International Journal of Pharmaceutics 388 (2010) 181–189

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Physicochemical properties of extruded and non-extruded liposomes containing the hydrophobic drug dexamethasone

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ARTICLE INFO

Article history:
Received 5 August 2009
Received in revised form
27 December 2009
Accepted 6 January 2010
Available online 14 January 2010

Keywords:
Extruded and non-extruded liposomes
Physicochemical properties
In vitro release
Hydrophobic drug
Dexamethasone
Sustained delivery

ABSTRACT

The physicochemical and release properties of non-extruded 'multilamellar' and small sonicated and extruded 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) liposomes containing hydrophobic drug dexamethasone were investigated. Non-extruded liposomes had similar diameter, however dexamethasone encapsulation decreased with increase in lipid chain length. Dexamethasone destabilized the liposome membranes as indicated by decrease in enthalpy and increase in the peak width of the main transition. Based on calorimetric analysis, it appeared that dexamethasone and cholesterol were heterogeneously distributed in the non-extruded liposomes. Sonication and extrusion reduced the diameter (DSPC > DPPC > DMPC) and decreased drug encapsulation (approximately 50%). Cholesterol incorporation decreased drug encapsulation in both extruded and non-extruded DMPC liposomes which appeared to be due to structural similarities between cholesterol and dexamethasone. Incorporation of dexamethasone and cholesterol in the same DMPC liposomes caused a marked perturbation in the phase transition. Dexamethasone release from extruded liposomes was fast, while non-extruded liposomes showed slower release. Release was fastest from DMPC liposomes and slowest from liposomes of high phase transition lipid DSPC. Incorporation of cholesterol did not decrease release from DMPC liposomes. These results indicated that change in the physicochemical properties and the phase transition behavior of liposomes, due to processing as well as incorporation of hydrophobic drug dexamethasone, changed their release properties.

1. Introduction

Dexamethasone is a potent anti-inflammatory and immunosuppressive glucocorticoid used for the treatment of inflammatory and autoimmune conditions such as rheumatoid arthritis, edema, multiple myeloma, as an adjunct with chemotherapy and for nasal and eye allergies (Schimmer and Parker, 2001). Systemic use of glucocorticoids causes a number of adverse and toxic effects (*e.g.* withdrawal symptoms, suppression of hypothalamus-pituitary axis, electrolyte imbalance, *etc.*) (Schimmer and Parker, 2001). The encapsulation of dexamethasone in carrier systems such as liposomes and microspheres can diminish the adverse effects and reduce the total amount of drug required. Liposomal and microsphere dexamethasone formulations have been investigated for hypersensitivity pneumonitis (Tremblay et al., 1993); ophthalmic anti-inflammatory action (Taniguchi et al., 1987, 1988; Al-Muhammad et al., 1996; Al-Muhammed et al., 1996); oral ulcers (Farshi et al., 1996); arthritis (Bonanomi et al., 1987; Koning et al., 2006); atherosclerosis (Chono et al., 2005a,b; Chono and Morimoto, 2006); dermatological conditions (Cevc and Blume, 2004); pulmonary diseases (Benameur et al., 1995; Suntres and Shek, 1998); silica-induced pulmonary toxicity (DiMatteo and Reasor, 1997); localized anti-inflammatory effect for metallic stents (Kallinteri et al., 2002); brain edema (Eroglu et al., 2001); and controlling the inflammatory response to implantable devices (Hickey et al., 2002b,a).

Liposomes are used to encapsulate both hydrophilic and hydrophobic small molecules as well as macromolecules such as proteins and genes (Katragadda et al., 1999; Kaiser et al., 2003; Patil and Burgess, 2005a; Sezer et al., 2007). However, most of the literature in this area is focused on hydrophilic drugs. In particular, liposomes have been used intravenously (IV) for chemotherapeutic agents and antibiotics to alter tissue distribution, pharmacokinetics and toxicology, as well as to reduce adverse effects (Szoka et al., 1987; Mayer et al., 1990; Chono and Morimoto, 2006; Sezer et al., 2007). The potential of liposomes for controlled release of hydrophobic drugs *via* subcutaneous (SC) or intramuscular (IM) administration has not been investigated extensively. The formulation and process parameters required for controlled delivery of hydrophobic drugs from liposomes is likely to differ with the route

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doi:10.1016/j.ijpharm.2010.01.003

of administration. For example, small unilamellar vesicles (SUV) are preferred for IV delivery in order to prevent macrophage uptake and extend circulation time, whereas this might not be required for SC/IM applications. Moreover, hydrophobic drugs such as dexamethasone are incorporated in the bilayer structure of liposomes, whereas hydrophilic drugs are encapsulated in the internal aqueous chamber. Incorporation of drugs or other additives in the liposome membrane might change the phase transition properties (Biltonen and Lichtenberg, 1993) which in turn might affect drug release.

There are a number of literature reports concerning liposome formulations of dexamethasone and other glucocorticoids (Bonanomi et al., 1987; Taniguchi et al., 1987; Tremblay et al., 1993; Benameur et al., 1995; Kulkarni and Vargha-Butler, 1995; Vargha-Butler and Hurst, 1995; Farshi et al., 1996; Lopes de Menezes and Vargha-Butler, 1996; Al-Muhammad et al., 1996; Al-Muhammed et al., 1996; DiMatteo and Reasor, 1997; Saarinen-Savolainen et al., 1997; Suntres and Shek, 1998; Kallinteri et al., 2002; Metselaar et al., 2002; Cevc and Blume, 2004; Chono and Morimoto, 2006; Koning et al., 2006; Tsotas et al., 2007). However, the physicochemical properties of these liposomes vary considerably. For example, both increases and decreases in drug loading and release profiles have been reported following cholesterol incorporation in liposomes (Taniguchi et al., 1987; Al-Muhammad et al., 1996; Tsotas et al., 2007). Most of the published literature focuses on the pharmacodynamic aspect of delivery with less emphasis on physicochemical properties. Much of the knowledge about the physicochemical properties of liposomes has been derived from studies using liposomes as a model for biological membranes.

The objective of this work was to investigate the effects of process (sonication and extrusion) and formulation (lipid type, incorporation of cholesterol and dexamethasone) parameters on the physicochemical properties and thermotropic behavior of dexamethasone-loaded liposomes. For this, liposome formulations with immediate, intermediate and slow release kinetics were developed. A homologous series of lipids: 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC); 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC); and 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) was selected in order to minimize the complexity introduced by the use of lipids with different head groups, unsaturation or backbones. The phase transition temperatures (Tm) of DMPC, DPPC and DSPC are 23.5, 41.4 and 55.1 °C, respectively (Taylor and Craig, 2003). At 37 °C (body temperature), DMPC will be in the fluidic liquid crystalline state, and DPPC will be in a less fluidic gel like state, whereas DSPC will be in the gel state. Therefore, it is expected that three different release profiles may be achieved using these three lipids. Non-extruded large multilamellar (MLV) and sonicated and extruded small oligo/unilamellar liposomes were investigated. This study will help in designing liposome formulation(s) of dexamethasone.

2. Materials and methods

2.1. Materials

Dexamethasone, sodium azide, sodium dodecyl sulfate (SDS) and HEPES sodium salt were purchased from Sigma–Aldrich (St. Louis, MO). DMPC, DPPC, DSPC and cholesterol were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Chloroform, acetonitrile and methanol were purchased from Fisher Scientific (Pittsburgh, PA). Spectra®/Por CE DispoDialyzer membranes (50 kDa molecular weight cut off (MWCO; volume 2 ml)) were purchased from Spectrum labs (Rancho Dominguez, CA). Nanopure™ quality water (Barnstead, Dubuque, IA) was used for all studies.

2.2. Preparation of liposomes

A thin film hydration method was used to prepare dexamethasone-loaded liposomes as reported previously with slight modification (Patil et al., 2005b). Briefly, a chloroform solution of lipid and a methanol solution of dexamethasone were mixed in a pear-shape flask and evaporated in a Büchi® rotary evaporator at a temperature above the phase transition temperature(s) of the lipids (*ca.* 37 °C for DMPC, 55 °C for DPPC and 70 °C for DSPC liposomes) to form a thin film (lipid:drug ratio—1:0.2 M). For liposomes containing cholesterol, a chloroform solution of cholesterol was also added. This film was dried overnight under vacuum for complete removal of the solvents. The lipid film was then hydrated in 10 mM HEPES buffer, pH 7.4 (with 0.1%, w/v, sodium azide as a preservative; at $T > Tm$ (same as used in the drying step above)) followed by vortexing for 2 min (final lipid concentration 1.2 mg/ml). These vortexed vesicles were used as large multilamellar 'non-extruded' liposomes (referred to as 'non-extruded liposomes' henceforth). For preparation of small 'extruded' liposomes, non-extruded liposomes were sonicated (for 4 min) using an Avanti Ultrasonic cleaner® bath sonicator ($T > Tm$) followed by extrusion (11 times) through a 400 nm polycarbonate membrane ($T > Tm$) (Avanti Polar Lipids, Inc., Alabaster, AL) using an Avanti MiniExtruder® for size homogenization (referred to as 'extruded liposomes' henceforth). The thin film hydration method was selected as it is a simple laboratory scale method and does not introduce any contamination/impurities that can affect the phase behavior and release properties of liposomes (as may occur in the case of the reverse phase evaporation method or the dialyzed octyl glucoside vesicle (DOV; detergent dialysis) method) (Parente and Lentz, 1984).

The non-entrapped dexamethasone was removed using the Amicon Ultra-15® centrifugal filtration devices (Millipore, Billerica, MA) 30 kDa MWCO. Briefly, 5 ml liposome suspension was added to the upper chamber of the ultrafiltration tube and filtered to 500 μl by centrifugation at $\sim 3800 \times g$ (10 °C) using a Beckman Coulter Allegra® X-15R centrifuge. Non-entrapped drug filtered into the bottom chamber of ultrafiltration tube and the purified liposome suspension was collected from the upper chamber.

2.3. Liposomes characterization

2.3.1. Drug loading

Five millilitre of liposome suspension was centrifuged at $4700 \times g$ for 2 h using a Beckman Coulter Allegra® X-15R centrifuge to form a pellet and the supernatant was removed. To determine dexamethasone loading, the pellet was dissolved in 6 ml of 0.5% (w/v) SDS solution in HEPES buffer using vortexing and sonication. Dexamethasone was analyzed using a high performance liquid chromatography (HPLC) method with acetonitrile/water/phosphoric acid (35:65:0.5, v/v/v) mobile phase on a Zorbax® Rx C_{18} column (4.6 mm × 15 cm) at flow rate of 1 ml/min. Dexamethasone was detected using a Perkin Elmer 785A UV–Vis detector at 242 nm. The percent drug encapsulation was calculated as: percent encapsulation = (amount in liposomes/total amount used) × 100. The results were reported as mean ± standard deviation (SD) ($n = 4$).

2.3.2. Liposomes size analysis

Liposome particle size (diameter) was measured using a dynamic light scattering particle size analyzer (Nicomp™ 380 submicron particle size analyzer, Santa Barbara, CA) equipped with a laser (wavelength 632.8 nm) set at an angle of 90° and at 25 °C. Liposome suspensions were diluted tenfold in 10 mM HEPES buffer before size estimation. The results were reported as mean ± SD of the Gaussian number weighed average diameter ($n = 4$).

2.3.3. Zeta potential analysis

The zeta potential was measured using a ZetaPlus™ zeta potential analyzer (Brookhaven Instrument Corporation, NY) equipped with 35 mW solid state red laser (wavelength 660 nm) at 25 °C. Liposome suspensions were diluted tenfold in 10 mM HEPES buffer before zeta potential estimation. The results were reported as mean ± SD ($n = 4$).

2.3.4. Differential scanning calorimetry (DSC)

The phase transition behavior of different liposomes was analyzed using a VP-DSC® microcalorimeter (Microcal™, Inc., Northampton, MA). Fivefold diluted liposome samples (lipid concentration *ca.* 0.3 μM) were subjected to thermodynamic studies at a scan rate of 60 °C/h. Degassed HEPES buffer (10 mM, pH 7.4) was used as a reference. MicroCal™ Origin software was used for fitting a non-two state model. Phase transition temperature (Tm), excess calorimetric enthalpy (ΔH), Van't Hoff enthalpy (ΔHv), and co-operative units (CU) were estimated. The co-operative unit reflects intermolecular interactions between phospholipids in the bilayer (McElhaney, 1982). An increase in CU indicates higher interaction between the lipid molecules. The CU is calculated as a ratio of Van't Hoff enthalpy to calorimetric enthalpy (CU = ΔHv/ΔH). The results were reported as mean ± SD ($n = 3$).

2.4. In vitro release from liposomes

In vitro release testing from liposomes was determined using a dialysis sac method (Chidambaram and Burgess, 1999). Liposome suspensions were added to Spectra®/Por CE DispoDialyzer 50 kDa MWCO membranes. The dialysis sacs containing the liposome suspensions were placed in glass tubes (Kimax glass culture tubes; 25 mm × 200 mm) containing 50 ml HEPES buffer maintained at 37 °C/25 °C in a shaker water bath (New Brunswick, Edison, NJ) and rotated at 50 rpm. One millilitre aliquots were withdrawn at each time point for release estimation and replaced with fresh buffer. Dexamethasone was analyzed using the HPLC method as described above. In case of incomplete release or if a plateau was reached, SDS was added to a final concentration of 0.5% (w/v) to disrupt the liposomes and confirm complete recovery. Addition of SDS is indicated by an arrow in all figures. The results were reported as mean ± SD ($n = 3$).

2.5. Statistical analysis

Statistical analyses to evaluate significant differences between different groups were carried out using JMPIN® software (SAS Institute, Inc.). Results were analyzed by one-way analysis of variance (ANOVA). Any significant difference was further analyzed by Tukey's HSD (Honestly Significantly Different) post hoc test, a multiple range test to determine significant difference between more than two groups. For comparison between two groups, Student's *t*-test was used. The level of significance was accepted at $p < 0.05$.

3. Results

3.1. Liposome characterization

3.1.1. Liposome size

The mean diameter of non-extruded liposomes of DMPC, DPPC and DSPC was approximately 1 μm and did not differ significantly between the three lipids (*p*-value 0.25; Table 1). The extruded liposomes had significantly smaller mean particle diameter than the non-extruded liposomes (*p*-value 0.0006) for all except the DSPC liposomes. The largest change in diameter after sonication and extrusion was observed for the low transition temperature DMPC liposomes (1015.35 nm before and 43.54 nm after sonication and extrusion). The mean particle diameter of the extruded liposomes of DMPC, DPPC and DSPC was significantly different between the three lipids (*p*-value 0.0001). The particle diameter increased with increase in acyl change length and transition temperature (DSPC > DPPC > DMPC). In fact, the mean diameter of the extruded DMPC liposomes (43.45 nm) was considerably smaller than that of the extruded DPPC (433 nm) and DSPC (728 nm) liposomes.

The incorporation of cholesterol into DMPC liposomes (at 0.1 M or 0.2 M ratio) did not result in any significant change in the mean diameter of non-extruded samples (*p*-value 0.07). However, for extruded liposomes the incorporation of cholesterol at the higher molar ratio (0.2 M) caused a significant change in the mean particle diameter (43.45 nm without cholesterol *vs.* 116.27 nm with cholesterol). Interestingly, this did not occur at the lower molar ratio (0.1 M) of cholesterol (the mean particle diameter was 50.39 nm with cholesterol).

3.1.2. Zeta potential

Saturated phosphatidylcholines are neutral zwitterionic molecules. The zeta potential of all the liposomes was close to neutrality and no significant difference was observed between different liposomes due to lipid type, sonication, extrusion and the incorporation of cholesterol and dexamethasone (Table 1).

3.1.3. Drug encapsulation

Usually, a gel filtration, dialysis or high speed centrifugation method is used for the separation of non-entrapped drug from liposomes (Peschka et al., 1998; Lasch et al., 2003). However, these methods have limitations such as: time consuming (dialysis), dilution of sample (gel filtration) and change in particle size due to pellet formation (centrifugation) (Ozer and Talsma, 1989). An ultrafiltration method was developed which provides an easy and fast

Table 1
Liposomes formulations and their physical properties.

Liposome	Diameter (nm)	Zeta potential (mV)	Percent encapsulation
Extruded			
DMPC:Dex (1:0.2 M)	43.45 ± 1.29	0.55 ± 2.2	12.53 ± 1.66
DMPC:Chol:Dex (1:0.1:0.2 M)	50.39 ± 7.17	−0.77 ± 0.58	7.50 ± 0.86
DMPC:Chol:Dex (1:0.2:0.2 M)	116.27 ± 40.37	0.77 ± 0.26	5.24 ± 0.68
DPPC:Dex (1:0.2 M)	433.0 ± 174.5	1.21 ± 1.23	9.20 ± 0.54
DSPC:Dex (1:0.2 M)	728.30 ± 125.32	1.31 ± 1.13	11.51 ± 1.01
Non-extruded			
DMPC:Dex (1:0.2 M)	1015.35 ± 131.96	−0.24 ± 1.34	26.6 ± 2.9
DMPC:Chol:Dex (1:0.1:0.2 M)	1166.09 ± 172.08	−1.12 ± 1.98	15.24 ± 4.04
DMPC:Chol:Dex (1:0.2:0.2 M)	1403.8 ± 284.4	0.6 ± 2.07	11.56 ± 1.42
DPPC:Dex (1:0.2 M)	1121.95 ± 113.06	−0.64 ± 1.8	19.55 ± 1.33
DSPC:Dex (1:0.2 M)	929.48 ± 200.15	−0.85 ± 1.30	16.01 ± 1.81

Results shown as mean ± SD; Chol: cholestrol; Dex: dexamethasone.

Table 2
Thermodynamic parameters of extruded and non-extruded liposomes of DMPC, DPPC and DSPC.

Liposome	Tm_1	ΔH_1	ΔHv_1	CU_1	Tm_2	ΔH_2	ΔHv_2	CU_2
Extruded								
DMPC	23.7 ± 0.06	3.9 ± 0.3	0.98 ± 0.01	250 ± 20	–	–	–	–
DMPC:Chol:(l:0.1 M)	23.27 ± 0.06	2.6 ± 0.2	0.6 ± 0.02	231 ± 28	24.82 ± 0.11	1.6 ± 0.01	0.11 ± 0.01	63.72 ± 9.1
DMPC:Chol(l:0.2 M)	23.2 ± 0.11	0.98 ± 0.08	0.4 ± 0.006	414 ± 38	25.66 ± 0.52	1.8 ± 0.08	0.07 ± 0.00	37.04 ± 2.4
DMPC:Dex (1:0.2 M)	23.1 ± 0.06	3.46 ± 0.21	0.74 ± 0.009	215 ± 13	23.74 ± 0.83	2.3 ± 0.99	0.12 ± .03	62.64 ± 33
DPPC	41.2 ± 0.03	6.5 ± 0.2	1.08 ± 0.11	167 ± 13	–	–	–	–
DPPC:Dex (1:0.2 M)	40.74 ± 0.14	2.4 ± 0.22	0.56 ± 0.08	237 ± 44	41.02 ± 0.03	4.53 ± 0.26	1.39 ± 0.1	306 ± 16
DSPC	54.4 ± 0.07	9.8 ± 0.34	1.24 ± 0.098	126 ± 14	–	–	–	–
DSPC:Dex(l:0.2 M)	53.89 ± 0.02	4.23 ± 0.6	0.765 ± 0.11	227 ± 102	54.25 ± 0.01	6.85 ± 1.2	1.44 ± 0.17	215 ± 39
Non-extruded								
DMPC	23.56 ± 0.05	5.15 ± 0.2	1.63 ± 0.15	316 ± 2	–	–	–	–
DMPC:Chol:(l:0.1 M)	23.08 ± 0.02	2.15 ± 0.2	1.42 ± 0.04	669 ± 78	24.1 ± 0.25	1.9 ± 0.03	0.12 ± 0.03	62.18 ± 9.7
DMPC:Chol(l:0.2 M)	21.6 ± 0.06	0.3 ± 0.05	0.98 ± 0.13	3343 ± 0.9	23.86 ± 0.09	2.8 ± 0.38	0.98 ± 0.11	35.26 ± 8.7
DMPC:Dex (1:0.2 M)	22.89 ± 0.07	3.68 ± 0.5	1.13 ± 0.06	310 ± 49	23.15 ± 0.05	2.0 ± 0.24	0.17 ± 0.03	85.08 ± 10
DPPC	41.32 ± 0.01	8.25 ± 0.5	2.8 ± 0.25	338 ± 10	–	–	–	–
DPPC:Dex (l:0.2 M)	40.6 ± 0.2	5.2 ± 0.1	0.57 ± 0.06	114 ± 35	41.02 ± 0.02	3.2 ± 0.7	2.5 ± 0.9	765 ± 164
DSPC	54.46 ± 0.01	15.8 ± 5.7	4.37 ± 1.15	282 ± 32	–	–	–	–
DSPC:Dex(l:0.2 M)	53.72 ± 0.06	9.56 ± 1.45	0.63 ± 0.04	67 ± 15	53.92 ± 0.78	1.08 ± 1.23	5.95 ± 4.65	27,677 ± 41,237

1 refers to first peak fit parameters and 2 refer to second peak fit parameters. Tm: phase transition temperature (°C); ΔH: experimental enthalpy change (kcal/mole/°C); ΔHv: Van't Hoff enthalpy change (Mcal/unit); CU: co-operative unit ($n = 3$; mean ± SD).

method (approximately 1 h) of separation of non-entrapped drug. Moreover, this method allows the preparation of concentrated liposome suspensions. Since only low speed centrifugation is used, liposome particle size is not affected by this process.

For non-extruded liposomes, drug encapsulation decreased with increase in phospholipid acyl chain length (Table 1) and was significantly different between the three lipids (DMPC > DPPC > DSPC; p-value 0.0001). Sonication and extrusion of the liposomes reduced drug encapsulation. Except for DSPC liposomes, the amount of drug encapsulated in the extruded liposomes was almost half of that in non-extruded liposomes. In the case of DSPC liposomes, the percent drug entrapment decreased from 16.01% to 11.51% on sonication and extrusion.

The incorporation of cholesterol significantly decreased dexamethasone encapsulation in DMPC liposomes for both extruded (p-value 0.0003) and non-extruded samples (p-value 0.0005; Table 1). This effect was more pronounced at the higher cholesterol ratio (0.2 M). Similar to other liposomes, sonication and extrusion caused a reduction in drug encapsulation of cholesterol containing liposomes by a factor of two.

3.2. Phase transition behavior

The phase transition of blank liposomes (no drug or cholesterol) was best modeled using a 'one-peak' fit, whereas for dexamethasone and cholesterol containing liposomes, the main transition peak was resolved into two peaks. The thermodynamic parameters are marked 1 and 2 for the two peaks in Table 2. The non-extruded blank liposomes showed a characteristic endotherm with a pre-transition peak followed by a sharp main transition peak. The main transition temperatures of DMPC, DPPC and DSPC were 23.56, 41.32 and 54.46 °C, respectively (Fig. 1 and Table 2). Both the main transition temperature and the enthalpy (ΔH) increased with increase in acyl chain length (Table 2). These results are in agreement with that reported earlier by others for MLVs (Kruijff et al., 1975; van Dijck et al., 1978b; Parente and Lentz, 1984; Biltonen and Lichtenberg, 1993). Sonication and extrusion of blank liposomes led to abolition or reduction of the pre-transition peak, broadening of the main transition, as well as a marked reduction in the peak height and ΔH values (Fig. 1 and Table 2). The size of the co-operative unit (CU) was also smaller for the extruded liposomes.

Incorporation of dexamethasone (0.2 M) led to abolition of the pre-transition peak, reduction in the main peak height and the ΔH values as well as main peak broadening (Fig. 1 and Table 2). These effects were more pronounced for non-extruded compared to extruded liposomes. The main transition peak of drug-loaded non-extruded liposomes had a shoulder (DMPC and DPPC; Fig. 1A and B) or split peak (DSPC Fig. 1C). The transition temperature of one of the fitted peaks was close to that of blank liposomes, while the second peak temperature was shifted downwards (Table 2).

Sonication and extrusion of liposomes resulted in a reduction in the main peak broadening or splitting. The main peak temperature shifted towards higher temperatures for extruded liposomes compared to non-extruded liposomes (Fig. 1). This was more evident in the case of DPPC and DSPC where the area of the second peak increased at the expense of the first (3.2 kcal/mole/°C *vs.* 4.53 kcal/mole/°C for DPPC and 1.08 kcal/mole/°C *vs.* 6.85 kcal/mole/°C for DSPC; Table 2).

Incorporation of cholesterol in blank DMPC liposomes caused a decrease in peak height and enthalpy as well as abolition of the pre-transition peak (Fig. 2). Increase in the cholesterol content (from 0.1 to 0.2 M) intensified these effects. Non-extruded liposomes containing the higher mole ratio (0.2 M) of cholesterol showed two distinct but smaller peaks whereas extruded liposomes showed only one broad peak. The transition temperature of the second fitted peak (Tm_2) shifted towards higher temperatures. The incorporation of both dexamethasone (0.2 M) and cholesterol (0.1 M) together in DMPC liposomes reduced the phase transition temperature and the peak height, while increasing the peak width. The non-two state model used could not fit the peak satisfactorily due to its broad shape.

3.3. In vitro release

3.3.1. Release from extruded liposomes

At 37 °C, release from the extruded DMPC and DPPC liposomes was similar with most of the drug releasing in the first 12 h, *ca.* 92% and *ca.* 96%, respectively (Fig. 3A). Drug release was slower in the case of extruded DSPC liposomes (*ca.* 78% at 12 h and *ca.* 90% at 48 h), which has the highest phase transition temperature. Drug release was also tested at 25 °C which is below the transition temperature of DPPC and DSPC but not that of DMPC (Fig. 3B). Three distinct release profiles were obtained at 25 °C. At 12 h, dexamethasone release from DMPC, DPPC and DSPC liposomes was 86.74%, 70.23% and 49.14%, respectively. This trend is in agreement with the transition temperatures of the lipids. The addition of SDS resulted in complete release of dexamethasone from liposomes.

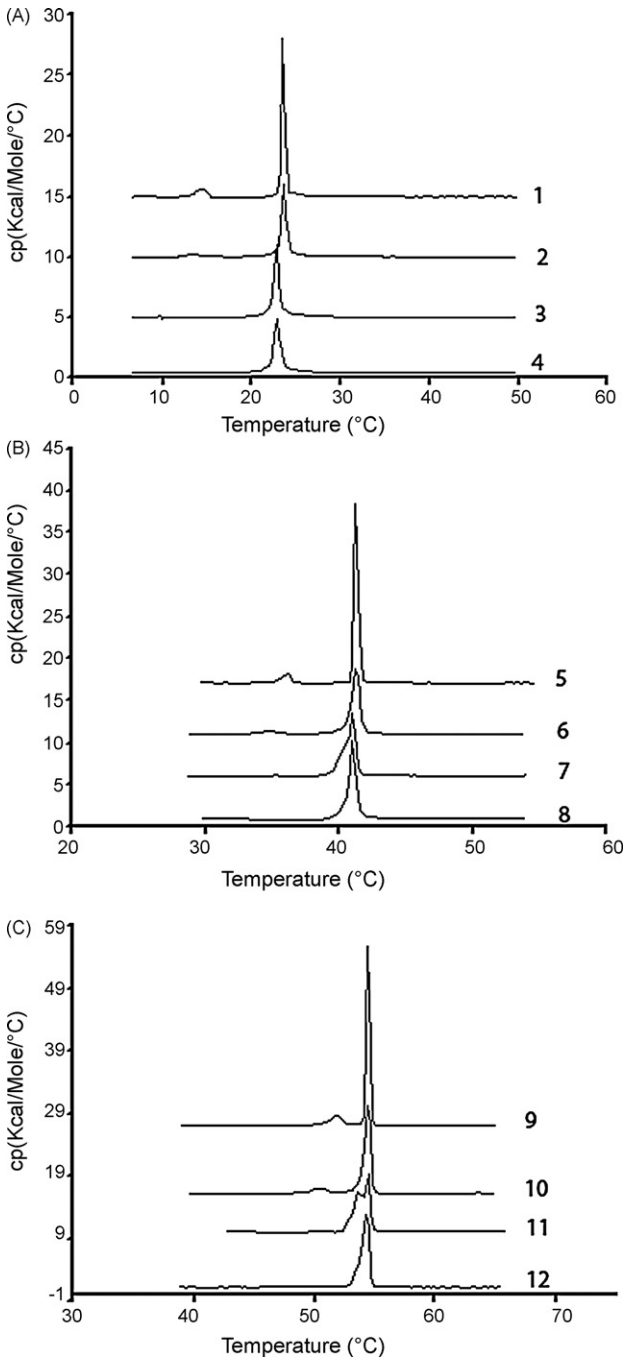

Fig. 1. Effects of extrusion and dexamethasone incorporation on phase transition behavior of (A) DMPC, (B) DPPC and (C) DSPC liposomes ((1) DMPC non-extruded liposomes; (2) DMPC extruded liposomes; (3) DMPC:dexamethasone (1:0.2 M) non-extruded liposomes; (4) DMPC:dexamethasone (1:0.2 M) extruded liposomes; (5) DPPC non-extruded liposomes; (6) DPPC extruded liposomes; (7) DPPC:dexamethasone (1:0.2 M) non-extruded liposomes; (8) DPPC:dexamethasone (1:0.2 M) extruded liposomes; (9) DSPC non-extruded liposomes; (10) DSPC extruded liposomes; (11) DSPC:dexamethasone (1:0.2 M) non-extruded liposomes; (12) DSPC:dexamethasone (1:0.2 M) extruded liposomes.).

3.3.2. *Release from non-extruded liposomes*

At 37 °C, dexamethasone release profiles for non-extruded DMPC and DPPC liposomes were similar while release from DSPC liposomes was slower (Fig. 4A). During the initial 12 h, 68.8%, 64.25% and 30.5% of dexamethasone were released from non-extruded DMPC, DPPC and DSPC liposomes, respectively. At 25 °C, a better discrimination was observed between non-extruded DMPC, DPPC and DSPC liposomes for dexamethasone release compared to

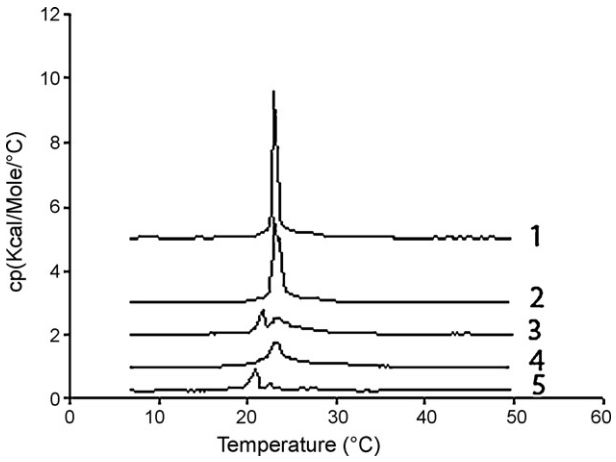

Fig. 2. Effects of cholesterol incorporation on phase transition behavior of DMPC liposomes ((1) DMPC:cholesterol (1:0.1 M) non-extruded liposomes; (2) DMPC:cholesterol (1:0.1 M) extruded liposomes; (3) DMPC:cholesterol (1:0.2 M) non-extruded liposomes; (4) DMPC:cholesterol (1:0.2 M) extruded liposomes; (5) DPPC:cholesterol:dexamethasone (1:0.1:0.2 M) non-extruded liposomes.).

that at 37 °C. At 12 h, 64.88%, 40.14% and 24.72% of dexamethasone were released from DMPC, DPPC and DSPC liposomes, respectively. The order of release was similar to that for the extruded liposomes at 25 °C *i.e.* DMPC > DPPC > DSPC. At both 25 °C and 37 °C, release from the non-extruded DPPC and DSPC liposomes showed a fast initial phase followed by a slow release phase till a plateau was reached. The non-extruded DPPC liposomes released over a period

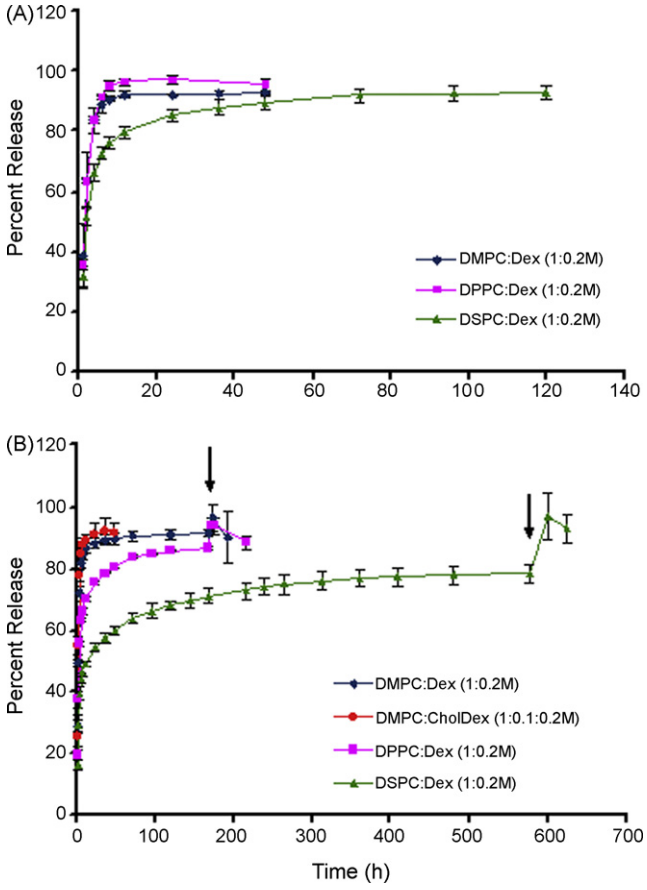

Fig. 3. *In vitro* release of dexamethasone from sonicated and extruded DMPC, DPPC and DSPC liposomes at (A) 37 °C and (B) 25 °C in 10 mM HEPES buffer, pH 7.4. The addition of SDS is indicated by an arrow. Each value represents mean ± SD ($n = 3$).

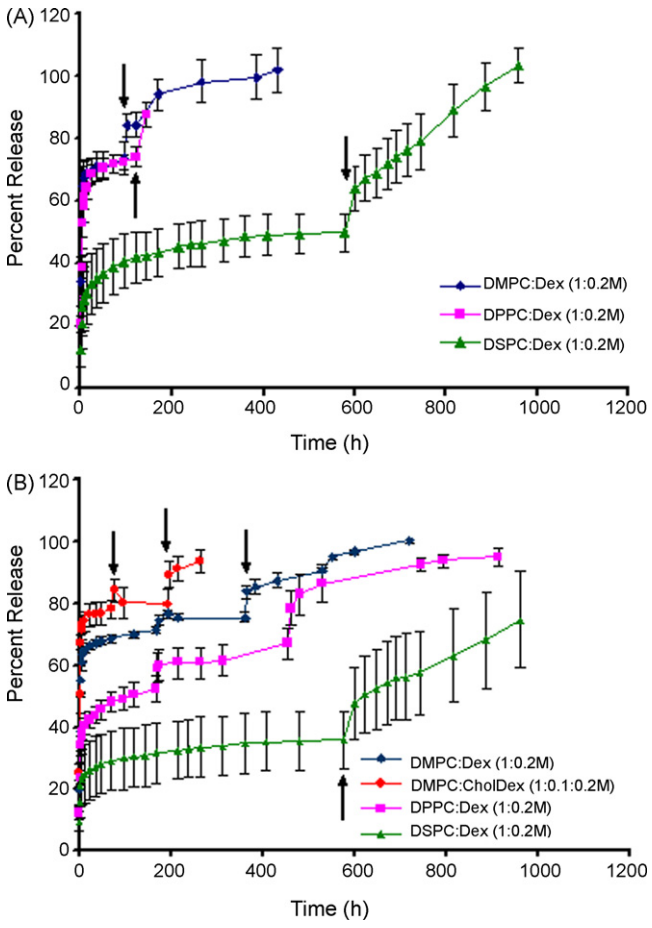

Fig. 4. *In vitro* release of dexamethasone from non-extruded DMPC, DPPC and DSPC liposomes at (A) 37 °C and (B) 25 °C in 10 mM HEPES buffer, pH 7.4. The addition of SDS is indicated by an arrow. Each value represents mean ± SD ($n = 3$).

of 4–5 days, whereas for DSPC, a plateau was reached around 20 days. The addition of SDS increased the rate of dexamethasone release at both 25 and 37 °C.

At 25 °C, the release profile from extruded DMPC:cholesterol liposomes was similar to extruded DMPC liposomes without cholesterol (Fig. 3B). Interestingly, cholesterol incorporation in non-extruded DMPC liposomes resulted in faster release of dexamethasone (Fig. 4B).

3.3.3. *Effect of temperature on release*

Drug release from all liposomes investigated was faster at 37 °C compared to that at 25 °C (Fig. 5). The effect of release medium temperature was more pronounced for non-extruded liposomes compared to extruded liposomes. The reduction in temperature from 37 to 25 °C had a minimal effect on drug release from DMPC liposomes. Release from both non-extruded and extruded DPPC and DSPC liposomes was slower at 25 °C.

3.3.4. *Effect of sonication and extrusion on release*

At a given temperature (25 °C or 37 °C), release from non-extruded liposomes was slower than release from extruded liposomes for all lipids studied (Fig. 5). The difference between the release profiles of non-extruded and extruded liposomes increased with the transition temperature of the phospholipid. Addition of SDS was required to achieve release of dexamethasone from non-extruded liposomes during the study time period.

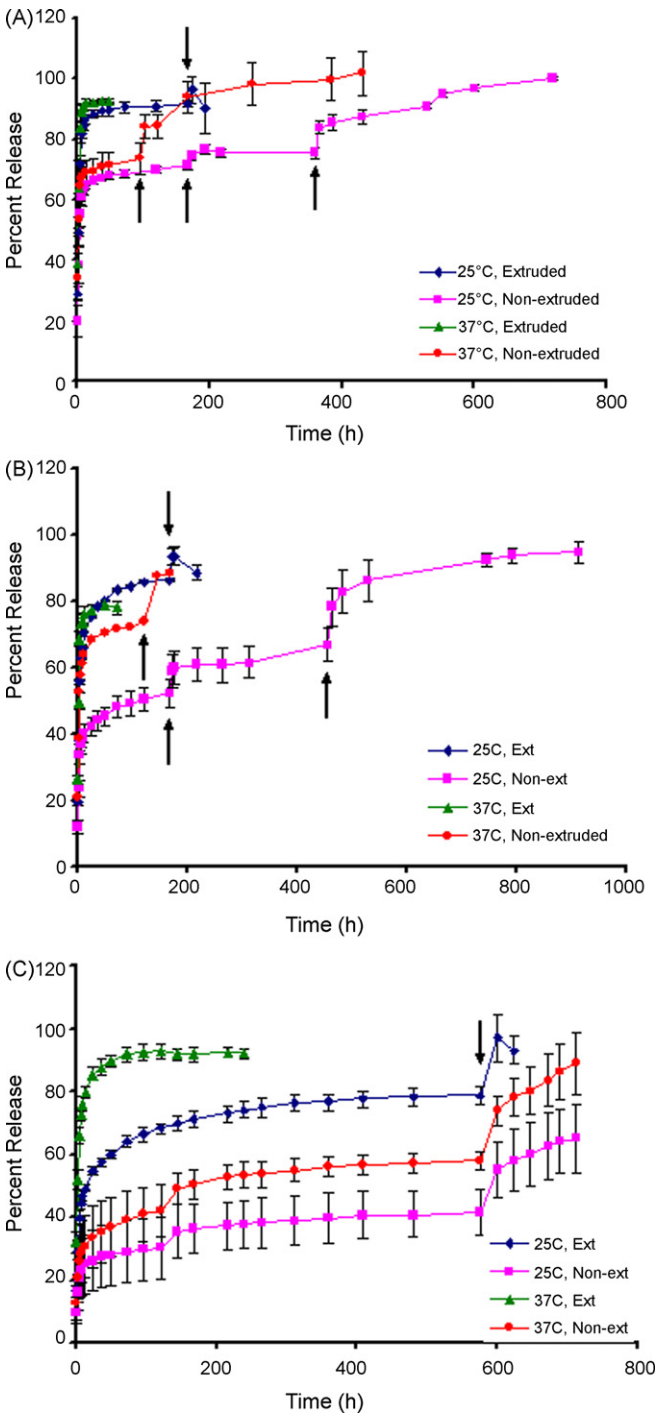

Fig. 5. Comparison of the effects of extrusion and release medium temperature on *in vitro* release of dexamethasone from (A) DMPC, (B) DPPC and (C) DSPC liposomes in 10 mM HEPES buffer, pH 7.4. The addition of SDS is indicated by an arrow. Each value represents mean ± SD ($n = 3$).

4. Discussion

The similar mean diameter of all the non-extruded liposomes indicated that particle size appeared to be dependent on the initial film thickness, since the concentration and volume of the lipid solutions was kept constant for all liposomes preparations. Sonication and extrusion led to a reduction in the mean particle diameter of the liposome formulations, due to structural rearrangement of their membranes as has been previously reported for the sonication processing (Kodama et al., 1993). The particle diameter of the

extruded liposomes was dependent on the lipid acyl chain length. The small acyl chain length of DMPC liposomes can acquire a higher curvature on sonication and this may be responsible for the small particle size of these liposomes (Kruijff et al., 1975; van Dijck et al., 1978a). The large size of the DMPC liposomes containing 0.2 M of cholesterol might be due to localization of cholesterol between the acyl chains, which would restrict the mobility of the lipids and inhibit high membrane curvature.

Dexamethasone encapsulation in the non-extruded liposomes was inversely related to the acyl chain length (Table 1). As the chain length increases, the van der Waals forces between the acyl chains increases and accordingly the space between the acyl chains decreases (Cevc, 1993). Therefore, less space would be available for dexamethasone incorporation in the DSPC liposome membranes compared to the DMPC liposome membranes, which may be responsible for the lower drug encapsulation. A similar phenomena has been observed with other hydrophobic drugs (Saarinen-Savolainen et al., 1997). Approximately half of the drug that was encapsulated in non-extruded liposomes was lost during the sonication and extrusion process. This may be due to breaking and structural rearrangement of the lipid bilayer during the sonication and extrusion process. Therefore, during this process dexamethasone embedded in the inner layers of non-extruded liposomes is lost to the aqueous media. In addition, the lipid molecules in the extruded liposomes are in a higher energy and permeability state due to their higher curvature. Interestingly, drug loading reduced the most for DPPC extruded liposomes (>50% of drug was lost from the non-extruded liposomes). DPPC has a higher tendency to form an interdigitated state, as compared to DMPC and DSPC (Demetzos, 2008). Sonication and extrusion induced interdigitation leads to a decrease in space between the acyl chains and the higher loss of dexamethasone from the extruded DPPC liposomes.

Cholesterol incorporation has been reported to increase membrane stability, decrease permeability and increase encapsulation of hydrophilic drugs (Papahadjopoulos et al., 1972; Gregoriadis and Davis, 1979). However, both cholesterol and dexamethasone have a similar hydrophobic steroidal molecular structure and compete for the same sites in the liposome membrane. Cholesterol is more lipophilic ($\log p \sim 7.17$) (Stanculescu et al., 2006) than dexamethasone ($\log p \sim 1.74$; Scifinder® Scholar) and therefore should be preferentially incorporated/partitioned in the liposome membranes, leading to a decrease in dexamethasone encapsulation.

A decrease in the peak height and enthalpy suggested that the lipid molecules in sonicated and extruded liposomes were in a higher energy state (Fig. 1 and Table 2). Sonication and extrusion are expected to induce high curvature in the extruded liposomes (van Dijck et al., 1978b; Kodama et al., 1993), which in turn would distort the packing of the acyl chains and increase the energy state of the lipid molecules. This is supported by the smaller CU for the extruded liposomes compared to the non-extruded liposomes. A shoulder (DMPC and DPPC; Fig. 1A and B) or split peak (DSPC Fig. 1C) in the main transition peak of the drug-loaded, non-extruded liposomes indicated a heterogeneous distribution of dexamethasone between the different liposomes or in-between different layers in the same liposome. However, sonicated and extruded liposomes had a more homogenous distribution of drug as suggested by the disappearance of the split peak and a sharper peak shape. The bilayer structure was destabilized after incorporation of dexamethasone, as indicated by a decrease in the peak transition temperatures (Tm_1 and Tm_2) compared to those of the blank liposomes (Table 2). This suggested that dexamethasone has a greater affinity for the liquid crystalline state of the lipids, reducing the van der Waals interaction between the acyl chains. Destabilization was more prominent for the non-extruded liposomes since heterogeneous distribution resulted in slightly lower Tm_1 values (Table 2). Interpretation of the effects of sonication and extrusion on the CU for DSPC liposomes was complicated by the heterogeneous distribution of dexamethasone in non-extruded liposomes (CU_2 value ~ 27,677).

At higher molar ratio (0.2 M), the cholesterol distribution in the non-extruded DMPC liposomes was heterogeneous (Fig. 2). Two distinct peaks indicated the presence of cholesterol-poor and cholesterol-rich domains/liposomes. It is speculated that in cholesterol-poor domains, lipid molecules are constrained in a smaller space which increases their energy ($\Delta H \sim 0.3$ kcal/mole/°C). The higher CU_1 value (~3343) supports this argument. Heterogeneous distribution of cholesterol in liposomes has been observed previously (McElhaney, 1982; Demetzos, 2008). Sonication partially negated this heterogeneous distribution as evident by the higher ΔH_1 and lower CU_1 values. A higher Tm_2 (compared to the Tm of the blank liposomes) suggested that cholesterol has more affinity for the gel state (Demetzos, 2008). Therefore, cholesterol incorporation would stabilize the DMPC liposome membrane as reported in the literature (McElhaney, 1982; Demetzos, 2008). The different stabilizing effect of cholesterol and dexamethasone on the liposomes might be due to differences in their interaction with phospholipid molecules. Highly lipophilic cholesterol gets incorporated between the acyl chains and reduces chain movement (increasing rigidity) above the phase transition temperature (Liu et al., 2000). Therefore, cholesterol shows membrane stabilization above the phase transition temperature. Dexamethasone, being more hydrophilic, may interact differently with phospholipid acyl chains and headgroups and destabilizes the membrane.

Dexamethasone release from all extruded liposomes was fast and most of the contents were released within 48 h at 37 °C (Fig. 3A). While the slower release from DSPC could be explained by the high transition temperature, the similar release profiles from the DMPC and DPPC liposomes were unexpected. At 37 °C, DPPC should be in a less permeable gel state while DMPC is in a more permeable liquid crystalline state. However, changes in the transition behavior of DPPC due to the incorporation of dexamethasone and the sonication and extrusion processing may explain the release profile. Dexamethasone incorporation and sonication and extrusion processing decreased the main transition onset temperature, increased lipid molecular energy and destabilized the DPPC membranes (Fig. 1B and Table 2). Therefore, extruded DPPC liposomes are in a more permeable state at 37 °C than expected and this may be responsible for the similar release profile to that of the DMPC liposomes. This was further corroborated by dexamethasone release studies at 25 °C from the extruded liposomes (Fig. 3B). At 25 °C, only DMPC is in the liquid crystalline state while DPPC and DSPC are in the gel state. Accordingly, dexamethasone release at 25 °C from extruded liposomes decreased with increase in the Tm of the lipids in the order DMPC > DPPC > DSPC (Fig. 3B).

At both 37 °C and 25 °C, the rank order of dexamethasone release from the non-extruded liposomes was similar to that from the extruded liposomes. Release profiles from DMPC and DPPC were similar at 37 °C. However, for non-extruded DPPC liposomes, heterogeneous distribution of dexamethasone reduced the main transition onset temperature even lower than that for the extruded liposomes. Nevertheless, dexamethasone release from non-extruded liposomes was slower than that from extruded liposomes at both 37 and 25 °C (Fig. 5). Therefore, it would appear that the rate controlling step for release of dexamethasone (to bulk) is diffusion across the multilamellar structure of the non-extruded liposomes. This is supported by the fact that the curvature in larger non-extruded liposomes is smaller (van Dijck et al., 1978b; Kodama et al., 1993) which would result in a close packing arrangement, a lower lipid molecular energy state and hence reduced drug permeability. Drug permeability is further decreased at lower temperature explaining the slower release at 25 °C (Fig. 5).

Moreover, larger non-extruded liposomes have a smaller surface area exposed to the aqueous media compared to the extruded liposomes. This will decrease the rate of diffusion across the membrane for non-extruded liposomes. On the other hand, the permeability of dexamethasone for extruded liposomes will be higher due to: (1) high curvature and energy state; (2) distorted acyl chain packing; (3) fewer lamellar barriers for dexamethasone diffusion; and (4) a marked increase in surface area. Hence, it appears that the ability of the lipid to affect release is masked by changes in the physical properties of the extruded liposomes.

There appears to be no advantage in adding cholesterol to obtain sustained release of dexamethasone. While the phase transition behavior showed that cholesterol stabilizes the DMPC membrane, cholesterol incorporation resulted in faster release from non-extruded liposomes. This appears to be mainly due to the structural similarity between dexamethasone and cholesterol, and consequent competition for the same sites in the lipid membrane. Similar effects have been observed in MLVs containing cholesterol and triamcinolone (Vargha-Butler and Hurst, 1995).

The results from this study suggest that sustained release of the hydrophobic drug dexamethasone can be achieved using MLVs composed of high phase transition temperature lipids such as DSPC. Non-extruded liposomes have higher encapsulation efficiencies and therefore, a smaller amount of liposomes may be required for therapeutic purposes. Extruded liposomes have a limited ability to control the release of dexamethasone. Therefore, an immediate release of dexamethasone can be achieved using extruded DMPC or DPPC liposomes. Whereas, intermediate and slow releases of dexamethasone can be obtained using non-extruded DPPC and DSPC liposomes. In addition, changes in the physicochemical and phase transition properties of liposomes due to the incorporation of hydrophobic drugs can impact their release behavior.

5. Conclusions

Sonication and extrusion process caused a marked change in liposomes size, dexamethasone encapsulation and phase transition behavior of DMPC, DPPC and DSPC liposomes. While dexamethasone and cholesterol were distributed heterogeneously in non-extruded liposomes, the extruded liposomes had a more homogeneous distribution. However, changes in the structure and energy state of the extruded liposomes due to processing and incorporation of dexamethasone increased the permeability. This undermined the ability of lipids to control release of dexamethasone from the extruded liposomes. Although, the non-extruded DPPC and DSPC liposome membranes were destabilized by dexamethasone incorporation, dexamethasone release from these liposomes was slow and dependent on the type of lipid used. This was due to the lower permeability of MLVs as a consequence of multilamellar structure and lower surface area. Moreover, the structural similarity between cholesterol and dexamethasone obviated the advantage of cholesterol in stabilizing the liposome membrane. Hence, sustained release of dexamethasone can be achieved using MLVs of high phase transition phospholipids.

Acknowledgements

The authors would like to thank Prof. Fotios Papadimitrakopoulos, Institute of Materials Science, University of Connecticut for valuable discussions regarding this work. Financial support for this work from the United States Pharmacopeia is greatly appreciated.

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