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Physico-chemical characteristics of methotrexate-entrapped oleic acid-containing deformable liposomes for *in vitro* transepidermal delivery targeting psoriasis treatment

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ABSTRACT

This study aimed to investigate the physico-chemical characteristics and *in vitro* permeability of methotrexate (MTX)-entrapped deformable liposomes prepared from phosphatidylcholine (PC) and oleic acid (OA), comparing with those of MTX-entrapped conventional liposomes prepared from PC and cholesterol (CH). Two formulations of MTX-entrapped PC2:CH1 and PC9:CH1 liposomes and one formulation of MTX-entrapped PC2.5:OA1 liposomes were prepared. The size, size distribution, zeta potential, thermal properties, entrapment efficiency, stability, and *in vitro* permeability across a porcine skin of the MTX-entrapped liposomes were evaluated. All liposome formulations showed a narrow size distribution with the size range of 80–140 nm which is appropriate for the skin permeability. The percentage of MTX loading, entrapment efficiency and the stability of MTX-entrapped PC2:CH1 and PC9:CH1 liposomes were slightly higher than those of MTX-entrapped PC2.5:OA1 liposomes. However, the MTX-entrapped PC2.5:OA1 liposomes enhanced the skin permeability characterized by the higher concentration and flux of MTX diffused across or accumulated in the epidermis and dermis layers of porcine skin. The enhanced permeability of MTX-entrapped PC2.5:OA1 liposomes was explained by 2 mechanisms: (1) the deformable and elasticity characteristics of OA-containing liposomes and (2) a property as a skin penetration enhancer of OA. This suggested that the PC2.5:OA1 deformable liposome was one of promising candidates to enhance the permeability of MTX for the treatment of psoriasis.

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1. Introduction

Psoriasis is a chronic autoimmune disease predominantly appeared on the skin and joint manifestations. It occurs when immune system mistakes the skin cells such as a pathogen, and transmits faulty signals that speed up the growth and division of skin cells, resulting in a well-defined erythematous together with red and white hues of scaly patches appearing on the top layer of the epidermis (Andrew et al., 2004; Linden and Gerald, 1999). Patients with psoriasis experience the increased levels of stress, anxiety, depression and anger. First-line treatments have been developed for the treatment of psoriasis, but they are often less effective due to the poor penetration, cosmetically unacceptable, inconvenient for long term use or associated with a significant toxicity (Mendonca and Burden, 2003; David et al., 1999).

Methotrexate (MTX) is one of the drug choices for the treatment of psoriasis. It can be administered as oral and parenteral routes. However, various side effects such as mucosal ulceration, stomatitis, bone marrow suppression, loss of appetite with drug induced hepatic fibrosis and cirrhosis can occur when applied over a long period (Frank and Alan, 2004). To reduce the adverse side effects, the topical administration of MTX is preferred (Trotta et al., 2004). Different methods have been introduced to deliver MTX topically to the psoriasis lesions, however MTX does not easily cross the stratum corneum which is recognized as the primary barrier to transepidermal drug delivery. The human stratum corneum intercellular lipids mainly consist of cholesterol (CH), ceramides and free fatty acids, which are structurally organized into multilamellar bilayers (Rougier et al., 1988; Lamp et al., 1983; Forster, 2002). These lipids strongly dictate the overall skin permeability properties. A promising approach to overcome the limited permeability of drug across the stratum corneum of skin is a liposomal delivery system. One conventional formulation of liposomes was produced from phosphatidylcholine (PC) phospholipid and CH (Constanzo

et al., 1994; Nagumo et al., 1991; Sentjurc et al., 1996; Vrhovnik et al., 1998; Coderch et al., 2000). It is reported that the addition of CH could modify the order and mobility of the phospholipids in the bilayer liposomes (Constanzo et al., 1994; Nagumo et al., 1991). The CH contents might be one of important parameters to obtain the effective delivery of substance-entrapped liposomes into the skin (Sentjurc et al., 1996; Vrhovnik et al., 1998; Coderch et al., 2000). The mechanism was explained in terms of the enhanced fluidity and stability of the CH-containing liposomes. However, as the target of MTX on the psoriasis treatment, a significant enhanced penetration of MTX across the remarkable thick epidermis is required. It is then necessary to develop MTX-entrapped liposome formulation with the better permeability to achieve the goal of treatment. Recently, the elastic vesicles composed of lipid materials and a surfactant that showed at least one inner aqueous compartment surrounded by a lipid bilayer, called deformable liposomes, were introduced (Trotta et al., 2004; Cevc et al., 1996; El Maghraby et al., 2000; Vanaja et al., 2008; Cevc and Gabriele, 2001). The unique characteristic of the deformable liposomes, compared with the conventional liposomes and other types of drug-laden lipid suspensions, is its flexibility. The flexibility of deformable liposomes has an advantage on the penetration capability even though the skin pores are much smaller than the diameter of liposomes (Cevc et al., 1995). Trotta et al. (2004) have formulated the deformable liposomes from the PC and a surfactant dipotassium glycyrrhizinate (KG) for dermal administration of MTX. The MTX amount permeated across porcine skin by using KG-containing liposomes were approximately 3–4-fold higher than that of water solution or conventional liposomes. This capability was explained by the self-regulating carrier deformability of KG. However, the deformable liposomes which are formulated from natural ingredients would be preferred and widely accepted by patients (Cevc and Gabriele, 2001). Oleic acid (OA) is a monounsaturated omega-9 fatty acid that is found in various animal and vegetable fats such as lipid bilayers of skin. It has been used as an emulsifying or solubilizing agent and a skin penetration enhancer in pharmaceutical products (Francoeur et al., 1990; Touitou et al., 2002). The increased skin permeability by OA is related to a selective perturbation of the intracellular lipid bilayers present in the stratum corneum (Francoeur et al., 1990). OA can dramatically change the morphology and the density of epidermal Langerhans cells and resulted in the generation of pores on the surface of epidermal corneocytes (Touitou et al., 2002). The deformable OA-containing liposomes have been investigated for the transepidermal drug delivery by some research groups (El Maghraby et al., 2000a,b; Choi and Maibach, 2005). Torchilin et al. (1992) reported that pH-sensitive OA-containing liposomes increased permeability and the release of fluorescent dye entrapped. The phenomena were explained by the destabilization of endosome membrane and intracytoplasmic mechanisms. However, based on our best knowledge, the effects of OA on the permeability of MTX entrapped in the OA-containing liposomes targeting the psoriasis treatment have not been elucidated.

In this study, the physico-chemical and skin permeation characteristics of MTX-entrapped deformable liposomes formulated from PC and OA were of our interest. The characteristics of the MTX-entrapped PC:OA deformable liposomes including size, size distribution, zeta potential, thermal properties, entrapment efficiency, stability, and in vitro permeability across a porcine skin using a Franz diffusion model were evaluated, compared with those of the MTX-entrapped PC:CH conventional liposomes.

2. Materials and methods

2.1. Materials

Soybean phospholipid Epikuron® 200 (PC, 95%, w/w phosphatidylcholine) was purchased from Degussa BioActives, Hamburg, Germany. Hydrogenated lecithin Phospholipon® 90H (HPC) was purchased from Nattermann phospholipid GmbH, Cologne, Germany. Methotrexate (MTX) and cholesterol (CH) were obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Oleic acid (OA) was purchased from Sigma–Aldrich Co., Ltd., Singapore. Other chemicals were obtained from Carlo Erba reagent, Italy and used without further purification.

2.2. Preparation of liposomes

Lipid vesicles containing PC or HPC (for thermal analysis) in the presence of CH or OA at different molar ratios were prepared by a thin film hydration method (Qina et al., 2011). Various molar ratios of lipid materials (PC:CH and PC:OA) were dissolved in diethyl ether and chloroform (1:1) at a final concentration of $10 \mu M$ (Table 1). The solvent was removed by a rotary evaporation at 30 rpm. The obtained lipidic thin film was then freeze-dried overnight to completely remove the residual organic solvent. The thin film was hydrated in acetate buffer solution (pH 5.5) containing various amounts of MTX solution (0.25, 0.5, or 1.0%, w/w) and shaken at 250 rpm, 25 °C for 1 h to obtain a stable and homogeneous suspension. Then, the MTX-entrapped liposomes were homogenized with a high pressure homogenizer at 1500 bar for 5 cycles to reduce particle size (approximately 120 nm) which is appropriate for transepidermal delivery (Verma et al., 2001). Free MTX was removed by ultracentrifugation at 7×10^4 rpm, 4 °C for 30 min.

Table 1
Different formulations of liposome entrapping various doses of MTX.

Formulation	PC (molar ratio)	CH (molar ratio)	OA (molar ratio)	MTX (%, w/w)
Formulation 1				
PC2:CH1	2	1	–	–
PC2:CH1 + 0.25% MTX	2	1	–	0.25
PC2:CH1 + 0.5% MTX	2	1	–	0.5
PC2:CH1 + 1.0% MTX	2	1	–	1
Formulation 2				
PC9:CH1	9	1	–	–
PC9:CH1 + 0.25% MTX	9	1	–	0.25
PC9:CH1 + 0.5% MTX	9	1	–	0.5
PC9:CH1 + 1.0% MTX	9	1	–	1
Formulation 3				
PC2.5:OA1	2.5	–	1	–
PC2.5:OA1 + 0.25% MTX	2.5	–	1	0.25
PC2.5:OA1 + 0.5% MTX	2.5	–	1	0.5
PC2.5:OA1 + 1.0% MTX	2.5	–	1	1

PC: phosphatidylcholine (Epikuron® 200); CH: cholesterol; OA: oleic acid; MTX: methotrexate.

2.3. Determination of size, size distribution, and zeta-potential of MTX-entrapped liposomes

The average size, polydispersity index (PDI), and zeta potential of different formulations of MTX-entrapped liposomes were determined by a particle size analyzer (Zetasizer ZS, Malvern, UK) at $25 \pm 0.5\,^{\circ}$C. The experiment was independently performed for 3 repeating samples per experimental group ($n = 3$).

2.4. Differential scanning calorimetry

Thermal properties of liposomes including melting temperature (T_m; the temperature at the peak) and the phase transition temperature range ($\Delta T_{m1/2}$; the width of the transition at half peak height) were determined by a differential scanning calorimetry (DSC, Mettler Toledo DSC 823^e, Switzerland). Liposomes (15.0 ± 1.0 mg) were loaded into the sample pan and an equal volume of acetate buffer was loaded into the reference pan. The samples were scanned from 40 to 80 $^{\circ}$C with a scanning rate of 5 $^{\circ}$C/min under nitrogen gas flow at 30 ml/min.

2.5. Phospholipid determination by phosphorus assay

The amount of phospholipid in liposomes was evaluated by a phosphorus determination through an acidic digestion (Rouser et al., 1970). Briefly, the phospholipid of liposomes was digested with 20% (v/v) perchloric acid at 180 $^{\circ}$C for 30 min to transform the phosphorous conformation to ortho-phosphate. The ortho-phosphate solution was reacted with 1% (w/v) ammonium molybdate under a phosphomolybdic acid condition of 10% (w/v) ascorbic acid at 100 $^{\circ}$C for 15 min. After cooling down, a strong blue color of molybdenum blue solution was vortexed and the absorbance of the solution was measured by an UV spectrophotometer at a wavelength of 830 nm. The phospholipid concentration was determined from the calibration curve of phosphorous at various concentrations. The experiment was independently performed for 3 repeating samples per experimental group ($n = 3$).

2.6. Loading percentage and entrapment efficiency of MTX in liposomes

MTX-entrapped liposomes were lyzed with 0.2% (v/v) Triton-X100 solution and centrifuged at 7×10^3 rpm, 25 $^{\circ}$C for 20 min. The supernatant was collected to analyze for MTX content by a high performance liquid chromatography (HPLC, Waters 2695, USA) using Symmetry© C_{18} column (column size 4.6 mm × 150 mm). Acetate buffer:acetronitrile solution (90:10) was used as a mobile phase at flow rate of 1 ml/min, an injection volume of 20 μl and at a wavelength of 302 nm. The MTX concentration was determined from the calibration curve of MTX at various concentrations. The experiment was independently performed for 3 repeating samples per experimental group ($n = 3$). Experimental and theoretical percentages of MTX loading were calculated from Eqs. (1) and (2), respectively:

$$\text{Experimental percentage of MTX loading} = \frac{\text{Mol}_{MTX}}{\text{Mol}_{PC}} \times 100 \quad (1)$$

$$\text{Theoretical percentage of MTX loading} = \frac{\text{TMol}_{MTX}}{\text{TMol}_{PC}} \times 100 \quad (2)$$

where Mol$_{MTX}$ and Mol$_{PC}$ represent mol of MTX entrapped and phospholipid used experimentally, respectively. TMol$_{MTX}$ and TMol$_{PC}$ represent mol of MTX entrapped and phospholipid used theoretically, respectively.

Entrapment efficiency of MTX in liposomes was calculated from Eq. (3):

Entrapment efficiency of MTX (%)

$$= \frac{\text{Experimental percentage of MTX loading from Eq. (1)}}{\text{Theoretical percentage of MTX loading from Eq. (2)}} \times 100 \quad (3)$$

2.7. Stability of MTX-entrapped liposomes

The stability of MTX-entrapped liposomes was evaluated after storage at 2 different temperatures, 4.5 ± 2.5 and $23.5 \pm 2.5\,^{\circ}$C, under nitrogen gas. At day 0, 7, and 14, size of liposomes was determined by a particle size analyzer as described previously. The experiment was independently performed for 3 repeating samples per experimental group ($n = 3$).

2.8. In vitro permeation study by a Franz diffusion cell

In vitro permeation study was conducted with a vertical Franz diffusion cell (IKA-WERKE-RO 5 power, Germany). The abdominal skin of newly born pig was kindly supplied from Faculty of Veterinary Science, Chulalongkorn University. The fresh skin was cut into 3.5 cm × 3 cm, dehaired and scraped to remove lipid layer. The skin prepared was kept in 0.9% (w/v) sterile normal saline solution and conserved frozen at −20 $^{\circ}$C for up to 1 week after arrival. The skin was thawed in acetate buffer (pH 5.5) at room temperature for 1 h before the following experiments. At a Franz diffusion cell, the square abdominal skin was carefully mounted onto the receptor compartment of the diffusion cell with the epidermis layer facing to the donor compartment. When the donor compartment was fastened to the receptor compartment, the skin acted as a seal between the two half-cells. The cells were then sealed with parafilm to allow the occlusive condition in order to minimize the evaporation and covered with aluminum foil to protect light. The receptor medium was an acetate buffer solution and maintained at 32 $^{\circ}$C under a constantly stirring at 600 rpm with a magnetic bar. A solution of MTX-entrapped liposomes (1 ml) was added into the donor compartment of each cell. An acetate buffer and free-MTX solution (0.5%, w/w) were used as controls of the experiment. At each time interval (0, 1, 3, 5, 10 and 24 h), the solution (700 μl) in the receptor compartment was collected and replaced with the equal volume of fresh buffer solution to ensure the sink condition. The concentration of MTX in the sampling solution was determined by HPLC as previously described and reported as the concentration of MTX across the skin (C_S). After 24 h, the skin was taken out from the cells and washed repeatedly with acetate buffer:ethanol (1:1), followed by several washes with double-distilled water (DDW) to remove excessive MTX. The skin of the epidermis layer was then repeatedly stripped by tape (Scotch® 3M, size 1.8 cm × 3.0 cm) for 20 times (Verma et al., 2001; Rouser et al., 1970; Pierre et al., 2001) to extract the MTX deposited in the stratum corneum. The skin after stripped was homogenized into small pieces. The MTX attached on the tape and remained in the homogenized skin was extracted with acetate buffer:ethanol (1:1) under shaking at 250 rpm, 25 $^{\circ}$C for 5 h. The solution was then centrifuged at 7×10^3 rpm for 10 min. The concentration of MTX in the supernatant was determined by HPLC and reported as the concentration of MTX deposited in the epidermis (C_E) and dermis layers (C_D), respectively. To prove the diffusion mechanism, the C_S values were plotted with time$^{1/2}$ ($t^{1/2}$) according to Higuchi equation (Gohel et al., 2000) as following:

$$C_S = kHt^{1/2} \quad (4)$$

where k_H represents constant Higuchi dissolution.

Flux of MTX diffused across the skin was calculated by dividing the values of C_S, C_E, and C_D by time and area of diffusion. The experiment was independently performed for 3 repeating samples per experimental group ($n = 3$).

2.9. Statistical analysis

All of the results were statistically analyzed by the analysis of variances: One-Way ANOVA (SPSS system for Windows version 17, USA) and $p < 0.05$ was considered to be statistically significant. Data were expressed as the mean ± the standard deviation.

3. Results

3.1. Size, size distribution, and zeta potential of MTX-entrapped liposomes

Average sizes of all liposome formulations were in the range of 82–150 nm (Table 2). For the empty liposomes without MTX, the PC2:CH1 and PC9:CH1 liposomes (104.10 and 93.73 nm) showed larger size than the PC2.5:OA1 liposomes (82.29 nm). When MTX entrapped, size of all liposomes tended to increase with the increasing MTX dose. It was also noticed that size of MTX-entrapped PC:CH liposomes increased with the increasing PC:CH ratio. PDI values showed the narrow size distribution of liposomes (Table 2). Zeta potentials of the PC2:CH1 and PC9:CH1 liposomes without MTX became positively charged at 2.56 and 2.20 mV, respectively (Table 2). With the increasing dose of MTX entrapped, charges of both the PC2:CH1 and PC9:CH1 liposomes were reduced. On the other hand, charge of the PC2.5:OA1 liposomes without MTX was −6.64 mV and that of MTX-entrapped ones showed more neutral values along the increasing MTX dose.

3.2. Thermal properties of MTX-entrapped liposomes

No difference in the T_m (∼68–69 °C) was observed for all formulations of HPC2:CH1 and HPC9:CH1 liposomes (Table 3). In contrast, HPC2.5:OA1 liposomes without or with 0.25% MTX showed the lower T_m at 67.01 and 65.56 °C, respectively. $\Delta T_{m1/2}$ values of HPC2:CH1 and HPC9:CH1 liposomes were markedly higher than those of the HPC2.5:OA1 liposomes, as well as increased with the increasing MTX dose (Table 3). The T_m and $\Delta T_{m1/2}$ values of HPC2.5:OA1 liposomes entrapping 0.5 and 1.0% MTX could not be analyzed in this study.

Table 2
Average size, polydispersity index (PDI), and zeta potential of 3 formulations of liposome encapsulating various doses of MTX at 25 °C (Mean ± SD).

Formulation	Average size (nm)	PDI	Zeta potential (mV)
Formulation 1			
PC2:CH1	104.10 ± 4.78	0.30 ± 0.02	2.56 ± 0.35
PC2:CH1 + 0.25% MTX	94.83 ± 13.97	0.22 ± 0.02	1.77 ± 0.52
PC2:CH1 + 0.5% MTX	109.20 ± 7.06	0.14 ± 0.02	2.12 ± 0.60
PC2:CH1 + 1.0% MTX	117.80 ± 7.13	0.22 ± 0.02	0.79 ± 0.39
Formulation 2			
PC9:CH1	93.73 ± 5.65	0.29 ± 0.02	2.20 ± 0.41
PC9:CH1 + 0.25% MTX	125.80 ± 10.42	0.22 ± 0.01	2.29 ± 1.21
PC9:CH1 + 0.5% MTX	134.70 ± 3.42	0.31 ± 0.01	1.87 ± 0.29
PC9:CH1 + 1.0% MTX	139.70 ± 6.34	0.25 ± 0.03	0.51 ± 0.18
Formulation 3			
PC2.5:OA1	82.29 ± 5.94	0.17 ± 0.01	−6.64 ± 0.73
PC2.5:OA1 + 0.25% MTX	149.70 ± 39.79	0.37 ± 0.05	−3.48 ± 0.29
PC2.5:OA1 + 0.5% MTX	117.80 ± 2.65	0.16 ± 0.02	−3.59 ± 0.96
PC2.5:OA1 + 1.0% MTX	133.70 ± 8.64	0.28 ± 0.09	−3.06 ± 1.18

Table 3
T_m and $\Delta T_{m1/2}$ of 3 formulations of liposome encapsulating various doses of MTX (Mean ± SD).

Formulation	T_m (°C)	$\Delta T_{m1/2}$ (°C)
Formulation 1		
HPC2:CH1	67.95	7.74
HPC2:CH1 + 0.25% MTX	68.18	5.59
HPC2:CH1 + 0.5% MTX	68.48	4.96
HPC2:CH1 + 1.0% MTX	69.46	19.10
Formulation 2		
HPC9:CH1	68.02	2.40
HPC9:CH1 + 0.25% MTX	68.48	3.15
HPC9:CH1 + 0.5% MTX	68.32	4.09
HPC9:CH1 + 1.0% MTX	68.18	6.68
Formulation 3		
HPC2.5:OA1	67.01	0.28
HPC2.5:OA1 + 0.25% MTX	65.56	1.90
HPC2.5:OA1 + 0.5% MTX	N/A	N/A
HPC2.5:OA1 + 1.0% MTX	N/A	N/A

HPC: Phospholipon® 90H.

3.3. Loading percentage and entrapment efficiency of MTX in liposomes

The percentages of MTX loading were increased with the increasing MTX dose for all formulations of liposomes (Table 4). The percentages of MTX loading could be achieved up to 18.23, 16.53, and 10.62 mol% for the 1.0% MTX-entrapped PC2:CH1, PC9:CH1, and PC2.5:OA1 liposomes, respectively. On the other hand, the entrapment efficiency of MTX tended to decrease with the increasing MTX dose for all formulations (Table 4). The high entrapment efficiencies of MTX were obtained for the 0.25% MTX-entrapped PC2:CH1 (7.5%), PC9:CH1 (10.20%), and PC2.5:OA1 liposomes (7.4%).

3.4. Stability of MTX-entrapped liposomes

After stored at 4.5 °C for 7 and 14 days, sizes of the empty liposomes without MTX were approximately 2-times reduced for all formulations (Fig. 1A–C). On the other hand, sizes of most MTX-entrapped liposomes were similar to their initial sizes, although some significant differences were observed in some formulations. Sizes of the PC2:CH1 and PC2.5:OA1 liposomes entrapping 0.5% MTX were significantly larger than their original sizes after stored for both 7 and 14 days. The PC9:CH1 liposomes entrapping 0.5 and 1.0% MTX showed a significantly reduced size when stored up to 14 days. In contrast, the sizes of PC2.5:OA1 liposomes entrapping 1.0% MTX were increased after the storage. When stored at 23.5 °C, the

Table 4
Percentage of MTX loading and entrapment efficiency of MTX of 3 formulations of liposome encapsulating various doses of MTX (Mean ± SD).

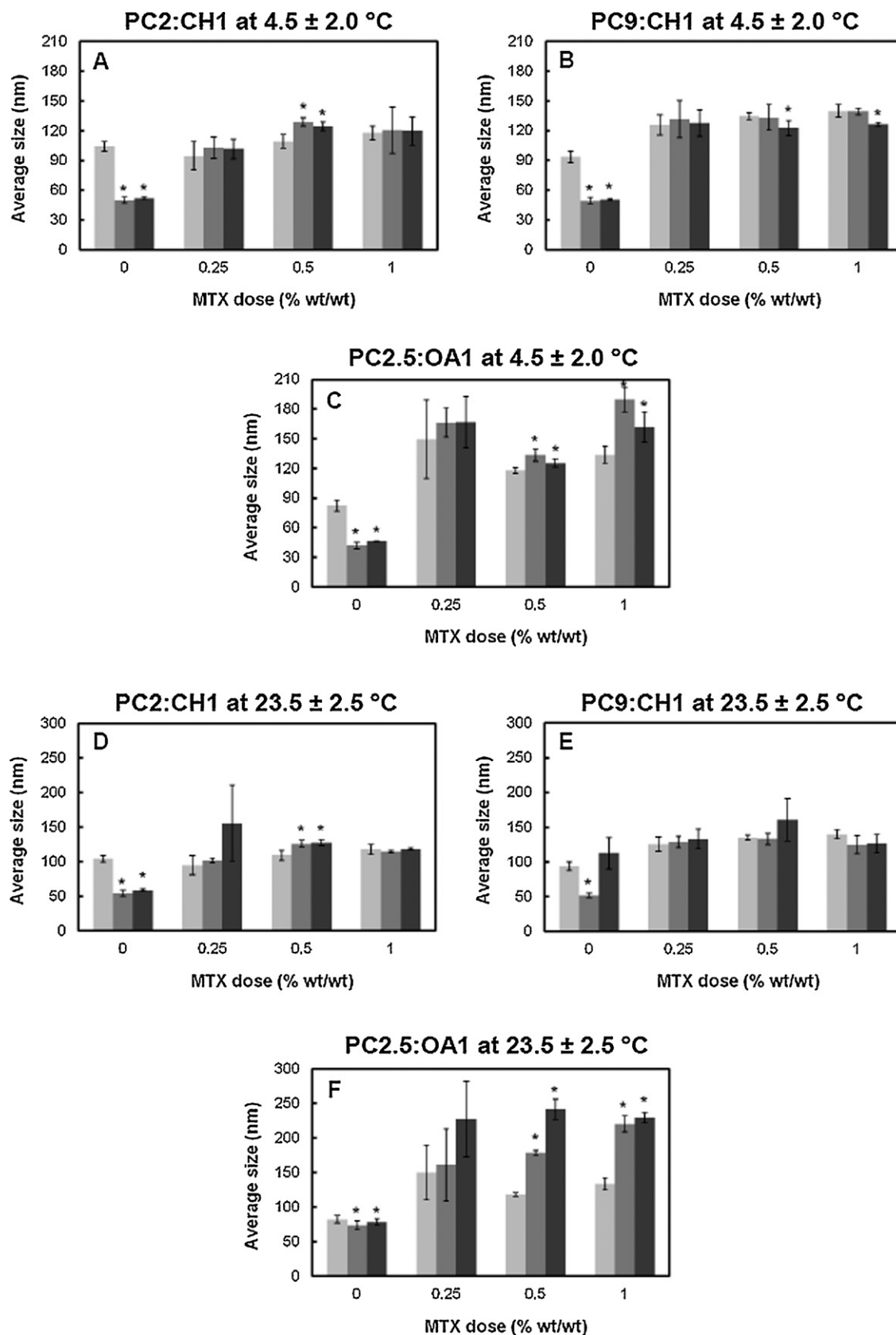| Formulation	MTX loading (mol%)	Entrapment efficiency of MTX (mol%)
Formulation 1		
PC2:CH1	–	–
PC2:CH1 + 0.25% MTX	8.21 ± 1.03	7.50 ± 1.57
PC2:CH1 + 0.5% MTX	11.65 ± 1.73	5.32 ± 1.96
PC2:CH1 + 1.0% MTX	18.23 ± 1.84	4.16 ± 2.41
Formulation 2		
PC9:CH1	–	–
PC9:CH1 + 0.25% MTX	9.43 ± 1.47	10.20 ± 1.62
PC9:CH1 + 0.5% MTX	12.76 ± 1.26	6.89 ± 1.34
PC9:CH1 + 1.0% MTX	16.53 ± 2.24	4.46 ± 1.93
Formulation 3		
PC2.5:OA1	–	–
PC2.5:OA1 + 0.25% MTX	8.16 ± 2.75	7.42 ± 1.45
PC2.5:OA1 + 0.5% MTX	9.09 ± 1.96	4.50 ± 2.03
PC2.5:OA1 + 1.0% MTX	10.62 ± 2.42	2.41 ± 4.20

Fig. 1. Average size of PC2:CH1 (A and D), PC9:CH1 (B and E), and PC2.5:OA1 (C and F) liposomes encapsulating various doses of MTX after storage at $4.5 \pm 2.0\,°C$ (A–C) and $23.5 \pm 2.5\,°C$ (D–F) for 0 (▨), 7 (▨), and 14 days (■) (*$p < 0.05$; significant against the values of initial average size at $t = 0$ at the corresponding MTX dose).
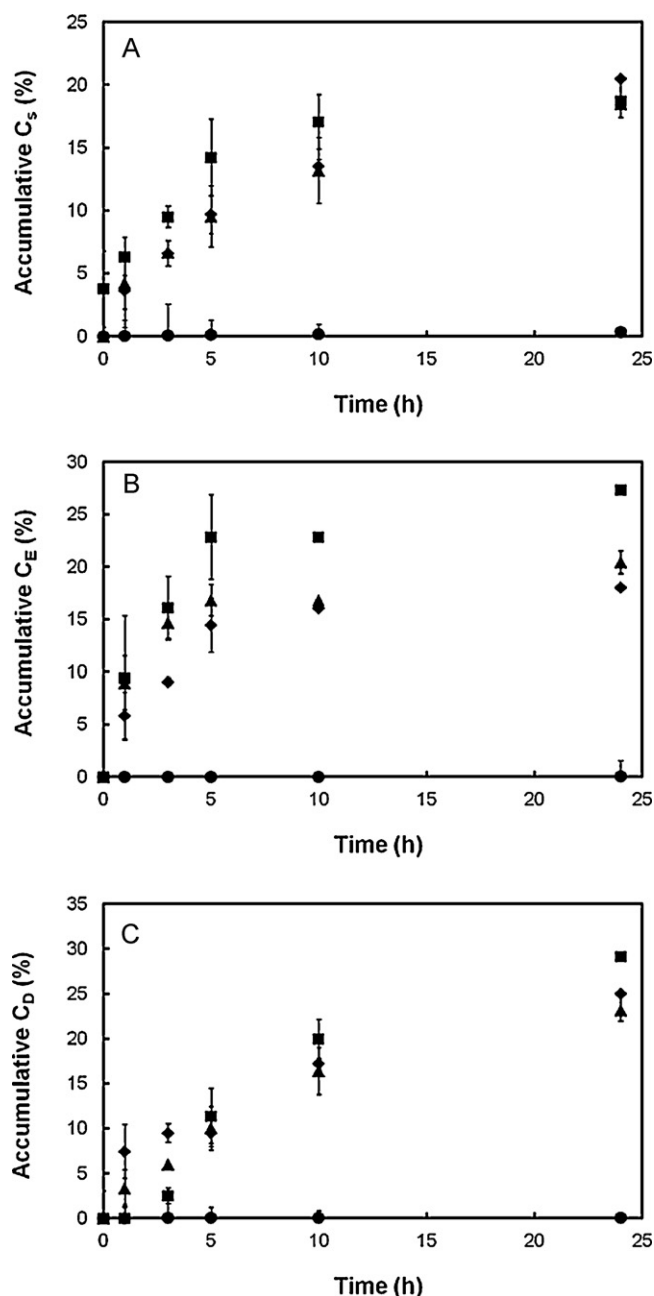
Fig. 3. Relationship profile of the percentage of accumulative concentration of MTX diffused into a receiver of Franz diffusion cell, C_S and time$^{1/2}$ of 0.5% MTX solution (●), PC2:CH1 (♦), PC9:CH1 (▲), and PC2.5:OA1 (■) liposomes encapsulating 0.5% MTX, based on Higuchi diffusion equation.

the porcine skin along the test period. The higher amount of MTX-entrapped PC2.5:OA1 liposomes was accumulated in the epidermis (C_E) and dermis layers (C_D), comparing with that of MTX-entrapped PC2:CH1 and PC9:CH1 liposomes. The free-MTX solution was not detected in both epidermis and dermis layers over 24 h (Fig. 2B and C). When plotted between the concentration of MTX diffused across the porcine skin (C_S) and time$^{1/2}$ based on the Higuchi equation (Gohel et al., 2000), a good linear relationship was found for the MTX-entrapped PC2:CH1 and PC9:CH1 liposomes ($R^2 \sim 0.99$) (Fig. 3). The diffused MTX-entrapped PC2.5:OA1 liposomes was not well correlated with the time$^{1/2}$ ($R^2 \sim 0.69$). However, the slope obtained from the relationship was highest for the MTX-entrapped PC2.5:OA1 liposomes ($k = 4.66$ ng/(ml h$^{1/2}$)), followed by the MTX-entrapped PC2:CH1 ($k = 4.19$ ng/(ml h$^{1/2}$)) and PC9:CH1 liposomes ($k = 3.95$ ng/(ml h$^{1/2}$)), and MTX-free solution ($k = 0.07$ ng/(ml h$^{1/2}$)), respectively.

When reported as flux of MTX, the highest flux of MTX was found in the epidermis layer for all formulations, followed by that diffused across the skin and accumulated in the dermis layer, respectively (Fig. 4). Comparing among formulations, flux of MTX-entrapped PC2.5:OA1 liposomes tended to be higher than that of MTX-entrapped PC2:CH1 and PC9:CH1 liposomes, and MTX-free solution, respectively.

4. Discussion

In this study, the physico-chemical and *in vitro* skin permeation characteristics of MTX-entrapped PC2.5:OA1 deformable liposomes were investigated, compared with those of MTX-entrapped PC:CH conventional liposomes. The molar ratios of lipid materials were selected based on our preliminary study and those reported by other research studies (Coderch et al., 2000; El Maghraby et al., 2000a,b; Stuhne-sekalec and Stanacev, 1989; Taylor et al., 1990). The concentrations of MTX entrapped were selected based on those clinically tested for the psoriasis treatment reported elsewhere, although the different routes of MTX administration were applied (Wienstein and Olsen, 1989; Eskicirak et al., 2006; Lakshmi et al., 2007; Syed et al., 2001).

All formulations of MTX-entrapped liposomes of this study were prepared by a thin film hydration technique which produced the multilamellar vesicles (MLVs) of liposomes (Betageri et al., 1993). Thereafter, high pressure homogenizer was used to reduce average size and size distribution of liposomes. The optimization of homogenization cycles has been performed in our preliminary study. Five-cycle was selected as it provided the appropriate size and size distribution of liposomes and cost effective (data not shown).

Fig. 2. Time profile of the percentage of accumulative concentration of MTX diffused into a receiver of Franz diffusion cell, C_S (A), accumulative concentration of MTX remained in the epidermis layer, C_E (B), accumulative concentration of MTX remained in the dermis layer, C_D (C) of 0.5% MTX solution (●), PC2:CH1 (♦), PC9:CH1 (▲), and PC2.5:OA1 (■) liposomes encapsulating 0.5% MTX.

overall tendency was similar to that observed at 4.5 °C for all formulations, although the less change in size of the empty PC2.5:OA1 liposomes without MTX was noticed after storage (Fig. 1D–F).

3.5. In vitro permeation of MTX-entrapped liposomes

The MTX-entrapped PC2.5:OA1 liposomes were diffused across the porcine skin with the higher amount than that of PC2:CH1 and PC9:CH1 liposomes over 24 h (C_S, Fig. 2A). At 24 h, the concentration of MTX-entrapped PC2.5:OA1 liposomes detected in the receptor compartment was up to 20%. The diffusion of MTX-entrapped PC2:CH1 and PC9:CH1 liposomes showed the similar profiles. In contrast, free-MTX solution could not permeate across
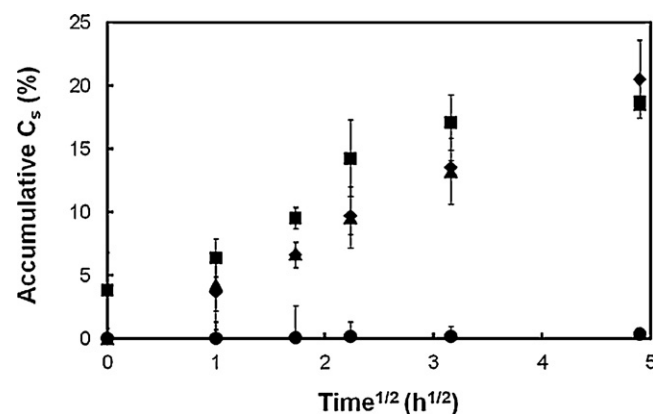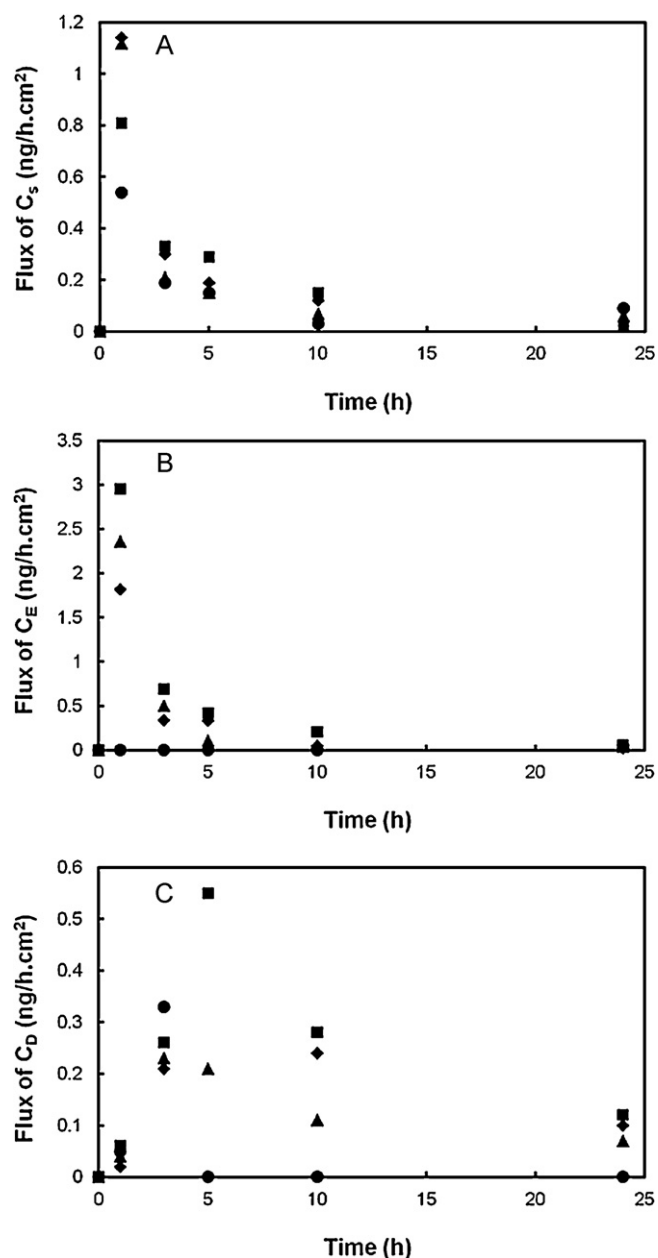
Fig. 4. Flux of concentration of MTX diffused into a receiver of Franz diffusion cell, C_S (A), concentration of MTX remained in the epidermis layer, C_E (B), and concentration of MTX remained in the dermis layer, C_D (C) of 0.5% MTX solution (●), PC2:CH1 (♦), PC9:CH1 (▲), and PC2.5:OA1 (■) liposomes encapsulating 0.5% MTX.

Average sizes of all liposomes were closed to 120 nm in diameter which showed the enhanced penetration into the skin compared to the larger ones (Verma et al., 2001). Considering liposomal structure, Betageri et al. (1993) showed that the liposomal structure was changed from MLVs to small unilamellar vesicles (SUVs) after the homogenization.

Average size of liposomes could be influenced by some factors such as type and molar ratio of lipid materials, MTX contents, and their net charges. The entrapment of MTX dose-dependently increased size of liposomes. It was reported that the entrapment of drug molecules could significantly modify size of liposomes (Lawrence et al., 1993). Considering net charge, the MTX entrapment neutralized net charges of all liposomes in a dose-dependent manner. The more neutral net charge resulted in the larger size of liposomes, possibly due to the aggregation. The relationship

between net charge and size of liposomes was reported elsewhere (Roy et al., 1998). It was showed that the surface net charge of liposomes affected on their physical stability and average size. The presence of surface net charge in liposomes protected them from aggregation.

In the evaluation of thermal properties of MTX-entrapped liposomes in this study, HPC was used as a lipid material to produce liposomes instead of PC because T_m of PC could not be analyzed at the conditions of this study and has not been reported by other researchers. Because the head group structure of HPC was the same as that of PC, thermal properties of HPC would closely represent the values of PC (Aboofazeli et al., 2000). No difference in the T_m for all formulations of HPC2:CH1 and HPC9:CH1 liposomes was observed. This indicated that MTX did not chemically react to the HPC:CH liposomes. On the other hand, the lower T_m values were observed for the HPC2.5:OA1 liposomes without or with 0.25% MTX, possibly being a result of some interactions occurred (Samuni et al., 1998; Taylor and Morris, 1995). Francoeur et al. (1990) have reported that the pretreatment of stratum corneum with OA shifted the T_m to lower values, confirming the effect of OA on the physical properties of stratum corneum lipids. The high $\Delta T_{m1/2}$ values of HPC2:CH1 and HPC9:CH1 liposomes represented a decrease in cooperativity of the transition, which could be caused by the contamination of sample with even minor amounts of lipid degradation products (Samuni et al., 1998). In addition, the high doses of MTX entrapped significantly decreased the cooperativity of HPC2:CH1 and HPC9:CH1 liposomes. On the other hand, HPC2.5:OA1 liposomes without or with 0.25% MTX showed a substantially low $\Delta T_{m1/2}$, indicating a negligible contamination with the degradation products. It is noted that, at 0.5 and 1.0% MTX-entrapped HPC2.5:OA1 liposomes, the chemical interaction between MTX and the PC2.5:OA1 liposomes might modify the arrangement of phospholipids so that their T_m and $\Delta T_{m1/2}$ values could not be analyzed.

When MTX was entrapped in liposomes, the low percentages of MTX loading were obtained for all formulations (8.1–18.2 mol%). This would be due to the small hydrophilic core of the SUVs liposomes to entrap MTX (Trotta et al., 2004). At the equivalent MTX dose, PC2.5:OA1 liposomes showed lower percentage of MTX loading than the PC2:CH1 and PC9:CH1 liposomes. This could be explained that the negatively charged OA might repulse the negatively charged MTX molecules and decreased the drug loading capability. In addition, the deformable conformation of PC:OA liposomes might modify the packing of hydrophilic core and its capability to entrap MTX.

In terms of stability, size of most MTX-entrapped liposomes did not markedly change after storage at both 4.5 and 23.5 °C up to 14 days, except some formulations of the MTX-entrapped PC2.5:OA1 liposomes. This indicated the stability of MTX-entrapped liposomes under these storage conditions. On the other hand, the empty liposomes without MTX revealed a considerably instability by approximately 2-time size reduction. Previous investigations reported that the entrapment of drug molecules could improve membrane integrity and stability of liposomes by modifying their surface net charges or average sizes (Schlieper et al., 1981; Kirilenko and Gregoriadis, 1993). The instability was also observed for the 0.5 and 1.0% MTX-entrapped PC2.5:OA1 liposomes. The reason for this phenomenon is not clear at present. One possibility would be the result of oxidation reaction at acryl chain of unsaturated hydrocarbon of lipid phosphate that changed the arrangement of liposomal structure and subsequently affected the stability of liposomes (Gallarate et al., 2006). Another possible reason was the aggregation of liposomes, resulting in the larger size. Liu et al. (1989) investigated the stability of liposomes composed of dioleoylphosphatidylethanolamine and OA. It was found that these liposomes were not stable at 37 °C in a simple buffered solution, but showed an unusual stability when incubated in normal human plasma

or serum due to lipid-protein interaction. In order to improve the stability of liposomes for further study, we here proposed some techniques for example, the addition of anti-oxidants (e.g. α-tocopherol, butyl hydroxyl toluene) or chelating agents (e.g. EDTA) into the solution during the stability test or storage under an inert gas (e.g. argon or nitrogen).

Based on the physio-chemical characteristics of liposomes and the literatures reviewed, 0.5% MTX-entrapped liposomes were selected for further in vitro permeation study. Porcine skin was chosen as a model for this study because its structure and properties are similar to that of human skin (Forster, 2002). The more amount of MTX-entrapped PC2.5:OA1 liposomes could penetrate across the skin as well as accumulate in the epidermis and dermis layers, compared with that of the MTX-entrapped PC:2:CH1 and PC9:CH1 liposomes. The underlying mechanisms for the enhanced permeation of MTX-entrapped PC2.5:OA1 liposomes were not clearly understood at present. However, it could be explained by some possible mechanisms. First possibility would be the deformable characteristic of OA-containing liposomes that allowed MTX to flexibly and easily penetrate across the skin (Trotta et al., 2004; Vanaja et al., 2008; Cevc and Gabriele, 2001; Cevc et al., 1995). In addition, as OA is a skin penetration enhancer (Francoeur et al., 1990; Touitou et al., 2002), the enhanced permeation of MTX-entrapped PC2.5:OA1 liposomes would be directly related to the perturbation of OA on the stratum corneum lipids of skin. In the other words, OA would disorder the highly packed stratum corneum intercellular domain lipids, allowing the molecules accompany molecules to easily penetrate through.

Considering the diffusion mechanism based on the Higuchi equation, only the diffused MTX-entrapped PC2:CH1 and PC9:CH1 liposomes showed a good linear relationship with time$^{1/2}$. This demonstrated that their permeation across the skin was mainly controlled by the diffusion. On the other hand, the permeation of MTX-entrapped PC2.5:OA1 liposomes might be governed by other phenomena in addition to the diffusion. The combination of permeation mechanisms would result in the enhanced permeability of MTX-entrapped PC2.5:OA1 liposomes. The Higuchi dissolution constant (k) of MTX-entrapped PC2.5:OA1 liposomes was higher than that of MTX-entrapped PC2:CH1 and PC9:CH1 liposomes, confirming the higher permeation rate of MTX-entrapped PC2.5:OA1 liposomes across the skin.

In addition, flux of MTX-entrapped PC2.5:OA1 liposomes tended to be higher than that of MTX-entrapped PC2:CH1 and PC9:CH1 liposomes, and MTX-free solution. This was corresponded to the results reported by El Maghraby et al. (2000b). The liposomal formula containing OA, PC and incorporating 7% ethanol resulted in an enhanced flux of oestradiol entrapped due to the potential as a skin penetration enhancer of OA. This strengthened the enhanced permeability of MTX-entrapped PC2.5:OA1 liposomes. Furthermore, in this study, the highest flux of MTX was found at the epidermis layer which is the target site of MTX-entrapped liposomes for the treatment of psoriasis.

Taken together, although the MTX-entrapped PC2:CH1 and PC9:CH1 liposomes showed good physico-chemical characteristics, the skin permeability of MTX-entrapped PC2.5:OA1 deformable liposomes was superior. Another highlighted advantage of the MTX-entrapped PC2.5:OA1 deformable liposomes was the increased drug accumulation on the targeted epidermis layer. We here concluded that the PC2.5:OA1 deformable liposome was one of promising candidates to enhance the permeability of MTX for the treatment of psoriasis.

5. Conclusions

The physico-chemical characteristics and in vitro permeability of MTX-entrapped PC2.5:OA1 deformable liposomes were evaluated, comparing with those of MTX-entrapped PC2:CH1 and PC9:CH1 conventional liposomes. All liposomes showed the narrow size distribution (80–140 nm). The percentage of MTX loading, entrapment efficiency and the stability of MTX-entrapped PC2:CH1 and PC9:CH1 liposomes were higher than those of MTX-entrapped PC2.5:OA1 liposomes. However, the MTX-entrapped PC2.5:OA1 liposomes enhanced the skin permeability characterized by the higher concentration and flux of MTX diffused across or accumulated in the epidermis and dermis layers of porcine skin. The enhanced permeability of MTX-entrapped PC2.5:OA1 liposomes was due to the deformable characteristic of OA-containing liposomes and a skin penetration enhancer of OA.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpharm.2012.01.045.

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