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International Journal of Pharmaceutics 285 (2004) 23–34
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Liposome formulation of poorly water soluble drugs: optimisation of drug loading and ESEM analysis of stability

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Received 21 January 2004; received in revised form 7 June 2004; accepted 6 July 2004
Available online 12 September 2004

Abstract

Liposomes due to their biphasic characteristic and diversity in design, composition and construction, offer a dynamic and adaptable technology for enhancing drug solubility. Starting with equimolar egg-phosphatidylcholine (PC)/cholesterol liposomes, the influence of the liposomal composition and surface charge on the incorporation and retention of a model poorly water soluble drug, ibuprofen was investigated. Both the incorporation and the release of ibuprofen were influenced by the lipid composition of the multi-lamellar vesicles (MLV) with inclusion of the long alkyl chain lipid (dilignoceroyl phosphatidylcholine (C_{24}PC)) resulting in enhanced ibuprofen incorporation efficiency and retention. The cholesterol content of the liposome bilayer was also shown to influence ibuprofen incorporation with maximum ibuprofen incorporation efficiency achieved when 4 μmol of cholesterol was present in the MLV formulation. Addition of anionic lipid dicetylphosphate (DCP) reduced ibuprofen drug loading presumably due to electrostatic repulsive forces between the carboxyl group of ibuprofen and the anionic head-group of DCP. In contrast, the addition of 2 μmol of the cationic lipid stearylamine (SA) to the liposome formulation (PC:Chol – 16 μmol:4 μmol) increased ibuprofen incorporation efficiency by approximately 8%. However further increases of the SA content to 4 μmol and above reduced incorporation by almost 50% compared to liposome formulations excluding the cationic lipid. Environmental scanning electron microscopy (ESEM) was used to dynamically follow the changes in liposome morphology during dehydration to provide an alternative assay of liposome stability. ESEM analysis clearly demonstrated that ibuprofen incorporation improved the stability of PC:Chol liposomes as evidenced by an increased resistance to coalescence during dehydration. These finding suggest a positive interaction between amphiphilic ibuprofen molecules and the bilayer structure of the liposome.

Keywords: Liposomes; Environmental scanning electron microscopy; Ibuprofen; Poorly water soluble drugs; Stability

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doi:10.1016/j.ijpharm.2004.07.010

1. Introduction

Development of combinatorial chemistry has made a dramatic impact on drug discovery, making it possible to synthesize thousands of compounds per year. However, many small organic molecule (<500 Da) drugs synthesized tend to have limited solubility, e.g. <1.5 mg/ml in water and the 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. Depending on the intended route of administration and the nature of the drug molecule, various methods may be employed to improve drug solubility and hence clinical applicability. In the case of liquid formulations, there are a number of solubility enhancing technologies currently used including manipulation of pH (e.g. Levis et al., 2003), production of alternative water soluble salts (e.g. Ohwada et al., 2003), the use of co-solvent systems (whereby drug solubility is increased by the addition of a water miscible solvent in which the drug has good solubility) (e.g. Stella, 1996), and drug complexation with cyclodextrins (Ma et al., 1999). Despite this array of formulation techniques, often the choice of system is limited either by the intended route of administration or by the nature of the drug. For example, 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, 1993). In addition, not all drugs have structures which can be suitably manipulated to improve solubility, e.g. Paclitaxel, lacks functional groups which are ionisable within the physiological pH range (Straubinger, 1995).

Despite extensive investigations of liposomes as carrier systems for hydrophilic drugs, their application as solubilising agents has received limited attention despite their biphasic character and diversity in construction, offering wide scope for enhancing drug solubility. Liposome encapsulation efficiency of lipophilic drugs is known to be enhanced by using derivatives of compounds with increased $\log P$ and lipophilicity (Goundalkar and Mezei, 1983), indeed compounds such as octadecylcarbamoyl-5-fluorouracil can be incorporated into the lipid bilayer structure with an encapsulation efficiency of up to 100% (Fresta et al., 1993). However liposomal incorporation of poorly water soluble compounds is not only dependent on the physicochemical properties of the drug, factors including bilayer composition and their method of preparation have also been shown to be contributing factors (Walde and Ichikawa, 2001).

To study liposome morphology various techniques including scanning electron microscopy (SEM) (Lopez et al., 2001) and freeze fracture analysis (Egelhaaf et al., 2003) have previously been employed. However, SEM analysis requires samples to be dried or fixed before imagining 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. Environmental scanning electron microscopy (ESEM) can overcome this problem with its ability to image wet systems without prior sample preparation. Furthermore, 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 et al., 2000). ESEM is possible due to a system of differential pumping, which maintains the electron gun at high vacuum while the sample chamber can be kept at a constant pressure of 10–20 Torr. This is combined with an electrical current detector which can collect and process signals generated from secondary electrons backscattered from the sample surface (Donald, 1998). Owing to the presence of vapour, these electrons also undergo collisions, leading to ionisation of gas molecules which in turn produces a daughter electron and a resulting cascade reaction and an overall sample amplification which is dependent on the population of electrons leaving the surface (Donald, 1998).

There have been few systematic investigations of the compositional and physico-chemical characteristics of vesicles which control inclusion of poorly water soluble drugs within the lipid bilayer and subsequent liposome stability. The aim of this work was

to identify the key formulation parameters controlling encapsulation of poorly water soluble drugs. Ibuprofen, as a model was chosen and incorporated within MLV. Ibuprofen is characterized by a log *P* value of 3.9, a water solubility of approximately 60 μg/ml and a pKa of 5.2 (weak acid). 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/stability relationships.

2. Materials and methods

Egg phosphatidylcholine (PC) (grade I) which has a mixture of saturated and unsaturated alkyl chains 16–18 carbons in length, dimyristoyl phosphatidylcholine (DMPC) and distearoyl phosphatidylcholine (DSPC) were purchased from Lipid Products, Epsom, Surrey, UK. Dilignoceroyl phosphatidylcholine (C_{24}PC) was purchased from Avanti Polar lipids, Alabaster, USA. Stearylamine (SA), dicetylphosphate (DCP), cholesterol (Chol) and ibuprofen were obtained from Sigma-Aldrich Company Ltd., Poole, UK. All the chemicals used were of analytical grade.

2.1. Preparation of multilamellar vesicles

Multilamellar vesicles (MLV) were prepared using a technique based on the established film method (Bangham et al., 1965). Briefly the lipid components were dissolved in a 9:1 solvent mixture of chloroform and methanol with the required amount of ibuprofen (1.25 mg) and the solvent was evaporated on a rotary evaporator to obtain a dry film. The film was hydrated with 2 ml of distilled water to give final lipid concentration of 16–32 μmol/ml dependent on formulation. Drug free liposomes were prepared as controls.

2.2. Determination of ibuprofen entrapment efficiency in MLV

The drug loading of liposomes was determined by measuring the non-incorporated drug present in the hydration medium and wash media after separation of liposomes by centrifugation (Beckman J2 Centrifuge) at 27,200 g for 30 min. The drug content of the supernatant was analysed by UV spectroscopy (Unicam Helios) at 221 nm wavelength (Paavola et al., 2000). This method was validated by HPLC analysis of ibuprofen content within a random selection of washed liposome preparations.

2.3. Ibuprofen release from MLV

The release rate of ibuprofen from MLV was determined by incubating ibuprofen loaded liposomes in 50 ml PBS at 37 °C in a shaking water bath. At time intervals 10 ml of release medium was withdrawn and centrifuged at 27,200 g for 30 min. The supernatant was analysed spectrophotometrically at 221 nm wavelength and the amount of drug released was assayed by comparison with a calibration curve of ibuprofen in PBS.

2.4. Determination of vesicle volume distribution and zeta potential

MLV mean volume distribution was carried out by laser diffraction spectroscopy using a Malvern Mastersizer X at 20 °C. Zeta potential was determined by photon correlation spectroscopy using a Zetaplus (Brookhaven Instruments) in 0.001 M PBS at 25 °C.

2.5. ESEM analysis

Liposomes and dried lipid films were analysed using a Philips Electron Optics ESEM. The ESEM sample holder 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 controlled hydration of dried lipid films. The effect of the 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 analysed using controlled dehydration of samples to define a coalescence pressure. A working temperature of 5 °C was maintained in all experiments.

3. Results and discussion

3.1. The effect of the cholesterol content of liposomes on ibuprofen entrapment in MLV

Phosphatidylcholine (or its derivates) is a main lipid excipient of several commercially available liposome products due to its non-toxic biodegradable profile. The effect of incorporating cholesterol (which is known (Gregoriadis, 1993) to influence liposome stability) within the lipid composition of phosphatidylcholine (PC; 16 μmol) liposomes on ibuprofen entrapment was determined by varying the cholesterol contents from 0–50% mol/mol (Table 1). The presence of cholesterol within the liposomes bilayer had little effect on ibuprofen incorporation when liposomes contained 4 μmol of cholesterol compared to PC only liposomes (10.7% versus 10.1% mol/mol, respectively; Table 1). However, increasing the cholesterol content to 8 and 16 μmol resulted in major reductions in drug loading to 5.5 and 3.1% mol/mol, respectively (Table 1). Variation of the cholesterol content of drug-free and drug-loaded liposomes made no significant difference to either the vesicle size or zeta potential (Table 1).

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 50% mol/mol cholesterol within a liposome formulation 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 (Egelhaaf et al., 2003). This inclusion of cholesterol within liposomal bilayers has been shown to result in an increased packing densities of phospholipids molecules (Semple et al., 1996) which 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 which show a decrease in effective area per molecule as the cholesterol content of the monolayer is increased (Rogerson et al., 1987). 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; Papahadjopoulos et al., 1973). Biophysical studies (Bernsdorff et al., 1997) of phospholipid-cholesterol bilayers have also shown that the addition of 30–50 mol% cholesterol to phosphatidylcholine liposomes can increase the hydrophobicity in the interfacial region of the liposome bilayer, a factor which could influence the incorporation of drugs within the lipid bilayer. However, the results in Table 1 suggest that in the case of the poorly water soluble drug ibuprofen, minor improvements in drug loading occurred in PC liposomes containing 4 μmol cholesterol content (20% mol/mol) and major reductions in drug loading resulted when the cholesterol content was increased. This maybe a result of two conflicting factors; on the one hand the increased hydrophobicity (Bernsdorff et al., 1997), increased stability (Gregoriadis and Davis, 1979), and 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

Table 1
The effect of cholesterol content on encapsulation of ibuprofen in PC:Chol liposomes

Liposome cholesterol content (μmol)	MLV		MLV incorporating ibuprofen			
	Liposome size (μm)	Liposome zeta potential (mV)	Liposome size (μm)	Liposome zeta potential (mV)	Ibuprofen entrapment efficiency (%)	Concentration of drug in liposomes (% mol/mol)
0	4.9 ± 0.5	−5.9 ± 0.4	5.1 ± 0.7	−5.2 ± 0.8	29.5 ± 0.6	10.1 ± 0.2
4	4.3 ± 0.5	−6.1 ± 0.1	5.3 ± 0.4	−6.1 ± 0.7	39.3 ± 0.9	10.7 ± 0.2
8	4.2 ± 0.4	−6.3 ± 0.8	5.0 ± 0.3	−5.4 ± 0.9	23.2 ± 0.8	5.5 ± 0.2
16	4.2 ± 0.5	−5.2 ± 0.9	4.8 ± 0.9	−5.8 ± 0.5	17.1 ± 0.2	3.1 ± 0.1

Multi-lamellar vesicles incorporating ibuprofen were prepared from 16 μmol phosphatidylcholine (PC) and varying cholesterol content (0–16 μmol) (0–50% mol/mol ratio) in the presence of 1.25 mg of ibuprofen as described in Section 2.1. Ibuprofen encapsulation efficiency within liposomes was determined as described in Section 2.2. Values denote mean ± S.D. from at least three experiments.

compete with ibuprofen for packing space within the bilayer therefore excluding the drug as the amphiphiles assemble into liposomes. The local arrangement of amphiphiles into aggregates is dictated by the specific shape of the molecules (defined by their critical packing parameter (CPP) which is determined by the ratio of the chain volume to head area (Israelachvili et al., 1977)). Consequently, the formation of the liposome bilayers will dictate molecular volume packaging available for ibuprofen incorporation with increased concentrations potentially resulting in alternative aggregate structures forming.

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

The influence of lipid alkyl chain length on ibuprofen encapsulation was investigated using liposomes prepared from Egg 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 by longer alkyl chain lipids influenced both ibuprofen incorporation (Table 2) and release rates (Fig. 1). A trend of increasing ibuprofen loading and encapsulation efficiency with increasing lipid chain length was apparent in the order of C_{24}PC > DSPC > DMPC > PC, with ibuprofen drug loading increasing by around 50% when C_{24}PC was substituted for PC (Table 2). Previous work (Gregoriadis, 1973) 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. This increased drug loading capacity of longer alkyl chain lipid bilayers could be attributed to the increased hydrophobic area within the liposome bilayers, similar to the effects previously demonstrated with micellar solubilisation of drugs such as barbiturates (Arnarson and Elworthy, 1980; Ismail et al., 1970).

The ability of liposomes to retain ibuprofen during incubation in PBS at 37 °C also correlated with the alkyl chain length of the lipid component with retention values again in the order C_{24}PC > DSPC > DMPC > PC. After only 30 min incubation, PC-containing MLV released 15.5% of the ibuprofen load compared to 1.5% for C_{24}PC liposomes and major differences in release were evident after 24 h incubation (Fig. 1). Drug release from liposomes is known to be influenced by the phase transition temperature of the lipid excipients within the bilayer (Senior and Gregoriadis, 1982). There are several factors which directly affect the phase transition temperature including alkyl hydrocarbon chain length: as the hydrocarbon chain length is increased, van der Waals interactions between the lipid chains become stronger requiring more energy to disrupt the ordered packing, and the phase transition temperature increases. Therefore at 37 °C both PC (T_c < 0 °C) and DMPC (T_c = 23 °C) liposomes will be in the fluid state resulting in increased drug loss compared with the higher transition temperature lipids DSPC (T_c = 55 °C) and C_{24}PC (T_c = 80 °C). Indeed release of steroids from MLV has also been shown to be similarly influenced by transition temperature, with both hydrocortisone and budesonide being retained longer in DSPC liposomes than in PC MLV (Saarinen-Savolainen et al., 1997). The results in Fig. 1 also show that C_{24}PC liposomes release less ibuprofen than DSPC liposomes despite both systems being in the ordered gel phase.

Table 2
The effect of lipid alkyl chain length on liposome size, zeta potential and ibuprofen encapsulation

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

Multi-lamellar vesicles incorporating ibuprofen were prepared from either 16 μmol phosphatidylcholine (PC), dimyristoyl phosphatidylcholine (DMPC), distearoyl phosphatidylcholine (DSPC) or dilignoceroyl phosphatidylcholine (C_{24}PC) and 4 μmol cholesterol in the presence of 1.25 mg of ibuprofen as described in Section 2.1. Values denote mean ± S.D. from at least three experiments.

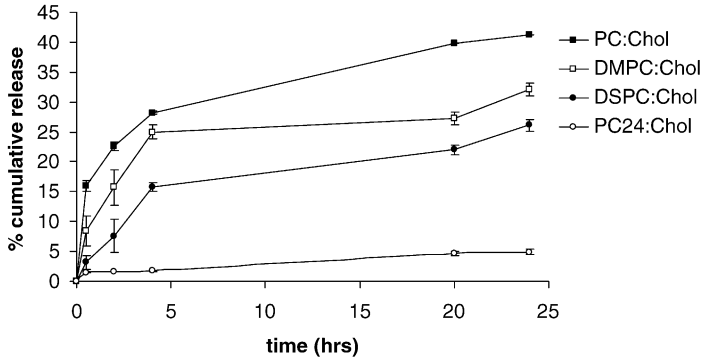

Fig. 1. The effect of lipid alkyl chain length on the release profile of ibuprofen from liposomes. Multilamellar vesicles incorporating ibuprofen composed of: 16 μmol PC, 4 μmol Chol (filled square); 16 μmol DMPC, 4 μmol Chol (open square); 16 μmol DSPC, 4 μmol Chol (filled circle); 16 μmol C_{24}PC, 4 μmol Chol (open circle) were incubated 0.01 M 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 percent of total incorporated. Results represent mean ± S.D., $n = 3$ of percentage cumulative release in PBS at 37 °C.

This behaviour may again be explained by the increased van der Waals interactions between the longer lipid chains and the increased lipid phase area within the liposomes enhancing the drug binding and bilayer stability.

3.3. Effect of charged lipids on ibuprofen encapsulation and release

The inclusion of charged lipids in the liposome formulations influenced ibuprofen incorporation, MLV size and zeta potential (Table 3). The addition of up to 2 μmol SA to the PC:Chol liposome formulation enhanced the incorporation efficiency of ibuprofen by approximately 8% to 47% (Table 3) although only a fairly minor change in drug loading was measured. The electrostatic attraction between the positively charged head group in stearylamine and the carboxyl group present in dissociated ibuprofen appears to have little effect on ibuprofen association with MLV. Indeed, further increases in SA content up to 6 μmol resulted in a major reduction of around 50% in ibuprofen entrapment efficiency and drug loading (Table 3) to below that of formulations excluding SA. Addition of 2 μmol of SA to the liposome formulation also resulted in a reversal of surface charge (due to the cationic head-group of SA) and an increase in vesicle size (based on mean volume distribution) of approximately 0.8 μm compared with the unmodified PC:Chol formulation (Table 3). The increased

Table 3
The effect of cationic lipid content on PC:Chol liposome size, zeta potential and ibuprofen encapsulation

Liposome SA content (μmol)	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

Multi-lamellar vesicles incorporating ibuprofen were prepared from 16 μmol phosphatidylcholine, 4 μmol cholesterol and varying stearylamine (SA) content (0–6 μmol) in the presence of 1.25 mg of ibuprofen as described in Section 2.1. Values denote mean ± S.D. from at least three experiments.

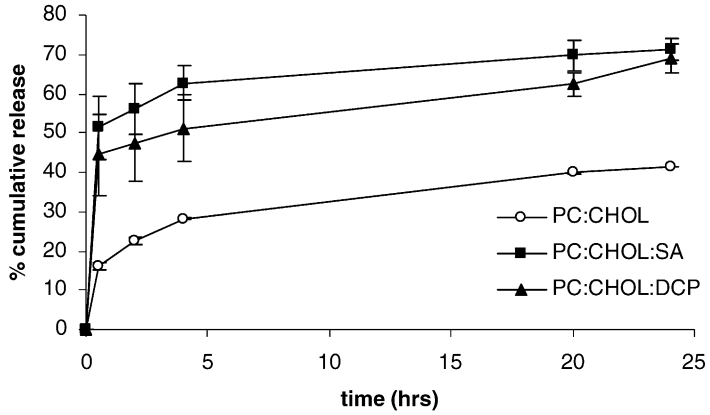

Fig. 2. The effect of incorporation of charged lipids on the release profile of ibuprofen from MLV. Multilamellar vesicles incorporating ibuprofen composed of: 16 μmol PC, 4 μmol Chol (circle); 16 μmol DMPC, 4 μmol Chol, 2 μmol SA (square); 16 μmol DSPC, 4 μmol Chol, 2 μmol DCP (triangle) were incubated 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 ± S.D., $n = 3$ of percentage cumulative release in PBS at 37 °C.

vesicle size of PC:Chol:SA (16 μmol:4 μmol:2 μmol) 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 drug-free PC:Chol:SA liposomes were notably smaller (∼1.3 μm; Table 3). Substitution of stearylamine with 2 μmol of dicetyl phosphate, an anionic lipid, resulted in reduced ibuprofen incorporation efficiency to 34% (8.6% mol/mol; results not shown) relative to PC:Chol liposomes (10.7% mol/mol; Table 3) suggesting that repulsive interactions between the negatively charged lipid and the drug molecule influence ibuprofen incorporation within lipid bilayers. The incorporation of charged lipids in PC:Chol liposomes also resulted in increased ibuprofen release compared with unmodified PC:Chol vesicles (Fig. 2). Both the 2 μmol SA and DCP containing liposomes released significantly more ($P < 0.001$) incorporated drug (71.2 ± 2.8% and 69.0 ± 3.7%, respectively) over 24 h compared with PC:Chol liposomes (41.4 ± 0.1%) suggesting that the presence of charged lipids within the liposome bilayer increases the permeability of the bilayer and/or lipid-drug binding.

Previous investigations into the characteristics of charged lipid membranes (Jahnig 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) (Jahnig et al., 1979). This electrostatically induced change in bilayer packing, combined with the electrostatic interactions between the ibuprofen carboxyl group and the charged headgroups of SA or DCP are both expected to influence the incorporation of ibuprofen within the liposomes. At lower concentrations of SA (1–2 μmol; 4–9% mol ratio) the opposing charges of the SA and ibuprofen head-groups may electrostatically interact thereby increasing ibuprofen–liposome association (Table 3). Indeed after incubation of MLV for 20 h with a solution containing 1.25 mg of ibuprofen, 14.5 ± 0.7% of the ibuprofen remained associated with cationic MLV (PC:Chol:SA – 16 μmol:4 μmol:2 μmol) after centrifugation compared with less than 4% for either PC:Chol (16 μmol:4 μmol) or PC:Chol:DCP (16 μmol:4 μmol:2 μmol) MLV. The marked decrease in ibuprofen encapsulation in liposomes containing SA concentrations above 4 μmol may be explained by electrostatic induced chain tilt and the subsequent changes in the lateral packing of the liposome bilayers (Jahnig et al., 1979). This may also be responsible for these variations in drug retention within the liposomal bilayers (Fig. 2).

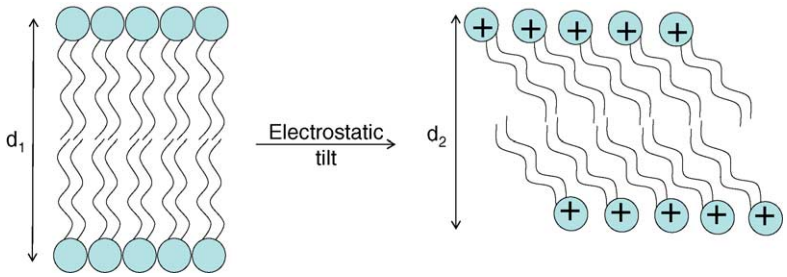

Fig. 3. Schematic representation of the electrostatically induced tilt of a lipid bilayer in the presence of charged surfactant head-groups. Electrostatic repulsion between charged-head groups increases the lateral packaging of the lipid polar heads. However, the ordered hydrocarbon chains, due to their attractive van der Waals interaction only partially follow this increase and in order to minimise a change in packing, the hydrocarbon chains tilt, which decreases bilayer thickness. d_1 and d_2 represent the bilayer thickness before and after induction of the electrostatic tilt.

3.4. ESEM assessment of liposome stability

ESEM was used to study liposome formation from lipid films and to provide an alternative assay of liposome stability based on controlled dehydration. The formation of liposomes by controlled hydration of a dry lipid film containing PC and Chol (4:1 molar ratio) was observed using ESEM 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. 4). Liposome formation 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). Early investigations using X-ray structural data (Small, 1967) demonstrated 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 studies suggested that nine molecules of water were

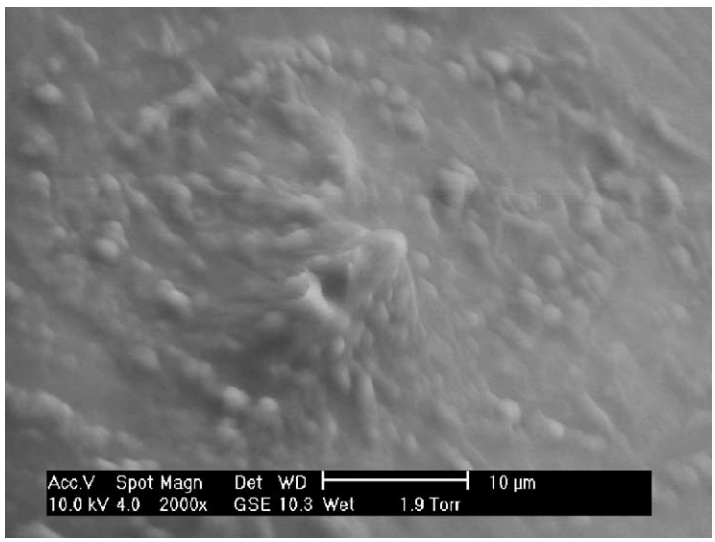

Fig. 4. Environmental scanning electron micrographs of a dry lipid film containing a mixture of PC and cholesterol (4:1 molar ratio) subjected to controlled hydration in the ESEM sample chamber under an operating pressure maintained at 1.9 Torr. The formation of spherical structures is clearly observed forming from the film.

associated with each lipid molecule at a lecithin: water weight fraction of 0.85:0.15. Further increases in the weight fraction of water (above 16%) 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).

Investigating their morphology and stability, MLV were also examined by ESEM. Drug-free MLV suspended in PBS (pH 7.4) at a pressure of 4.0 Torr appeared as spherical vesicles, superimposed on a background moisture film (Fig. 5a). Gradual reduction of the ESEM operating pressure to 3.4 Torr resulted in the liposomes starting to aggregate and at 2.9 Torr pressure (Fig. 5b) the liposomes were observed to be coalescing as evaporation of the surrounding aqueous environment continued. Small salt crystals were also visible interspersed between the lipid globules. Further reduction of pressure to 1.9 Torr (Fig. 5c) resulted in the liposomes losing their spherical shape as flattening and spreading occurred to form lipid patches. Partially spread, hemispherical structures are also visible in Fig. 5c. PC:Chol liposomes incorporating ibuprofen were also subjected to ESEM analysis: reduction of the ESEM operating pressure to 2.8 Torr again resulted in salt crystallisation from the PBS medium (Fig. 6a) as previously observed with drug-free MLV (Fig. 5b), however unlike the drug-free liposomes in PBS, which

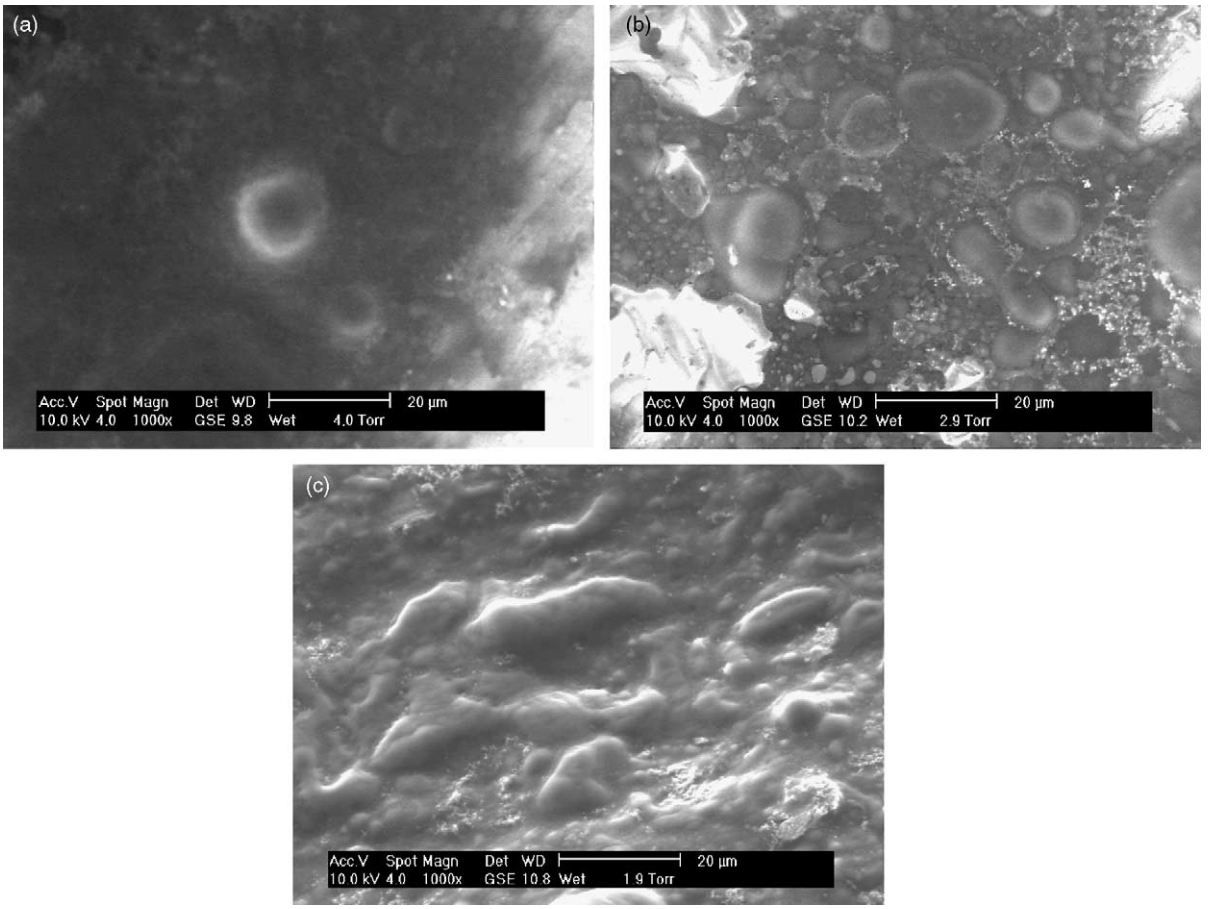

Fig. 5. Environmental scanning electron micrographs of preformed drug-free MLV (PC:Chol; 4:1 molar ratio) suspended in 0.01 M PBS (pH 7.4). At an operating pressure of 4.0 Torr (a) liposomes appear as spherical vesicles. Reduction of the ESEM operating pressure to 2.9 Torr (b) results in the liposomes coalescing as the aqueous media evaporates. Further reduction of pressure to 1.9 Torr (c) resulted in the liposomes losing their spherical shape as flattening and spreading occurred to form lipid patches.

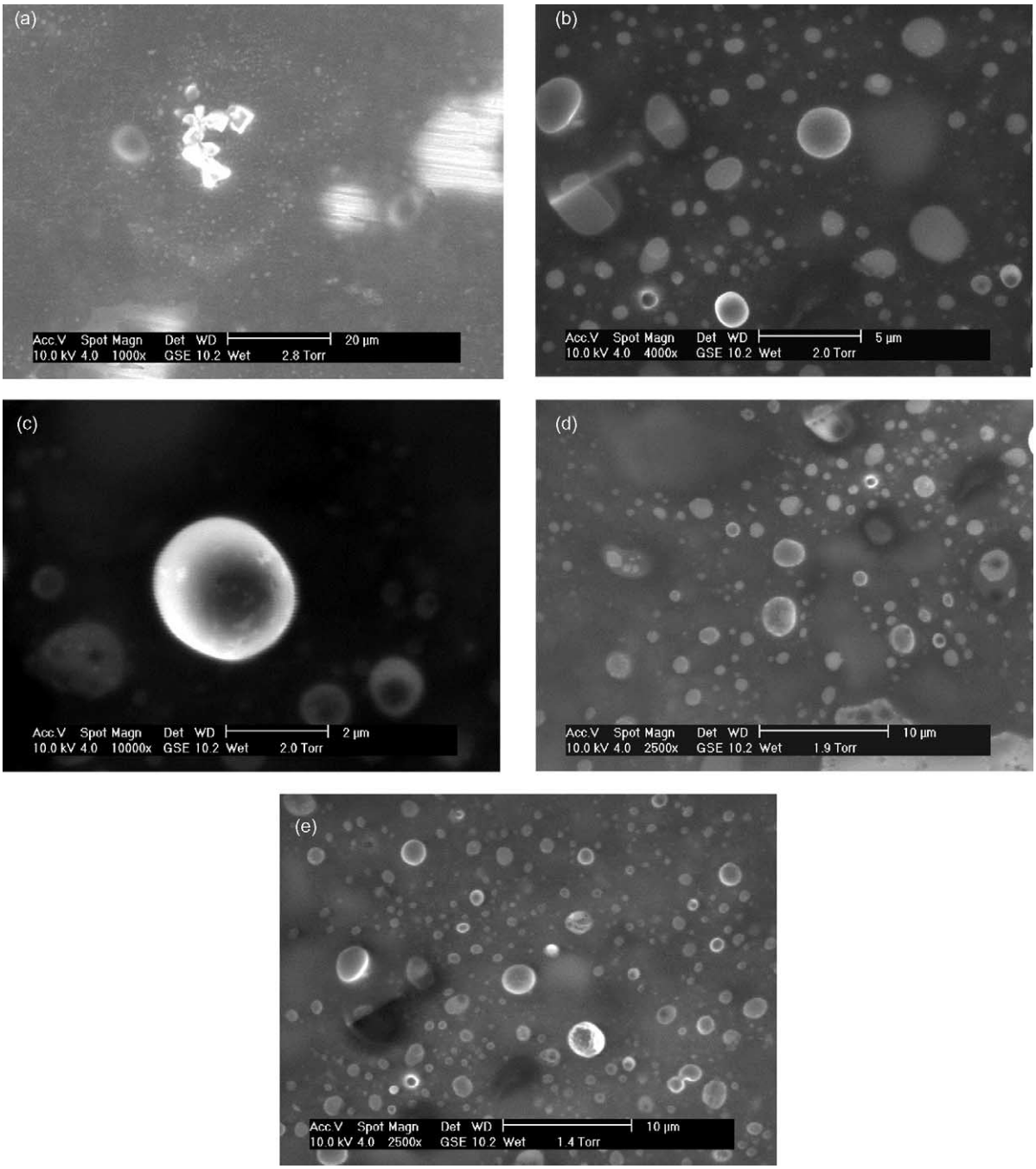

Fig. 6. Environmental scanning electron micrographs of ibuprofen-loaded MLV (PC:Chol; 4:1 molar ratio) suspended in 0.01 M PBS (pH 7.4). Vesicles were subjected to controlled dehydration in the ESEM sample chamber as in Fig. 5. At an operating pressure of 2.8 Torr spherical vesicles and salt crystallisation 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 reveals small grains or specks on the liposome surface (c).

showed signs of coalescence at 2.9 Torr (Fig. 5b), drug-loaded liposomes maintained their spherical morphology at 2.0 Torr (Fig. 6b). Closer examination of these liposomes shows small grains or specks on the liposome surface (Fig. 6c) which may be attributed to either PBS crystals or adsorbed ibuprofen. Attempts to remove these features by washing the drug loaded liposomes twice more were unsuccessful. 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. 6d) as opposed to drug-free liposomes, which tended to flatten at a similar pressure (Fig. 5c). Indeed ibuprofen loaded liposomes were able to withstand pressure reductions to 1.4 Torr without losing their spherical form (Fig. 6e).

Recent studies (Fatouros and Antimisiaris, 2002) have demonstrated the ability of membrane incorporated amphiphilic drugs to enhance membrane integrity and significantly stabilise liposomes. Liposomes composed of PC and either prednisolone, diazepam or griseofulvin were 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). The ESEM analysis reported here clearly demonstrates that ibuprofen incorporation improves the stability of PC:Chol liposomes as revealed by enhanced resistance to coalescence during dehydration compared to drug-free liposomes. Vesicle stability may be due to the presence of amphiphilic ibuprofen interdigitating between the lipid chains of the bilayer thereby increasing the rigidity of the PC:Chol (16 μmol:4 μmol) bilayer.

4. Conclusions

Incorporation of the poorly water soluble drug ibuprofen, into PC:Chol liposomes was shown to be influenced by the bilayer cholesterol content, lipid alkyl chain length, and the presence of charged lipid headgroups within the MLV formulation, respectively. Optimum drug loading was achieved by using MLV containing 20% (total lipid) cholesterol, 9% stearylamine or by substituting long alkyl chain lipids (dilignoceroyl phosphatidylcholine (C_{24}PC)) for PC. Retention of the ibuprofen was enhanced by employing long alkyl chain length PC but reduced by incorporation of charged lipids in the liposome formulation. Enhanced ibuprofen incorporation in PC:Chol liposomes tended to coincide with a small increase of approximately 10% in vesicle mean volume diameter; however the presence of ibuprofen did not significantly influence the zeta potential of any of the liposome formulations tested. ESEM analysis revealed that ibuprofen-loaded liposomes were structurally more resistant to destabilisation during dehydration than drug-free liposomes suggesting a direct effect of drug/lipid binding on liposome bilayer stability. These studies also confirm the utility of ESEM for monitoring the changes in liposome morphology in real time during dehydration, thereby providing an alternative assay of liposome formulation/stability relationships.

Acknowledgements

The authors gratefully acknowledge the financial supported of this research by Pfizer Global Research and The School of Life and Health Sciences, Aston University.

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