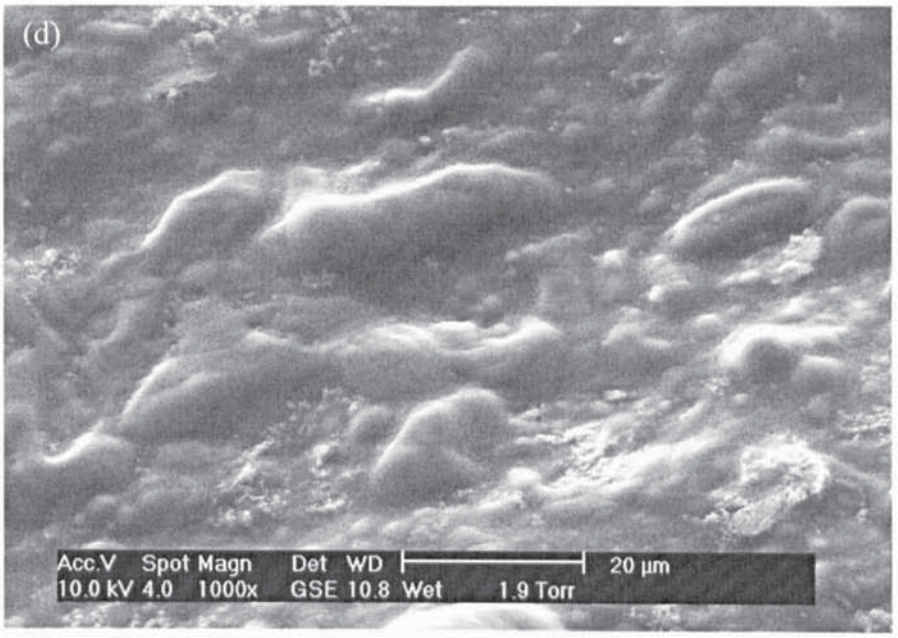
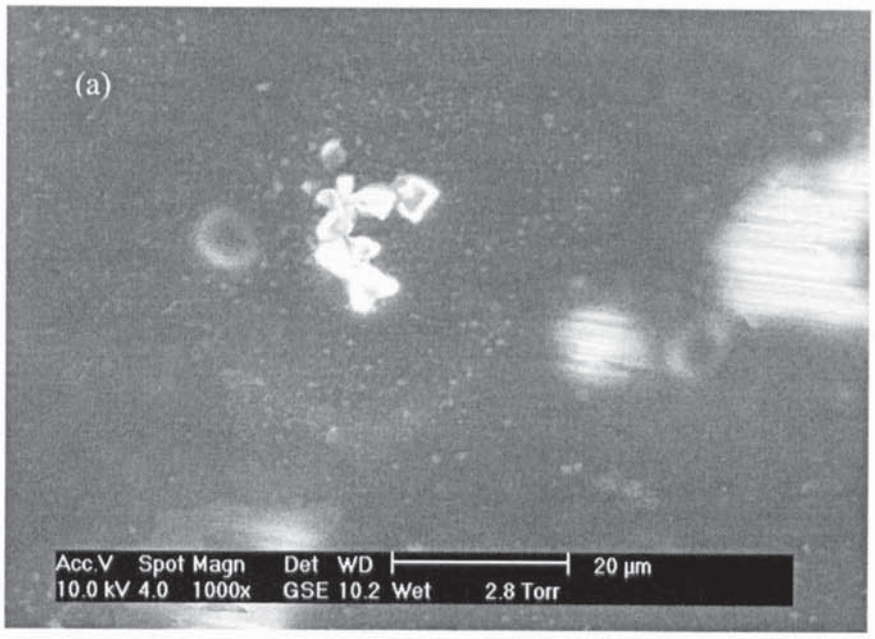


Fig 6.2: Environmental scanning-electron micrographs of preformed drug-free MLV (PC:Chol; 4:1 molar ratio) suspended in 0.01M PBS (pH 7.4). Vesicles were subjected to controlled dehydration in the ESEM sample chamber. At an operating pressure of 4.0 torr (a) liposomes appear as spherical vesicles. Reduction of the ESEM operating pressure to 3.4 torr (b) reveals spherical liposomes surrounded by salt crystals. At an operating pressure of 2.9 torr (c) liposomes are coalescing as the aqueous media evaporates. Further reduction of pressure to 1.9 torr (d) resulted in the liposomes loosing their spherical shape as flattening and spreading occurred to form lipid particles.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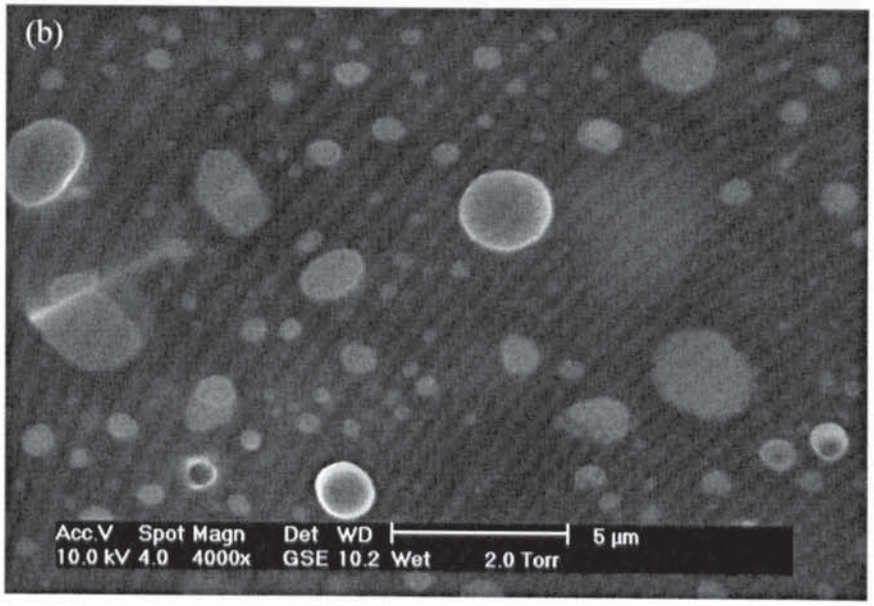
(b)
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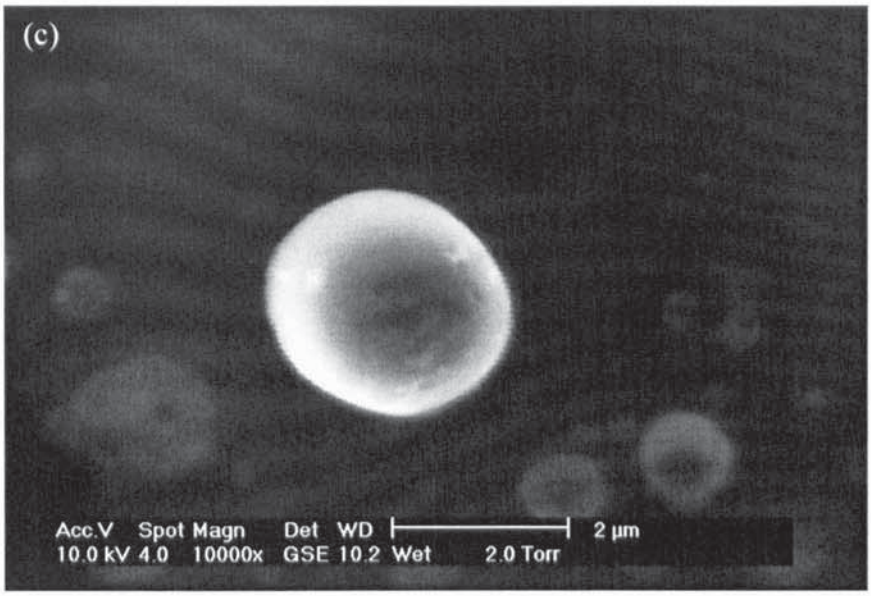
(c)
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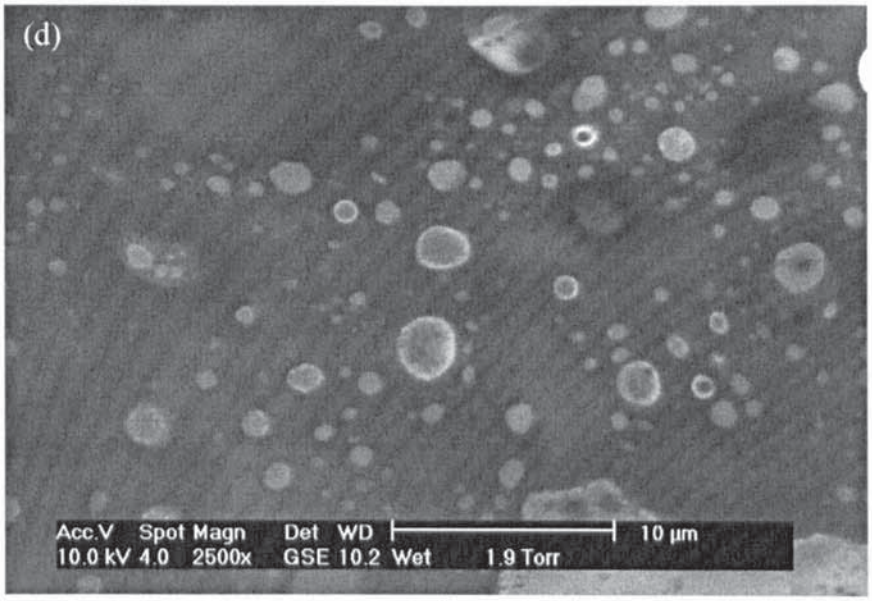
(d)
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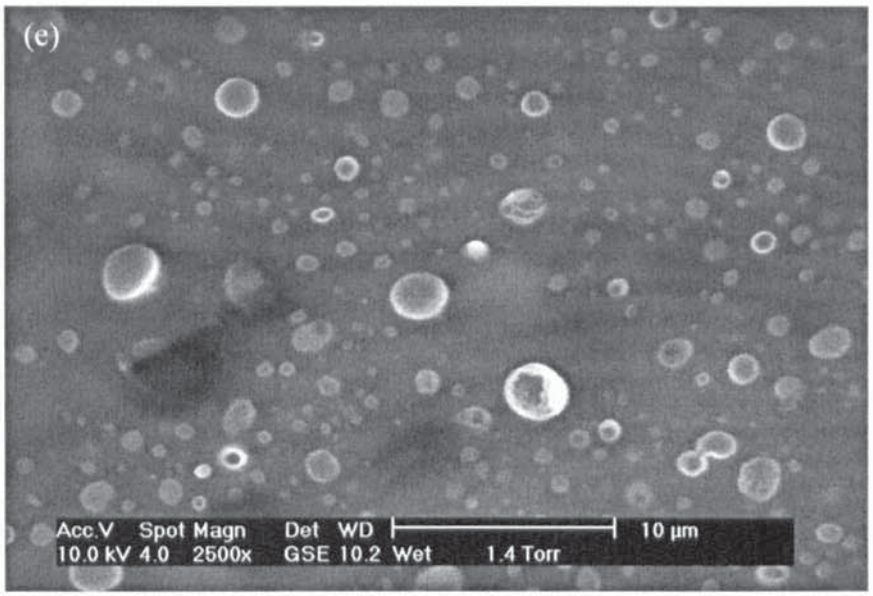


Fig6.3: Environmental scanning-electron micrographs of ibuprofen-loaded MLV (PC:Chol; 4:1 molar ratio) suspended in 0.01M PBS (pH 7.4). Vesicles were subjected to controlled dehydration in the ESEM sample chamber. At an operating pressure of 2.8 torr spherical vesicles and salt crystallisation is observed (a). Spherical liposomal structures remain stable at pressures of 2.0 torr (b), 1.9 torr (d) and even 1.4 torr (e). magnification of samples held at an operating pressure of 1.9 torr reveal small grains or specks on the liposome surface (c).

6.2 Does the presence of salts in the hydration medium affect liposome morphology?

The role of the ions in the dispersing medium has attracted attention to study the stability of the colloidal particles (Eastman et al., 1997; Bordi and Cametti, 2002). The concentration of salts, variations in zeta potential and changes in surface characteristics greatly influences the behaviour of the liposomal formulation.

To investigate the effect of the presence of buffers and salts on liposome stability during dehydration, drug free liposomes suspended in distilled water were compared to the previous studies involving liposomes suspended in PBS (section 6.1). Fig 6.4a shows drug free liposomes dispersed in water at a pressure of 4.7 torr. The lack of crystals in the ESEM micrographs is in clear contrast to the images of liposomes suspended in PBS where crystal structures are clearly seen (Fig 6.2 a, b). As before gradual reduction of pressure caused loss of surrounding dispersing water and at a pressure of 2.2 torr (Fig 6.4b) showed that liposomes originally suspended in distilled water remained dispersed and retained their spherical form whereas liposomes suspended in PBS began to collapse and flatten at 2.9 torr (Fig 6.2c). Indeed liposomes suspended in water began to coalesce and lose their spherical structure at 1.7 torr pressure (Fig 6.4c).

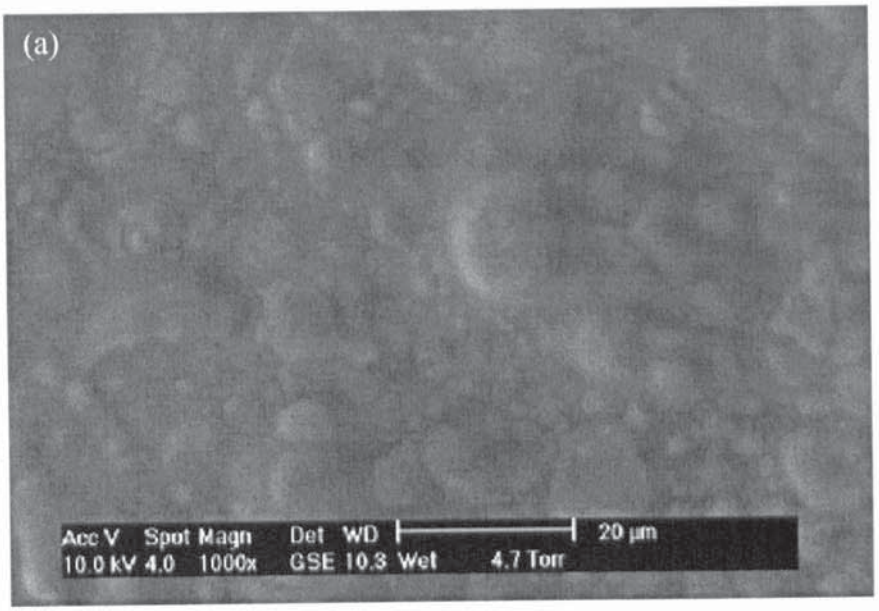
Therefore, it can be seen that drug free liposomes showed different characteristic behavior in the presence and absence of the electrolytes in the dispersing medium. Research has previously shown that the presence of ions in the dispersing medium induces disorder in the bilayer structure. Liposomal formulations with and without

cholesterol were used to entrap cyclosporin. In order to assess drug leakage, the samples were incubated in solutions of monovalent (sodium) and divalent (magnesium) ions. The results showed that the presence of ions in the cholesterol free liposomes caused a higher amount of drug leakage whereas addition of cholesterol minimized the drug leakage without preventing it completely. Thus the authors concluded that the bilayer disorder in the presence of ions in the surrounding medium was responsible for the drug leakage (Al-Angary et al., 1995). Another parameter that is influenced by changes in the concentration of the electrolytes in the surrounding medium is the zeta potential of the liposomes. An increase of ion concentration (for e.g. due to dehydration of the medium) can cause some of the lipid head groups to be either neutralized or acquire a charge depending on their characteristics and hence changes the zeta potential of the vesicles can occur. This alteration in zeta potential as a result of the dehydration of the media could also be contributing to the destabilization of the vesicles, as this has been a major cause of vesicle aggregation in stability studies (Schwarz and Mehnert, 1997).

Indeed the stability of the liposomes, as stated by Derjaguin-Landau-Verwey-Overbeek (DLVO theory) depends on the salt concentration, the counter ion valency, the surface potential and the liposome diameter. The liposomal colloidal system is stable as long as the potential barrier is higher than the thermal energy of the system. When the potential barrier of the system is reduced, possibly due to the addition of the salts, most collisions between the colloidal particles result in aggregation (Bordi and Cametti, 2002). However, investigations by Bordi and Cametti (2002), found that, as the concentration of the added salt (in this case sodium chloride) increased to over 0.5 mol/l, it resulted in the fusion of the particles giving rise to liposomes of two different

size ranges. This prompted suggestion that apart from ionic strength of the salts, the hydration forces and the surface geometry of the formulation also have a role to play in liposome aggregation. As already discussed, Small (1967) reported that hydration of the lipid molecules to form the vesicular structures requires at least 9 molecules of water per molecule of lipid suggesting that hydration forces play a role in vesicle formation. It can thus be suggested that the destabilisation of liposomes during dehydration associated with the presence of the salts in the dispersing medium could possibly be due to the increase in ionic strength of the surrounding environment (due to dehydration) and/or reduction in the hydration forces responsible for vesicle formation.

This provides an illustration of the 'salting out' mechanism, a source of instability of charge stabilised colloids and suggests a further application for ESEM in assessing the stability of liposomal formulations in drug delivery.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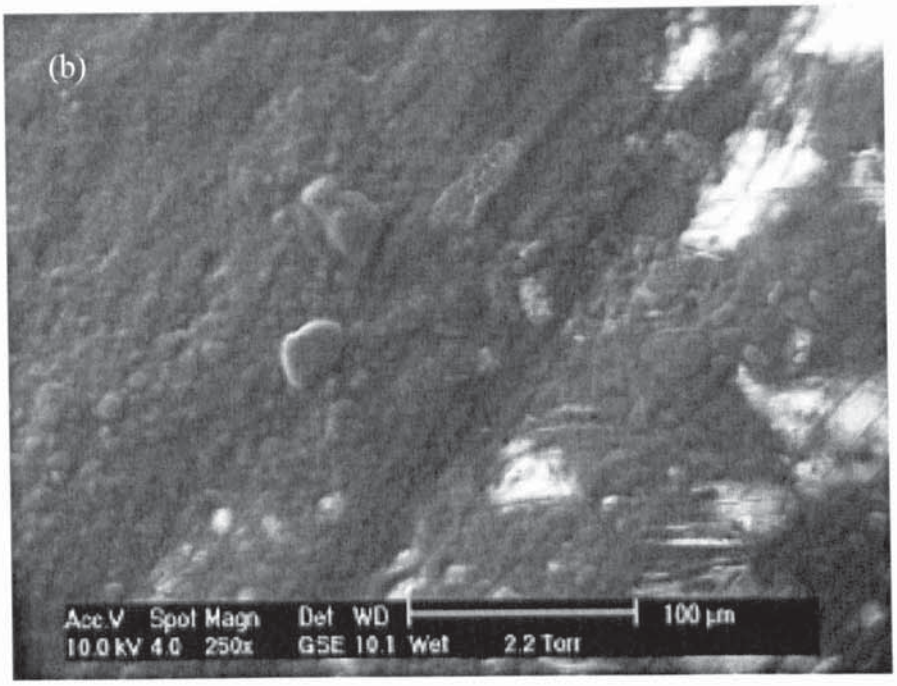
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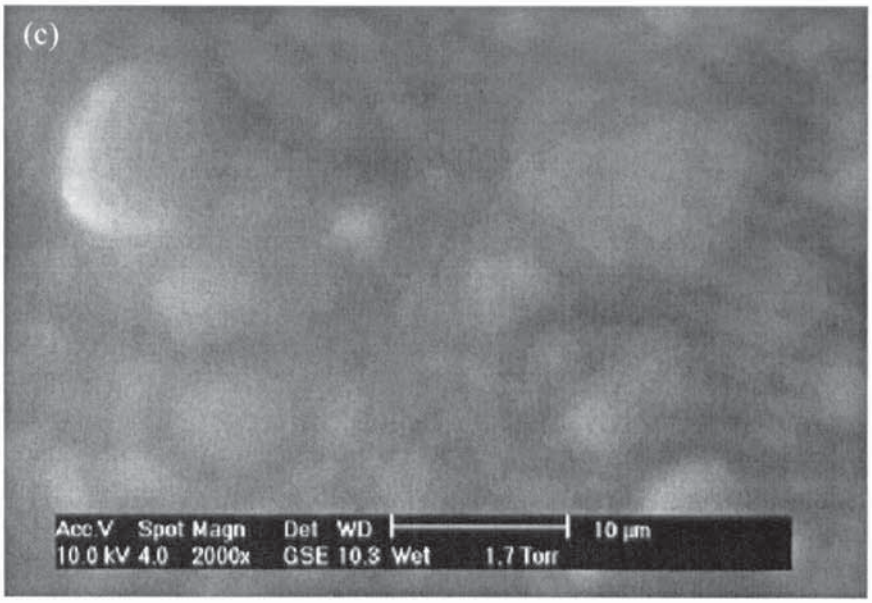


Fig 6.4: ESEM micrographs of drug free MLV (PC:Chol; 4:1 molar ratio) suspended in distilled water. Vesicles were subjected to controlled dehydration in the ESEM sample chamber. At an operating pressure of 4.7 torr (a) liposomes appear as spherical vesicles. Reduction of the ESEM operating pressure to 2.2 torr (b) reveals spherical intact liposomes. At an operating pressure of 1.7 torr (c) liposomes are coalescing as the aqueous media evaporates.

6.3 ESEM analysis of MLV made from high transition temperature lipids

The arrangement of the lipids in the bilayer of the liposomes is governed by the ratio of the area of the lipid head group to the ratio of the area of the cross section of the alkyl chain length, transition temperature, moisture content and geometry of the molecule (Israelachvili et al., 1977; Small, 1977; Swarbrick and Boylan, 1994).

To investigate the role of the transition temperature on the stability offered during dehydration of the liposomes, MLV made from DSPC:Chol with encapsulated flurbiprofen suspended in PBS were investigated and compared to similar low transition temperature lipid vesicles. To start with, the liposomal sample was investigated at an operating pressure of 5.1 torr. However due to the dense layer of moisture on the sample surface, no clear images could be taken. The first micrographs taken at 2.9 torr show clear, spherical liposomes (Fig 6.5a, b). The size of the MLV from the micrographs resembles the mean volume diameter measured on a Malvern Mastersizer (6.8 ± 0.6μm). The reduction of the pressure to 2.4 torr resulted in salt crystallisation presumably from the PBS as before (Fig 6.5c). Further reductions of the operating pressures from 2.4 torr to 1.7 torr (Fig 6.5d) showed no signs of morphological changes of the formulation. The liposomes were intact, spherical and resisted any variations in the pressure changes.

The results further strengthen the previous observations involving enhanced stability offered by the encapsulated drug into the MLV. PC:Chol liposomes loaded with ibuprofen did not yield to low pressures and resisted coalescence (Fig 6.3c). The presence of the drug in the liposomal bilayer thus seems to influence rigidification

and improves stability. This prompted further investigation to see if lipids of high transition temperature, without any added drug, would offer any different stability parameters.

The ESEM analysis of unloaded DSPC:Chol (16:4 μmoles) liposomes was carried out to study surface morphology and the behaviour under varying conditions of moisture. MLV were prepared as described in 2.2.1. The lipids were again hydrated with 1ml of PBS (pH 7.4). The surface morphology of the formulation revealed clear, spherical and intact liposomes in the size ranges recorded on the Malvern mastersizer in ddH_2O (Fig 6.6a). The decrease in pressure from 4.0 torr to 3.7 torr (Fig 6.6b, c respectively) revealed intact liposomes with the gradual growth of the crystals in the surrounding environment as was also observed with unloaded PC:Chol MLV (Fig 6.2b). However, upon further reduction of the operating pressure to 3.0 torr, DSPC:Chol liposomes were intact and spherical in contrast to the drug free PC:Chol MLV suspended in PBS, which began to flatten and loose their spherical morphology at an operating pressure of 2.9 torr (Fig 6.2c). Further reduction of the operating pressure to 2.4 torr (Fig 6.6e) revealed intact DSPC:Chol liposomes suggesting the ability of the lipids such as DSPC with high transition temperature to withstand low pressures even in the presence of the salts. The DSPC MLV resisted even lower pressures of 1.1 torr without compromising the spherical nature of the liposomes (results not shown).

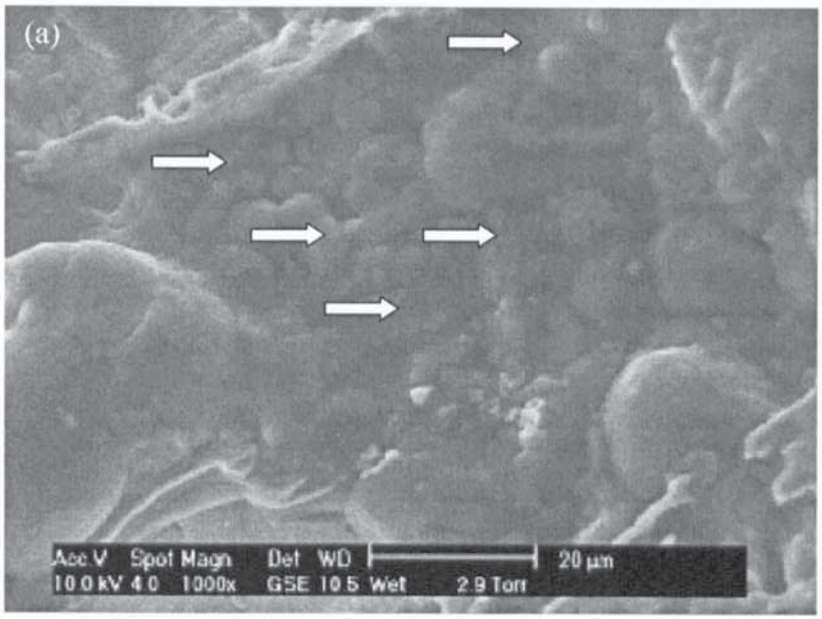
The results suggest that MLV made from DSPC:Chol offer spherical, and rigid liposomes which resist fusion at pressures down to 2.4 torr. The presence of salt crystals could be due to crystallisation as seen with the previous observations for

PC:Chol MLV suspended in PBS. However, the substitution of DSPC has highlighted that liposomes made from lipids with high transition temperatures would resist coalescence without any added drug below their transition temperature. PC:Chol liposomes collapsed at 2.9 torr when suspended in PBS and 1.9 torr when suspended in distilled water. Alternatively, the DSPC:Chol liposomes were intact and retained their spherical nature even at low pressures possibly because of the high transition temperature of the lipid (Tc 55°C). All the investigations were carried out at 5°C, which is below the transition temperature of DSPC but above that of PC.

The transition temperature is the temperature at which lipids change from a highly ordered gel state to a fluid state. Thus, the transition temperature governs the arrangement of the lipids in the liposome bilayer. Temperatures below the transition temperature result in the lipids being in the fluid ordered state whereas at higher temperatures rearrange into an ordered gel state. The ordered gel state is characterised by the presence of rigid bilayers with the lipids arranged closely to each other. Below the pre-transition temperature (temperature below the transition temperature) is characterised by the ordered gel state with all the alkyl chains in the trans configuration (L_{β}; Fig 6.7). This is a preferred mode of arrangement as the phosphatidylcholine head groups of the lipid molecules are bulky and occupy more space compared with the alkyl chain length. This results in the alkyl chains tilting relative to the plane of the membrane to account for the extra space formed by the big head groups. With the increase of temperature to the pre-transition state, the lipid molecules change into a two dimensional arrangement which is characterised by ripples in the alignment of the lipid molecules (P_{β}; Fig 6.7). Further increase of temperature to values greater than the transition temperature, the lipids revert back to

the one-dimensional arrangement. The increase in temperature causes an increase in the rotational speed of the alkyl chain length at the interface between the head group and the lipid chain thereby expanding the chains laterally resulting in a fluid arrangement of the lipids (L_{α}; Fig 6.7)

This could possibly explain that lower pressures of 2.4 torr did not affect the liposome morphology of the drug free DSPC:Chol liposomes essentially due to the rigid nature and the ordered structure of the bilayer. Although there might have been changes in zeta potential, salt concentration due to the evaporation of the hydration media, the MLV made from lipids of high transition (DSPC:Chol) retained the spherical nature and resisted any changes in the surrounding environment due to dehydration in the sample chamber.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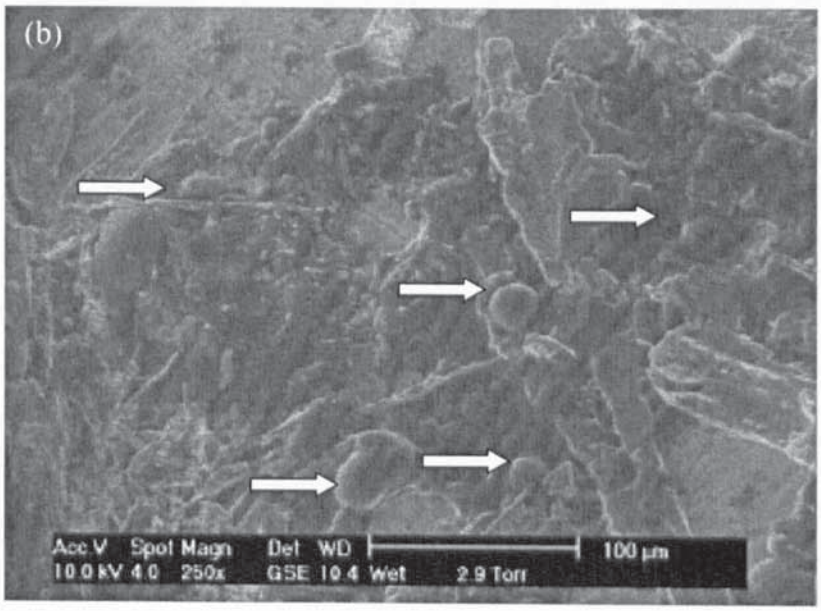
(b)
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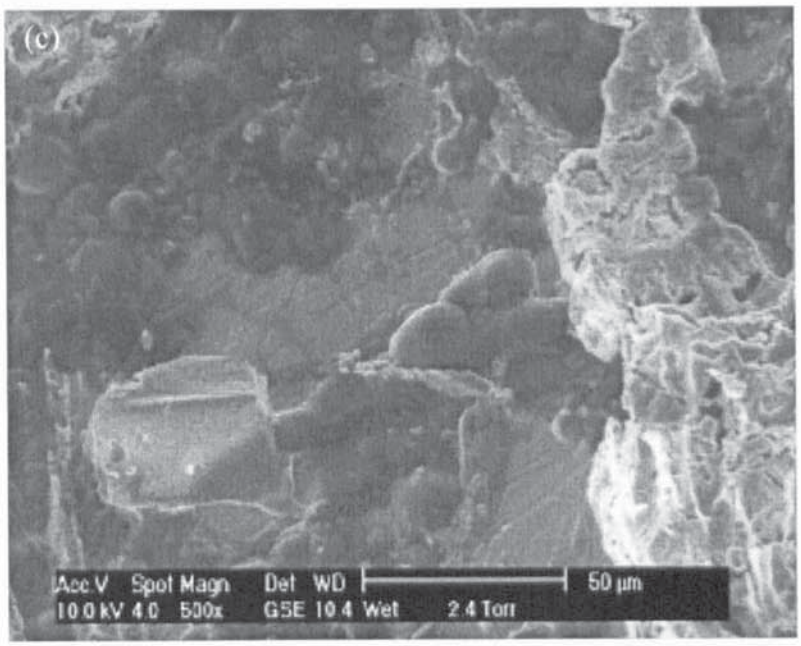


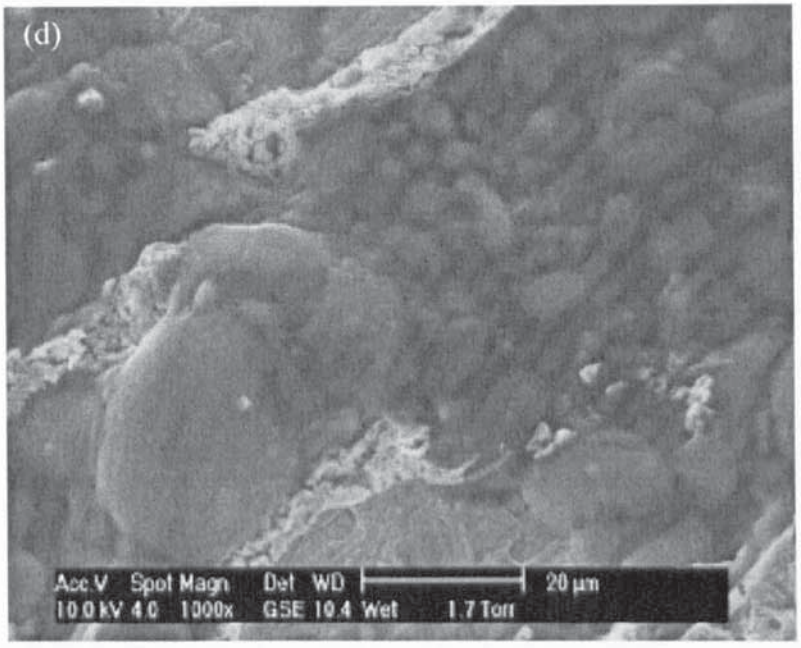
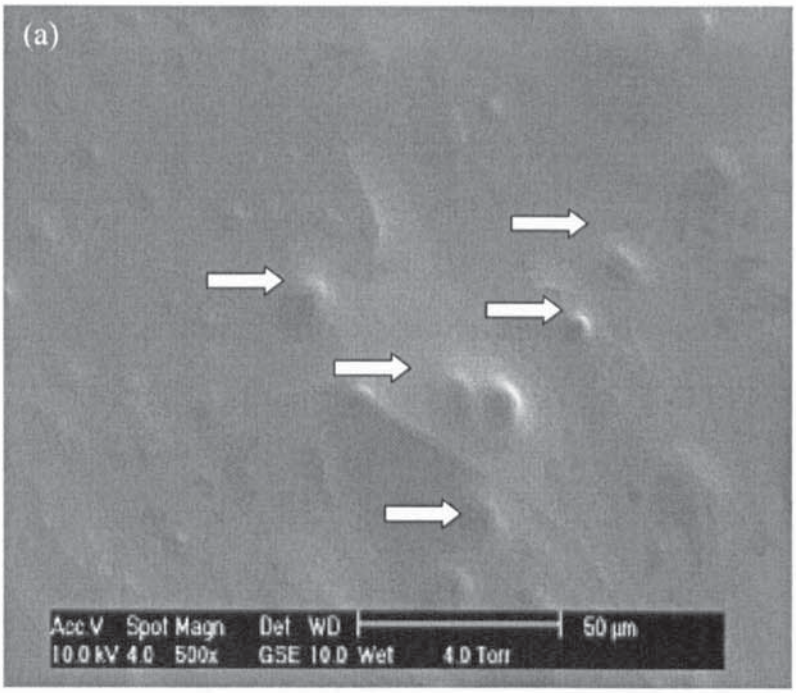


Fig 6.5: Environmental scanning-electron micrographs of flurbiprofen loaded MLV (DSPC:Chol; 4:1 molar ratio) suspended in 0.01M PBS (pH 7.4). The MLV were seen as spherical vesicles at an operating pressure of 2.9 torr (a) and (b). Reduction of the pressure to 2.4 torr did not change the morphology of the vesicles (c). The MLV resisted coalescence even at a pressure of 1.7 torr (d).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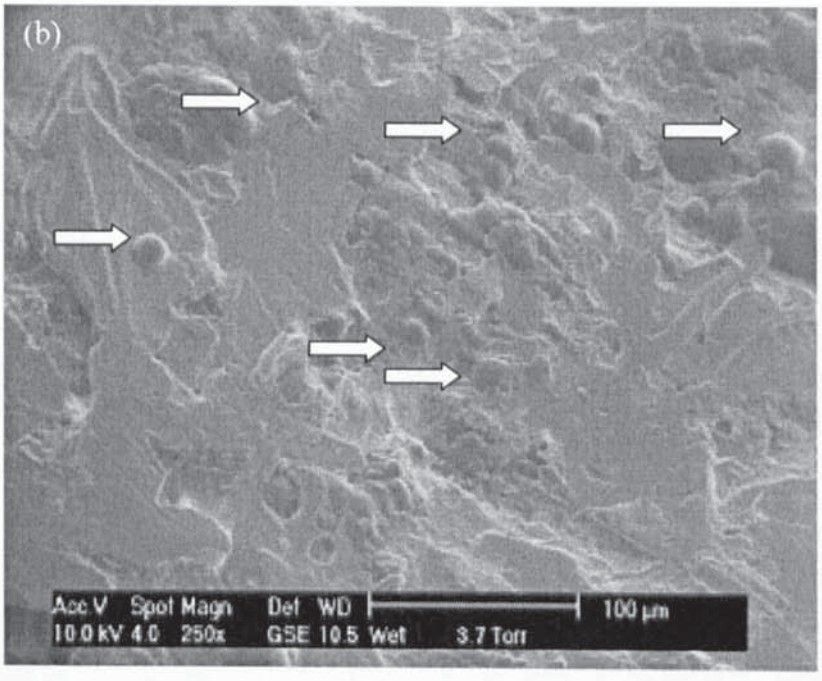
(b)
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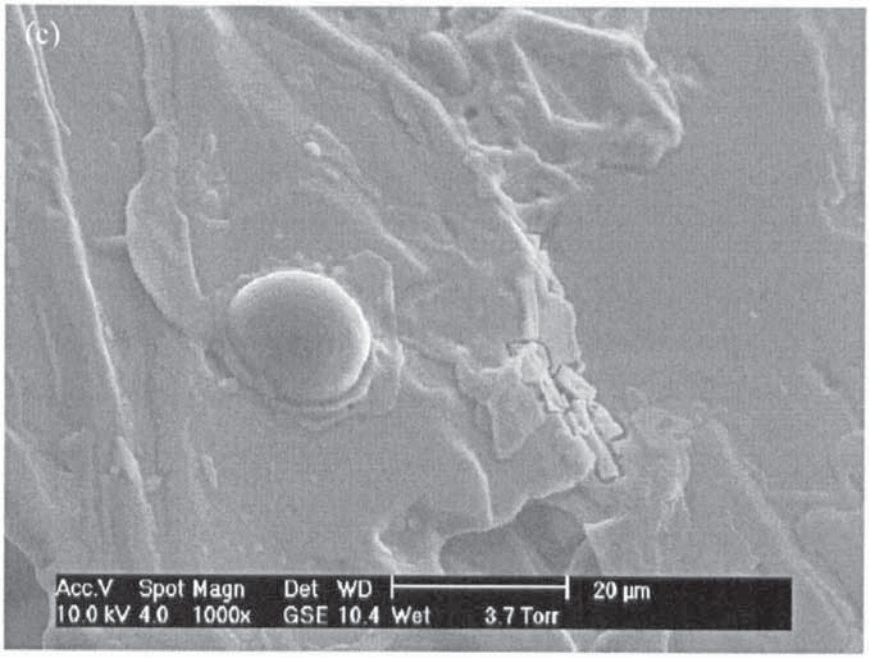
(c)
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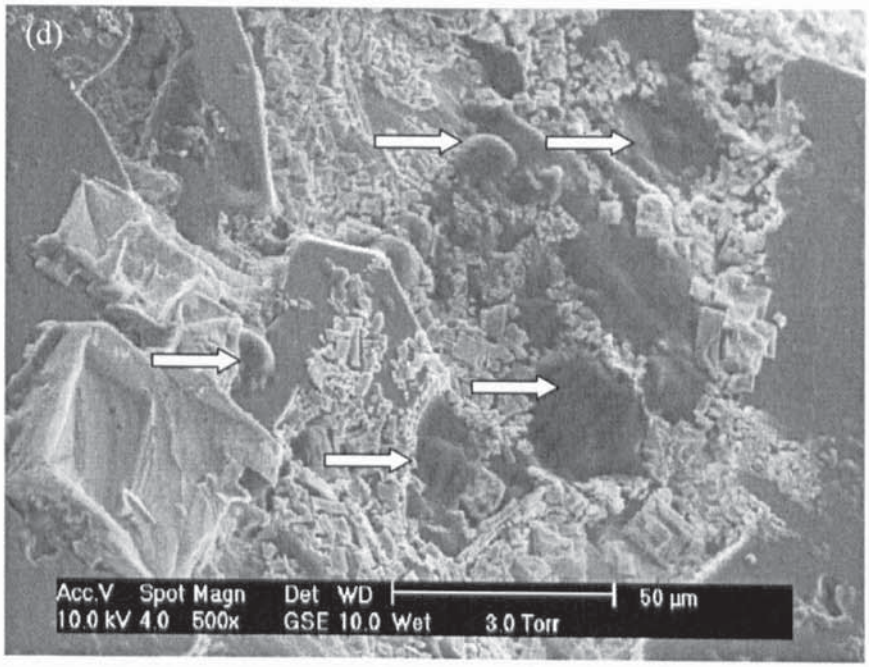
10.0 kV 4.0 1000x GSE 10.4 Wet 3.7 Torr


(d)
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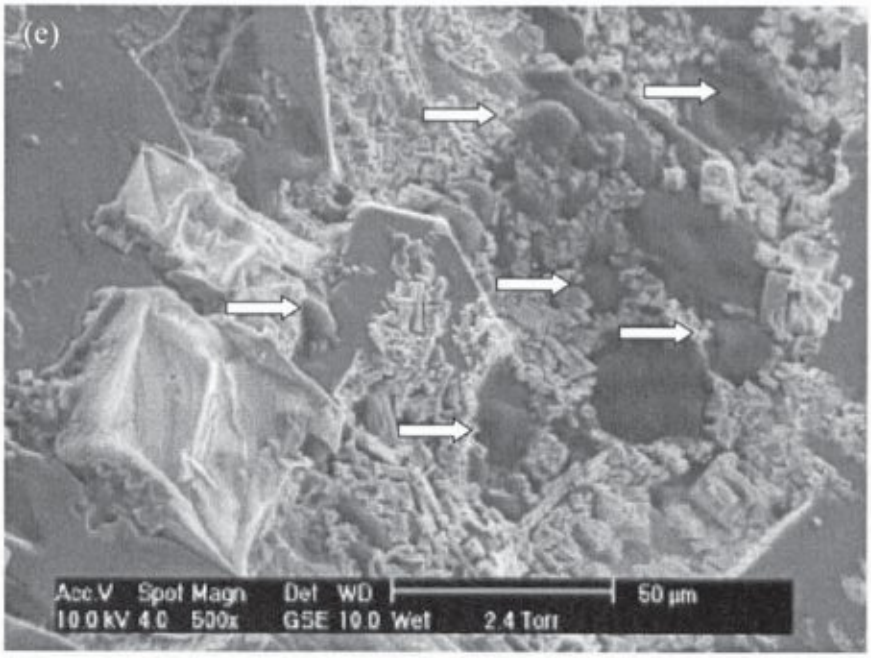


Fig 6.6: Environmental scanning-electron micrographs of drug free MLV (DSPC:Chol; 4:1 molar ratio) suspended in 0.01M PBS (pH 7.4). The MLV were seen to be densely covered with moisture (a) at a pressure of 4.0 torr. Reduction of pressure to 3.7 torr (b) (c) and 3.0 torr (d) showed spherical liposomes. The liposomes were intact and resisted any structural changes under reduced pressure of 2.4 torr (e).

Fig 6.7. Diagram showing the arrangement of lipid at temperatures below and above the transition temperature. The lipids are in a ordered gel state below the transition temperature and enter into a disordered state over the transition temperature (modified from Swarbrick and Boylan, 1994).

6.4 ESEM investigation of rehydration SUV previously freeze dried in the presence of amino acids

To study the morphology of the resuspended SUV, previously freeze dried in the presence of lysine as a cryoprotectant, ESEM investigation was done. SUV were made from PC:Chol (16:4) without the addition of drug. The investigation was initiated at a pressure of 5.0 torr to study the morphology, composition and stability of the formulation. Initial analysis showed small spherical structures freely suspended with size range of about 200 nm (Fig 6.8a & b), which confirms the size measurements recorded on the Zetaplus instrument (106 ± 7 nm). However, the sizes for SUV freeze dried without the cryoprotectants were in the range of 3988 ± 210 nm (section .1). Further investigations on the different areas of the stub revealed particles of fairly uniform size distribution at an operating pressure of 5.0 torr (Fig 6.8b, c). The formulation was again subjected to variations in the operating pressure. Reducing the pressure to 2.4 torr demonstrated liposomes resisting any structural changes (Fig 6.8d). This is in contrast to the previous investigations into the liposomal stability of the unloaded PC:Chol MLV which showed the inability of the system to prevent any coalescence (Fig 6.4.b).

The liposomal sample was also analysed for the elemental composition of the system. This was achieved by energy dispersive X ray analysis (EDXA). EDXA analysis is used to determine the elemental composition of the specimen under investigation. X-rays emitted by the sample after it has been exposed to high energy electrons is investigated. The x-rays emitted by the sample are characteristic of the parent atom in the sample which gives the elemental composition of the specimen. An EDXA of the

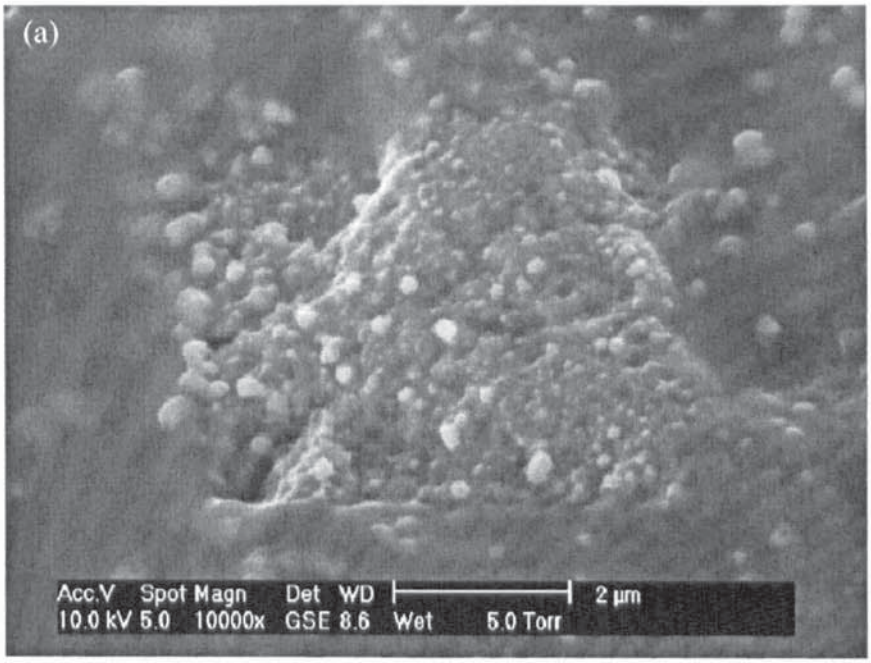
metal surface of the stub was performed to be used as a background for the EDXA of the sample. When the sample of liposome was exposed to the X-rays, a prominent peak was noticed for nitrogen possibly suggesting the presence of amino acid in the formulation.

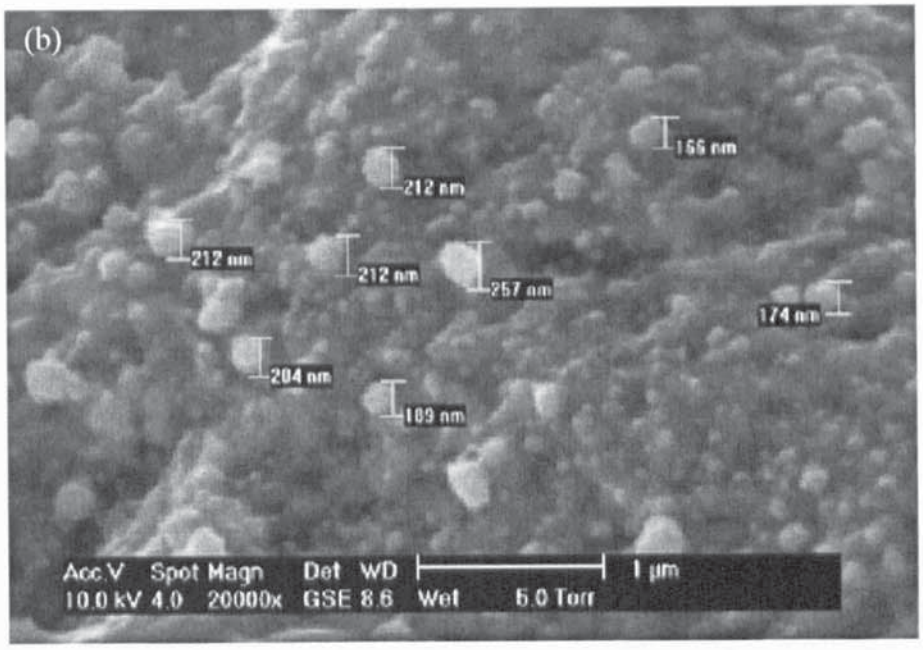
In another investigation, PC:Chol liposomes loaded with ibuprofen and previously freeze dried with lysine were analysed for morphology and stability. The ESEM micrographs showed a uniform size distribution of the SUV. Size distribution was consistent with structures in the size range of 300 nm to 550 nm similar to that recorded on the Zeta plus instrument (389 ± 50 nm). Investigations were started at 5.0 torr operating pressure (Fig 6.9a) with gradual reductions to lower pressures of up to 4.0 torr (Fig 6.9b, c).

To illustrate the role of the cryoprotectants, two hypotheses have been proposed. Firstly, the water replacement model, where water surrounding the head group of the lipid molecules is replaced by the cryoprotectants which is subsequently responsible for the prevention of aggregation and fusion of the liposomes (Crowe et al., 1988). The second hypothesis suggests that the lipid layer is surrounded by the glassy solid of the cryoprotectants whose interaction with the lipid layer decreases the surface tension of the liposomal surface leading to the stabilisation of the formulation (Koster et al., 1994).

The protective effect of lysine in the freeze dried liposomal formulation needs to be thoroughly investigated. However, the ability of the amino acid to effectively cryoprotect the formulation, giving it a porous appearance with the further capacity to

retain the liposome size upon hydration, demonstrates the new era of cryoprotectants that might be effectively used for the protection of not only encapsulated drugs but also proteins and other biologicals. The cryoprotection offered by the amino acids essentially could be due to the ability of the amino acids to act as water replacing moieties as suggested in chapter five (section 5.1).





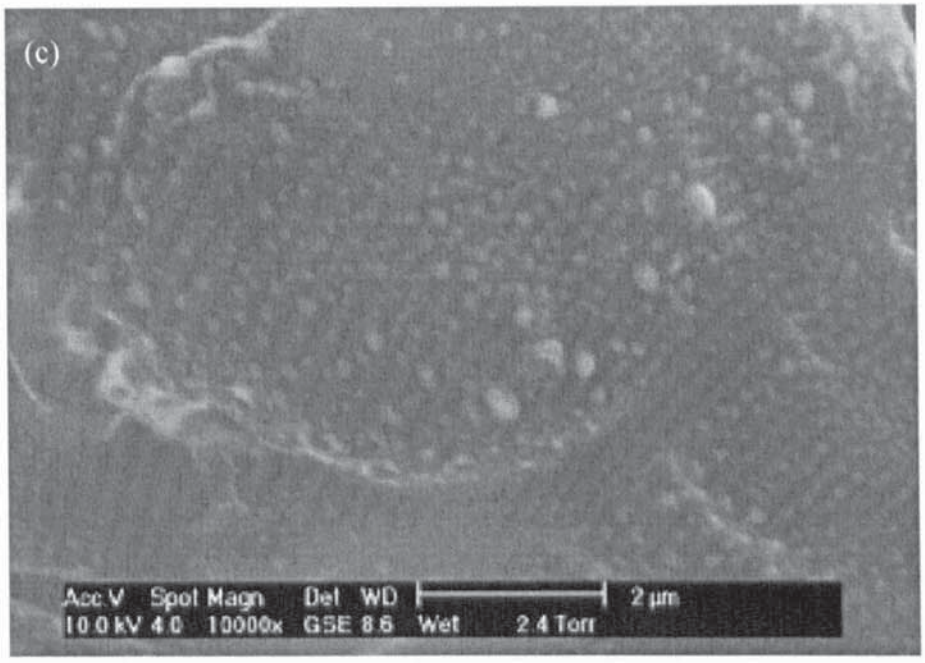


Fig 6.8: Environmental scanning-electron micrographs of SUV (PC:Chol; 4:1 molar ratio) suspended in distilled water. The SUV were previously freeze dried with lysine and the micrographs represent the rehydrated structures. The image reveals liposomes with spherical structure (a)and uniform size distribution (b) and resisted any structural changes upto an operating pressure of 2.4 torr (c).

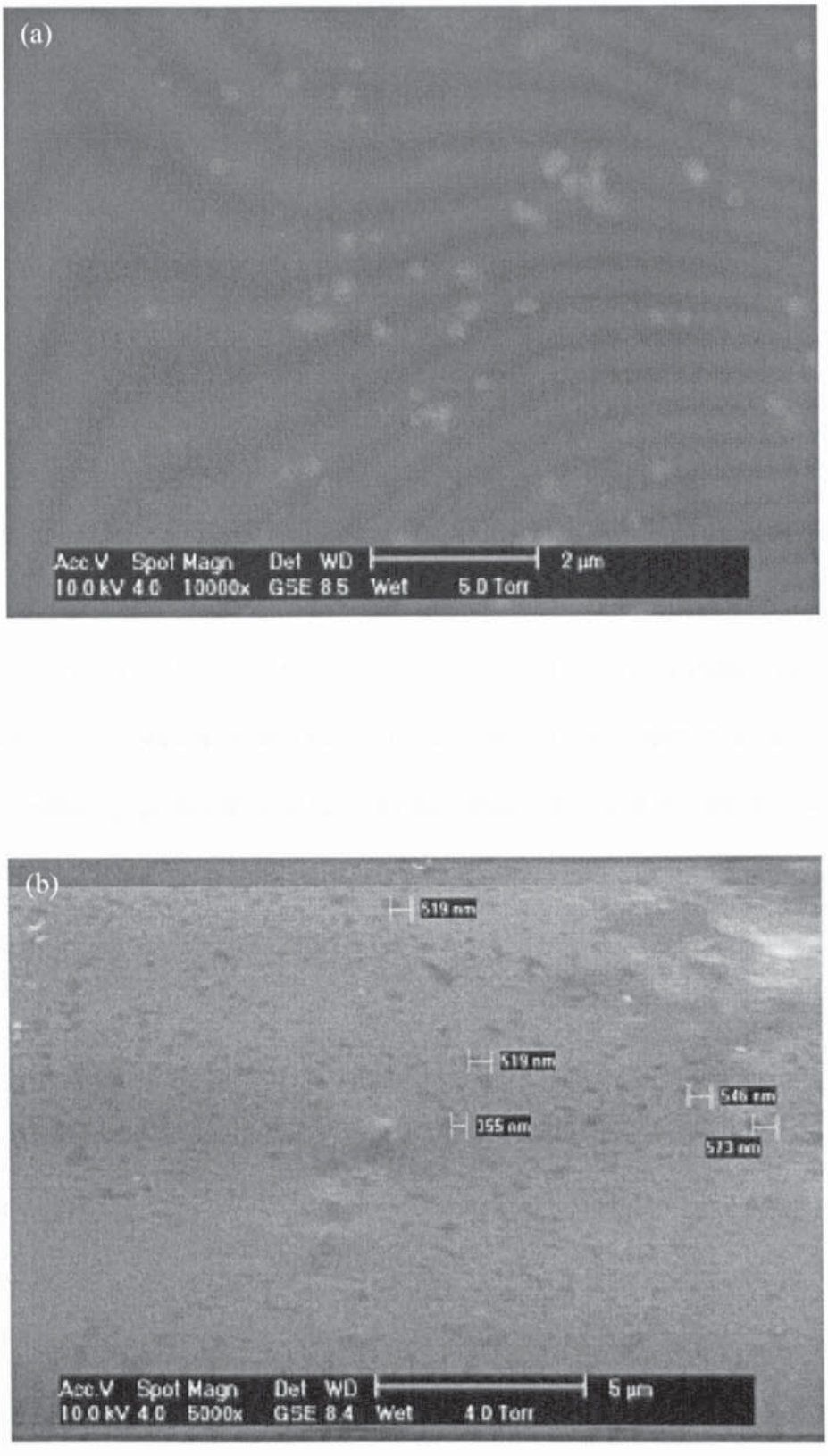


Fig 6.9: Environmental scanning-electron micrographs of SUV (PC:Chol; 4:1 molar ratio) with ibuprofen encapsulated and suspended in distilled water. The SUV were previously freeze dried with lysine and the micrographs represent the rehydrated structures (a). The image reveals liposomes with spherical structure and uniform size distribution (b).

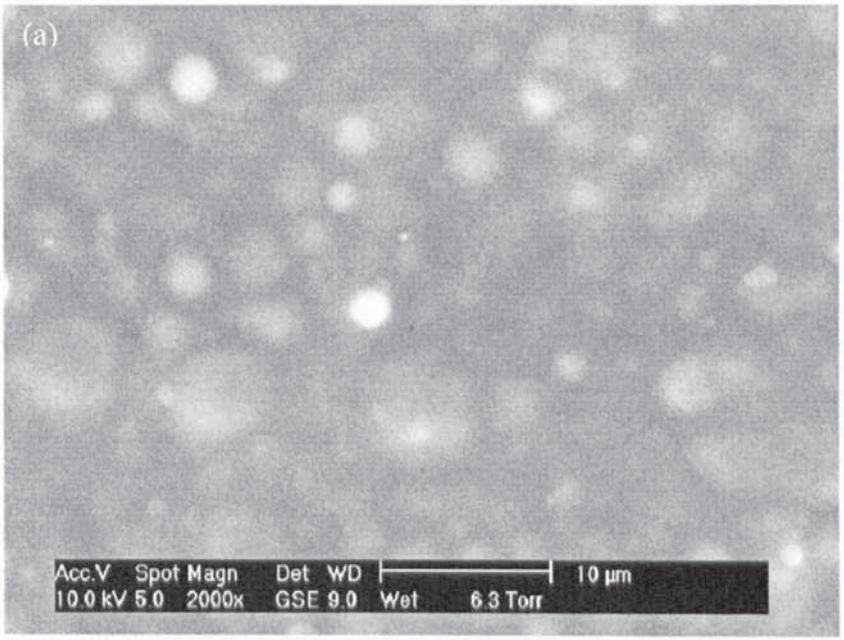
6.5 Can ESEM be used as a hot stage microscope?

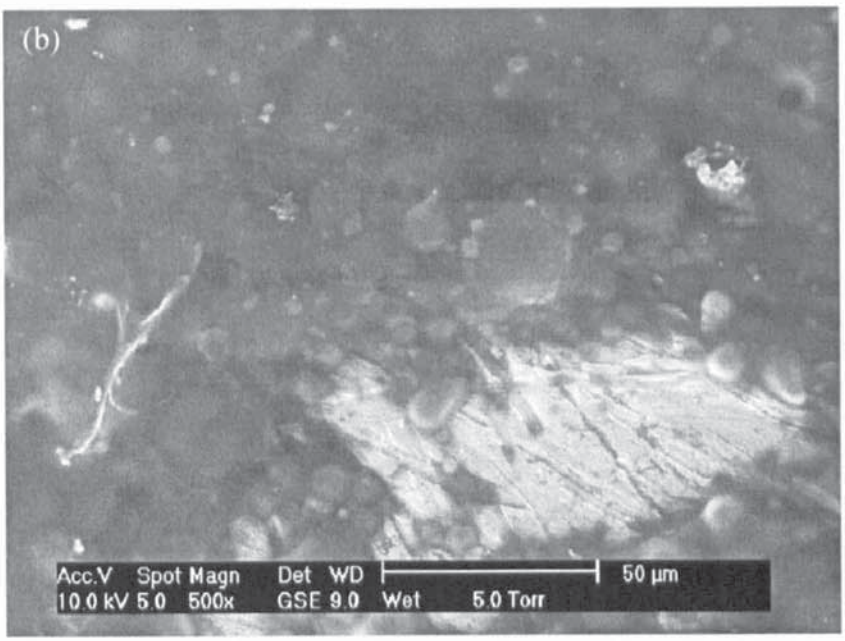
To illustrate the effect of temperature on the morphology of the liposomes, DMPC liposomes were subjected to increases in temperature and their morphology analysed. The samples were initially examined at 5.6 torr pressure and a temperature of 5°C. Upon gradual increase of temperature to 13.8°C, an increase in the surrounding pressure was recorded (6.2 torr) (Fig 6.10a). This increase could possibly be due to the pressure exerted by the evaporating water in the closed sample chamber. The size distribution of the MLV was in the range of 3-6μm in line with the size previously measured by laser diffraction. When the temperature was further increased to 17.3°C (5.0 torr) (Fig 6.10b) there was no change in the appearance of the spherical liposomes. However, with a continued increase in the temperature of the sample stub, the operating pressure was seen to rise essentially due to the evaporation of the surrounding water in the closed sample chamber (23.6°C operating pressure 3.0 torr) (Fig 6.10c), however some spherical structures can still be clearly seen.

The samples were then subjected to lowering of the pressure at high temperatures to see if the high temperature and low humidity in the sample chamber would have any interesting implications on the sample conditions. The MLV were subjected to higher temperatures of 27.7°C and 5.0 torr pressure (Fig 6.10c). The increase in temperature influenced the morphology of the liposomes with most of the liposomes retaining their spherical form and some beginning to change shape (Fig 6.10c, d). The pressure was further reduced to as low as 3.0 torr but this did not result in any morphological changes of the formulation. However, the sample was not held at low pressures and high temperatures for a longer period due to the difficulty that the sample would char

with low moisture and high heat. This possibly suggests that the MLV made from high transition lipids resist any changes offered by high temperatures and low operating pressures.

Investigations by Arunothayanun et al., (1999) have shown that morphological changes were observed when niosomes with differing composition were subjected to hot stage microscopy. Niosomes had a polyhedral shape below their transition temperature whereas temperatures above the transition temperature of the surfactants rendered them a spherical form.





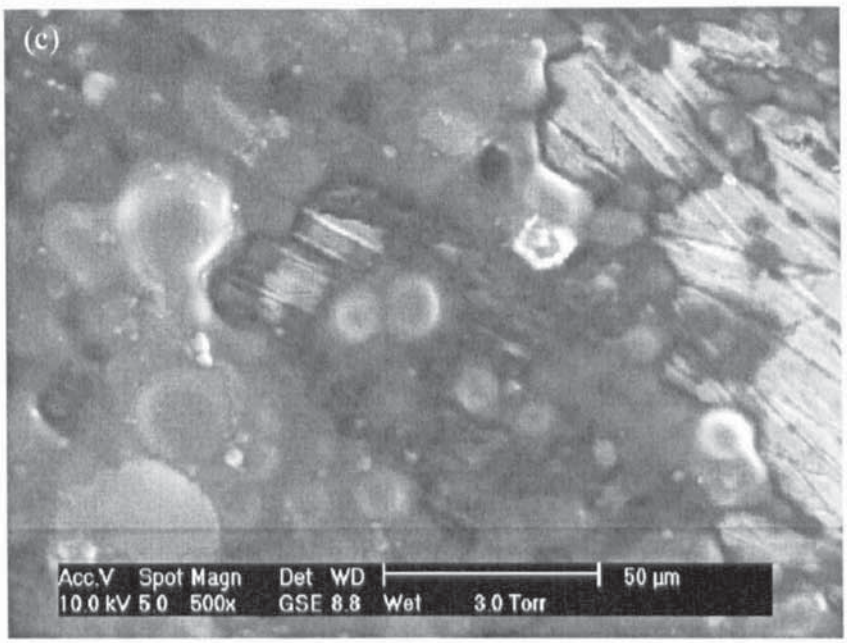


Fig 6.10: Environmental scanning-electron micrographs of drug free MLV (DMPC:Chol; 4:1 molar ratio) suspended in 0.01M PBS (pH 7.4). The liposomes were intact at 13.8°C (a), and resisted any structural changes at 17.3°C (b) under reduced pressure of 3.0 torr and a temperature of approx 28°C (c).

6.6 CONCLUSIONS

The ESEM provides a novel technique to study liposome morphology in moist conditions. The sample also carries the added advantage that no fixation procedure needs to be undertaken prior to analysis, as is the case with SEM and other techniques.

The results suggest that hydration of the lipid film results in the formation of the spherical liposomal structures, which can be done on the stub of the microscope.

The presence of salts in the hydration medium destabilises liposomes upon dehydration leading to their coalescence. Thus it can be concluded that the ions in the hydration medium play a vital role in the process of freeze drying which resembles the dehydration followed up in the sample chamber of the ESEM.

The enhanced resistance of Ibuprofen-loaded liposomes to structural breakdown during dehydration compared with drug-free MLV indicate a direct influence of drug incorporation on liposome bilayer stability. These studies also confirm the ability of ESEM to dynamically follow the changes in liposome morphology during dehydration in real time, thereby providing an alternative assay of liposome formulation/ stability relationships.

The MLV made from lipids with high transition temperature also offer a rigid structure resisting any coalescence under reduced conditions of the operating pressure suggesting that the physical state of the lipid also governs the rigidity of the

formulation with gel state offering a tougher bilayer (DSPC) when compared to the fluid liposomal bilayer (PC).

The ESEM technique also provides good quality resolution to study morphology of the liposomes. The resuspended SUV of the previously freeze dried liposomes have been analysed on the ESEM showing spherical and uniform structures upon rehydration. ESEM could also be exploited for study as hot stage microscope to decipher morphological changes with changes in temperature.

7.0 Concluding remarks and future work

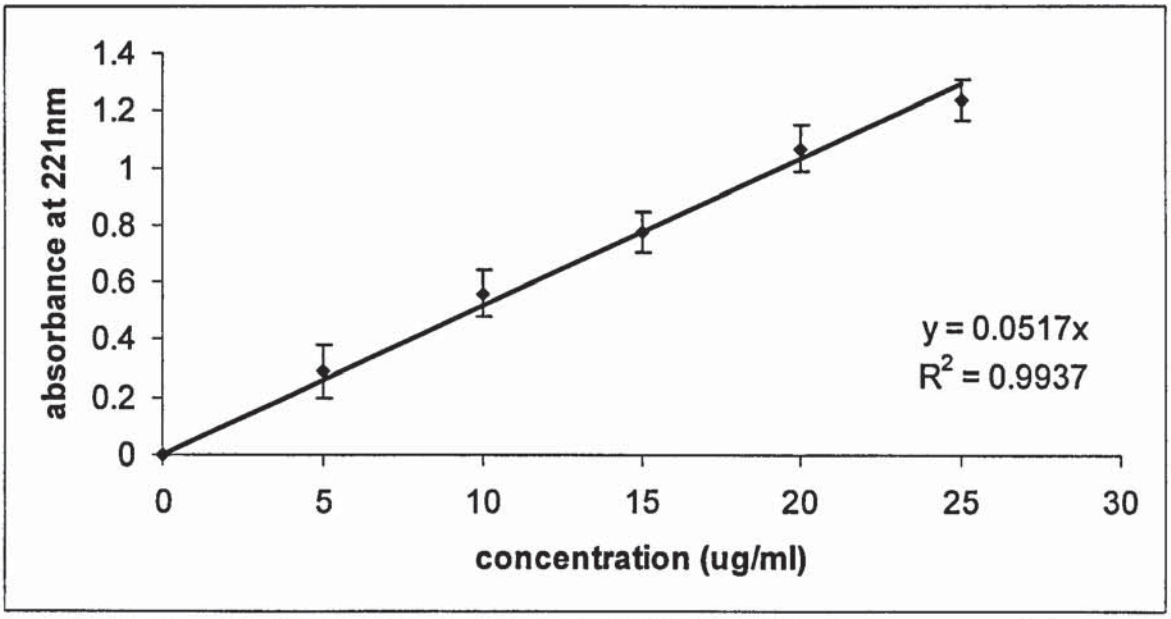
Investigations into the solubilisation of the poorly water soluble drugs have shown that liposomes are a promising delivery system. The results presented here demonstrate that both the drug characteristics as well as the liposomal bilayer composition must be considered collectively for the design of the solubilising systems. The optimisation of the cholesterol content plays a vital role in influencing drug encapsulation and possibly bilayer fluidity. The various bilayer properties that potentially influence drug entrapment include bilayer charge, length of the alkyl chain, presence of surfactants and alkyl chain unsaturation. The results show that the presence of oppositely charged lipids has a significant impact on the solubilisation of ionisable drugs with drugs carrying an opposite charge to that of the lipid improving the entrapment. Another potential approach to increase drug retention in the bilayer could be the careful control of the pH of the hydration media with pH favouring the unionised form of the drug candidate in improving drug encapsulation as noted for ibuprofen. However this must take into account the physiological applicability. The substitution of longer chain lipids has also been shown to enhance drug encapsulation and retention with values as high as ~90% as in the case of flurbiprofen and progesterone. Indeed the substitution of dilignoceroyl phosphatidylcholine ($C_{24}PC$) resulted in improvements of the encapsulation of ibuprofen by approximately two fold. The encapsulation of the insoluble drugs could possibly be improved with the addition of the single chain surfactant alpha tocopherol. However, the addition of tocopherol is limited in that a careful investigation of the concentration needs to be considered for enhanced encapsulation dependent upon the drug candidate to be encapsulated.

The drug properties however did not show a specific trend. Although log P and/or molecular weight of the drug could possibly partly influence drug encapsulation, a careful consideration of the individual drug properties needs to be considered for optimised solubilisation. An investigation using the X ray powder diffraction technique or NMR could possibly shed light on the location and interaction of the drug candidate within the liposomal bilayer. Surface tension measurements on the Langmuir trough could also potentially be reflective of drug interaction with the lipid bilayer thus adding to the investigations addressing the lack of clear correlation in the drug properties and liposomal loading.

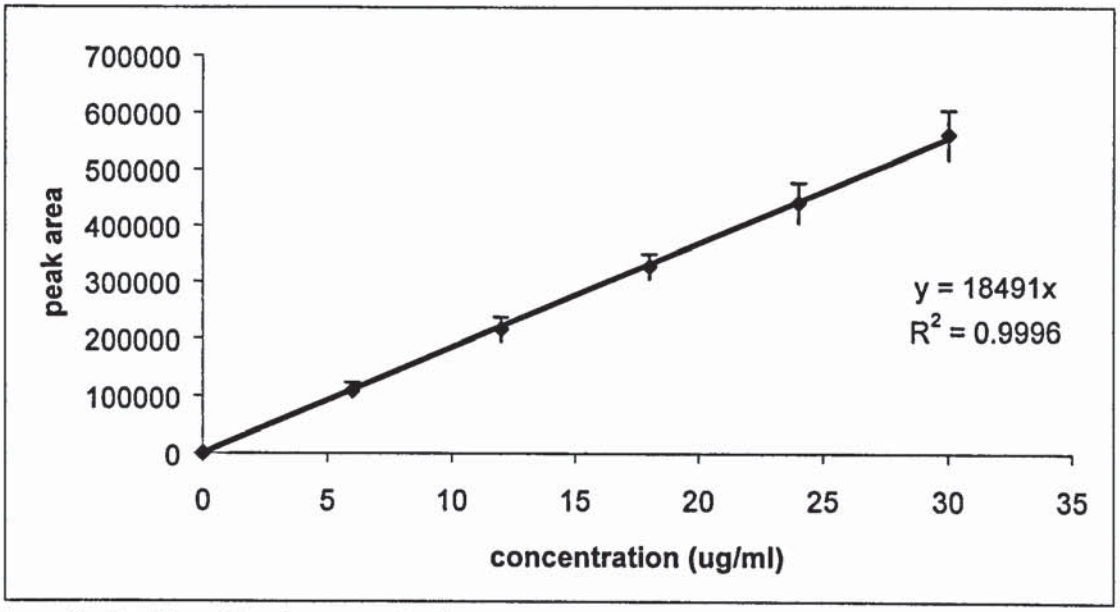
Environmental scanning-electron microscopy (ESEM) was used to dynamically follow the changes in liposome morphology in real time during dehydration thereby providing an alternative assay of liposome formulation and stability. The ESEM analysis clearly demonstrated that ibuprofen incorporation enhanced the stability of PC:Chol liposomes with drug-loaded liposomes showing enhanced resistance to coalescence during dehydration compared to drug-free liposomes. This behaviour suggests the incorporation of ibuprofen within the liposomal structure may enhance vesicle stability due to the presence of amphiphilic ibuprofen within the bilayer structure of the liposome. Further application of the ESEM involving PBS and water as the hydration media have shown that the presence of the salts in the media can be responsible for causing destabilisation of the liposomal structure under reduced pressure. The ESEM technique was also exploited to study the morphology of the liposomes at different temperatures. With the development of the higher sensitive

ESEM recently, it could be possible to image the anatomy of the liposomal formulation *in situ*.

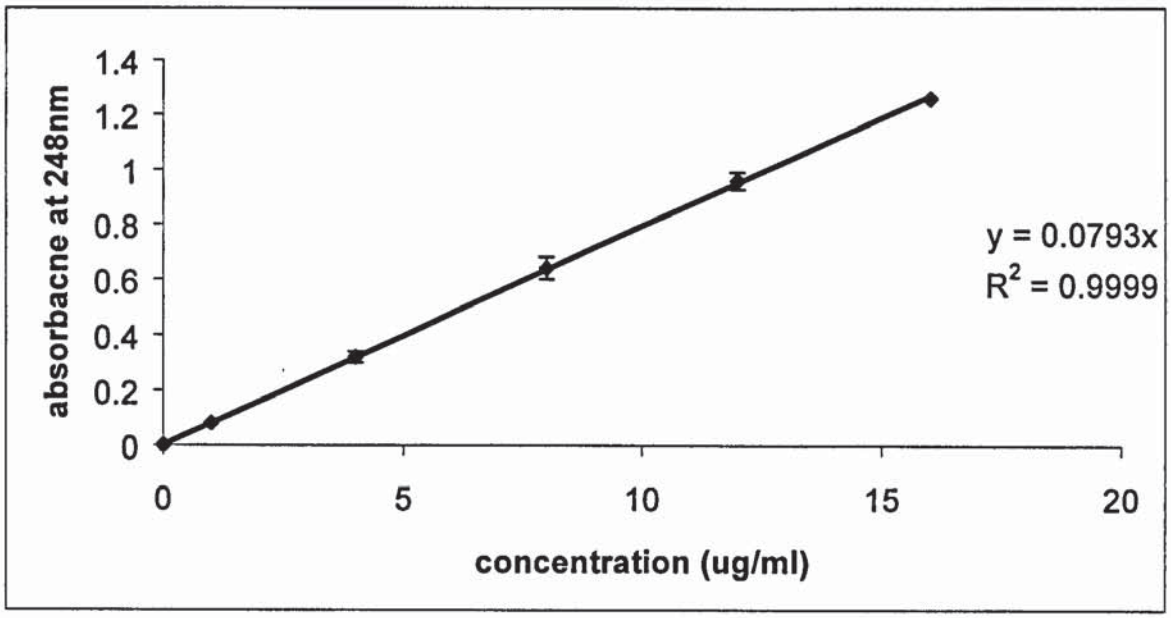
The freeze drying of liposomes using amino acids as cryoprotectants has shown potential for a new class of compounds to stabilise liposomal formulations. The three amino acids investigated have exhibited good drug retention properties and contributed to the stability of liposomal formulations. It may be possible that investigation into the other classes of amino acids could potentially add to the existing list of cryoprotectants.



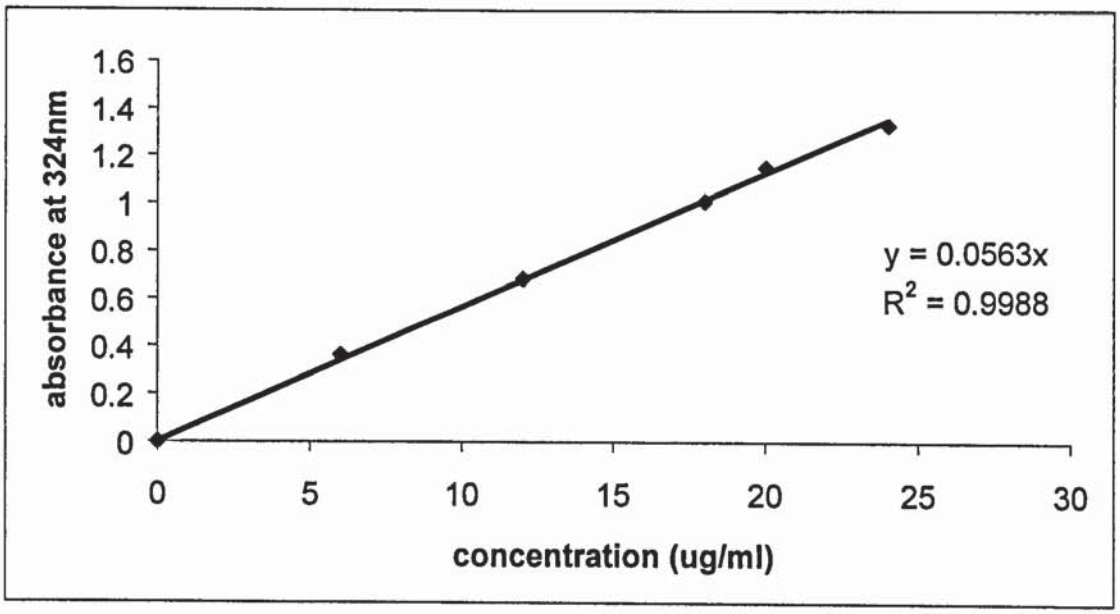
Appendix 1a: The calibration curve for ibuprofen in PBS (7.4). The calibration curve was made by dissolving ibuprofen in PBS and serial dilutions made to achieve concentrations in the range of 5-25μg/ml. The absorbance was measured at 221 nm. Values denote mean ± S.D. from at least three experiments.



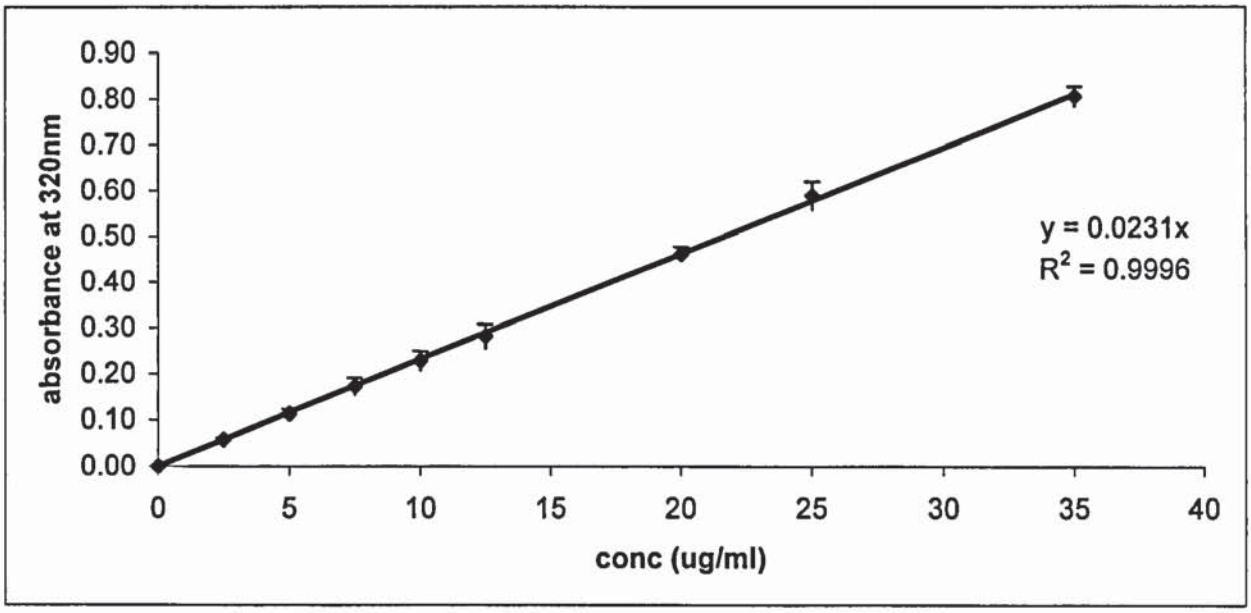
Appendix 1b: The calibration curve for ibuprofen made in methanol. The calibration curve was made by dissolving ibuprofen in methanol and serial dilutions made to achieve concentrations in the range of 0-30μg/ml. The absorbance was measured at 222 nm. The mobile phase was phosphate buffer:acetonitrile (70:30) (pH 7.4) at a flow rate of 1 ml/min. The sample injection volume was 15μg/ml. Values denote mean ± S.D. from at least three experiments.



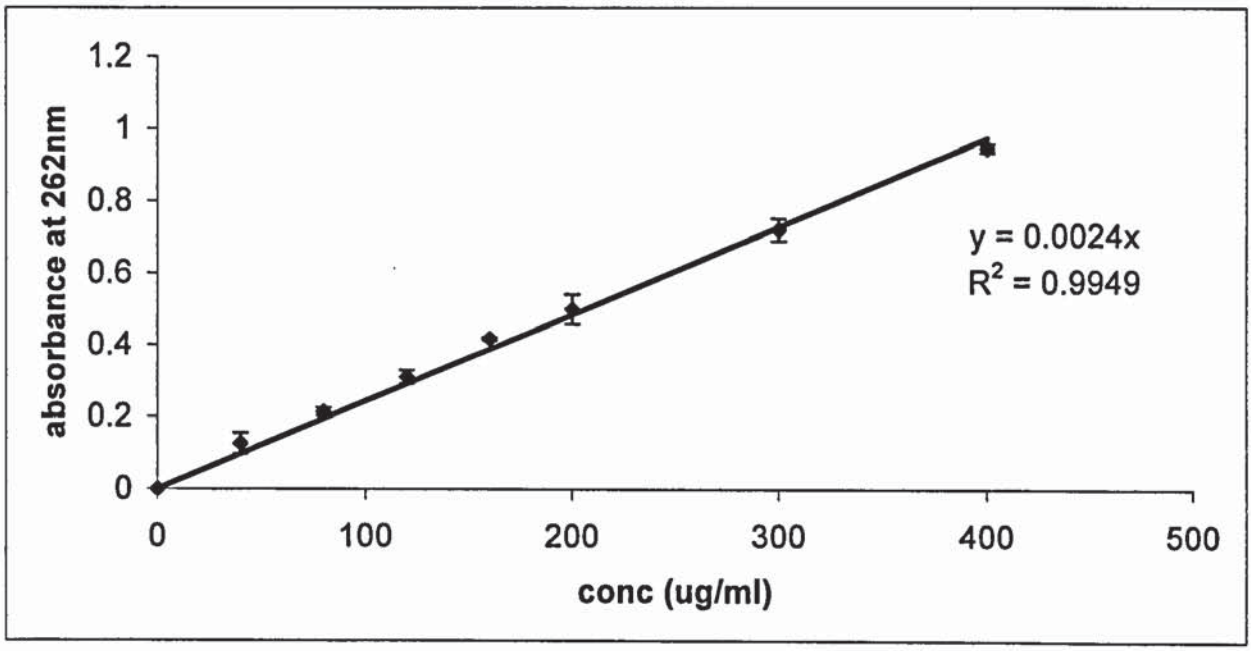
Appendix 1c: The calibration curve for flurbiprofen in PBS (7.4). The calibration curve was made by dissolving flurbiprofen in PBS and serial dilutions made to achieve concentrations in the range of 1-18μg/ml. The absorbance was measured at 248 nm. Values denote mean ± S.D. from at least three experiments.



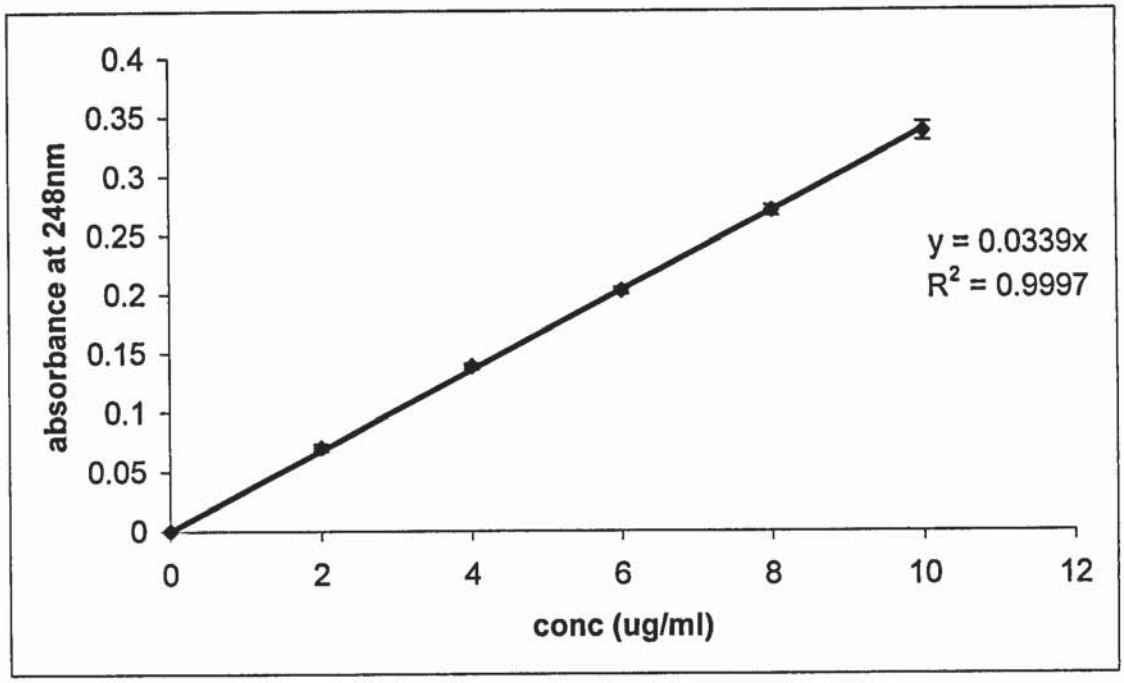
Appendix 1d: The calibration curve for sulindac in PBS (7.4). The calibration curve was made by dissolving sulindac in PBS and serial dilutions made to achieve concentrations in the range of 0-25μg/ml. The absorbance was measured at 324 nm. Values denote mean ± S.D. from at least three experiments.



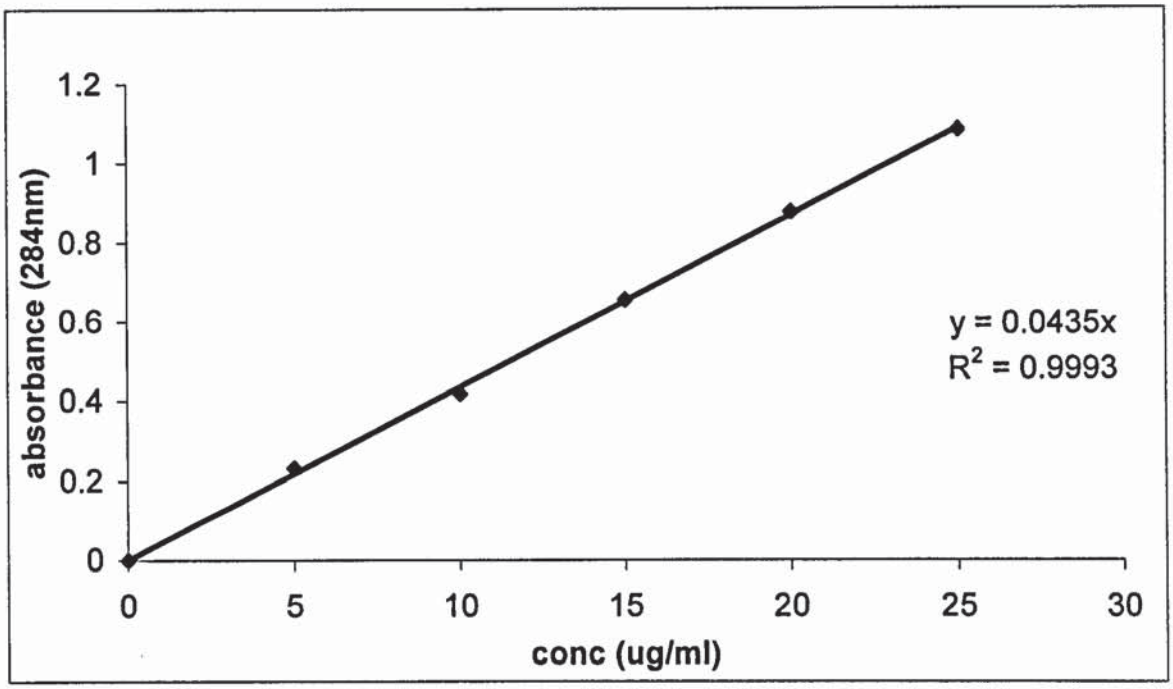
Appendix 1e: The calibration curve for indomethacin in PBS (7.4). The calibration curve was made by dissolving indomethacin in PBS and serial dilutions made to achieve concentrations in the range of 0-35μg/ml. The absorbance was measured at 320 nm. Values denote mean ± S.D. from at least three experiments.



Appendix 1f: The calibration curve for lignocaine in PBS (7.4). The calibration curve was made by dissolving lignocaine in PBS and serial dilutions made to achieve concentrations in the range of 50-400μg/ml. The absorbance was measured at 262 nm. Values denote mean ± S.D. from at least three experiments.



Appendix 1g: The calibration curve for progesterone in PBS (7.4). The calibration curve was made by dissolving progesterone in PBS and serial dilutions made to achieve concentrations in the range of 0-10μg/ml. The absorbance was measured at 248 nm. Values denote mean ± S.D. from at least three experiments.



Appendix 1h: The calibration curve for mefenamic acid in PBS (7.4). The calibration curve was made by dissolving mefenamic acid in PBS and serial dilutions made to achieve concentrations in the range of 5-25μg/ml. The absorbance was measured at 284 nm. Values denote mean ± S.D. from at least three experiments.

A.R. Mohammed, A.G.A Coombes, Bock. N, M. Fitzgerald and Y. Perrie (2004). Liposome formulation of poorly water soluble drugs: optimization of drug loading and ESEM analysis of stability. Int J Pharm; 285;1-2; 23-34.

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