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Selection of phospholipids to design liposome preparations with high skin penetration-enhancing effects

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ABSTRACT

This study attempted to identify a good selection method for phospholipids to design liposome preparations with high skin penetration-enhancing effects. Five kinds of phosphatidylcholines and phosphatidylglycerols each were selected. First, phospholipid aqueous dispersions and liposomes containing caffeine as a model drug were tested for their skin penetration-enhancing effects using excised hairless rat skin. Accordingly, 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol, sodium salt (DPPG) dispersions showed high penetration-enhancing ratios (*ERs*), whereas DPPG, 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC), and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) liposomes showed high *ERs*, suggesting that liposomes had different skin penetration-enhancing mechanisms from phospholipid dispersions. Next, two experiments were done to clarify the possible mechanism of liposome penetration; excised skin was pretreated for 1 h with caffeine-free phospholipid dispersions and liposomes, and caffeine solution was added to determine its skin permeation. Separately, caffeine permeation experiments were done using physical mixtures of blank liposomes and caffeine solution (caffeine-spiked liposomes) and caffeine-entrapped liposomes (caffeine was entrapped only in liposomes). DPPG was found to be a promising phospholipid candidate for liposome formulations with high skin penetration-enhancing effects, because DPPG phospholipid and liposome vesicles had a combination effect of disrupting the SC lipid barrier to carry both free and entrapped caffeine in the formulation through the skin.

1. Introduction

Liposomes, a type of classical vesicular drug delivery system, have received extensive attention in the field of skin drug delivery due to their ability to entrap drug(s) and enhance the skin penetration of both hydrophilic and lipophilic molecules [1–3]. The main component of liposomes is an amphiphilic molecule, phospholipids, which can spontaneously form closed bilayer vesicles as they confront water [4]. Mechanisms to account for the enhancement of skin penetration of drugs by liposomes have been proposed including (1) free drug operation, (2) intact vesicular penetration, (3) vesicle adsorption to and/or fusion with the stratum corneum (SC) and (4) their penetration-enhancing effect [5]. However, conflicting results for these mechanisms have been found in spite of extensive efforts made by many research groups.

One of the well-accepted mechanisms for the skin penetration-enhancement by liposomes is the penetration of the amphiphilic components into the skin barrier and their perturbatory actions on the packing of SC lipids [6,7]. From this reason, liposome composition must be an important parameter for the enhancing effect of liposomes on the skin penetration of drugs. In the formulation design of liposomes, many researchers have already focused on the optimization of liposomal characteristics; *i.e.*, morphology, vesicular size, surface charge, entrapment efficiency, transition temperature, or elasticity of liposomes by changing the liposome compositions or their preparation procedure [8–10]. Moreover, novel classes of vesicular carriers have been developed to obtain the high skin penetration-enhancing effect of liposomes, by addition of edge activators or chemical penetration enhancers into classical liposomes [11–13]. However, the skin penetration-enhancing effects of the main phospholipid compositions in liposomes have not been fully clarified.

In the present study, different types of phospholipids were first tested for their skin penetration-enhancing effects of a model hydrophilic drug, caffeine, through excised hairless rat skin using a currently established assay system comprised of multiple-diffusion cells as a screening approach, because phospholipids in the liposome composition can play an important role in the skin penetration-enhancing effect

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of liposomes. Then, several kinds of phospholipids were selected to prepare liposomes and tested for their skin penetration-enhancing effects, and the obtained results were compared with those for their phospholipid dispersions, in order to design suitable liposomes with high skin penetration-enhancing effects. Next, two further permeation experiments were carried out in order to clarify the possible modes of action of each phospholipid in their skin penetration-enhancing effects. 1) The effect of 1 h of pretreatment of excised skin was evaluated with caffeine-free phospholipid dispersions and liposomes. Caffeine solution was added after pretreatment to assess general skin permeation. 2) Caffeine permeation experiments were performed using a physical mixture of blank liposomes and caffeine solution (caffeine-spiked liposomes) and caffeine-entrapping liposomes (caffeine was entrapped only inside of the liposomes).

These results were used to survey phospholipid(s) to design liposomes having a high skin penetration-enhancing effect of a model hydrophilic drug, caffeine.

2. Materials and methods

2.1. Materials

Phospholipids including: 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC; as below); 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC); 1,2-dimyristoyl-*sn*-glycero-3-phospho-glycerol, sodium salt (DMPG); 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC); 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol, sodium salt (DPPG); 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC); 1,2-distearoyl-*sn*-glycero-3-phosphoglycerol, sodium salt (DSPG); 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC); and 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol, sodium salt (DOPG) were purchased from NOF Corporation (Tokyo, Japan). 1,2-Dilauroyl-*sn*-glycero-3-phosphoglycerol, sodium salt (DLPG) was obtained from Olbracht Serdary Research Laboratories (Toronto, ON, Canada). Table 1 summarizes the abbreviations, the number of carbon atoms and double bonds in the alkyl chains of these phospholipids .

Cholesterol was purchased from Sigma-Aldrich (St. Louis, MO, USA). Caffeine, chloroform, methanol, and ethanol were purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). These reagents were used without further purification.

2.2. Experimental animals

Male WBN/ILA-Ht hairless rats, weighing between 200 and 260 g, were obtained from the Life Science Research Center, Josai University (Sakado, Saitama, Japan) and Ishikawa Experimental Animal Laboratories (Saitama, Japan). Rats were bred in a room maintained at 25 ± 2 °C, in which the on and off times for the lighting were 07:00 and 19:00, respectively. Animals had free access to water and food (MF, Oriental Yeast Co., Ltd., Tokyo, Japan).

All breeding procedures and experiments on the animals were performed in accordance with the guidelines of the Animal Experiment Committee of Josai University.

Abdominal skin from hairless rats was excised under anesthesia by *i.p.* injection of anesthesia containing medetomidine (0.375 mg/kg), butorpharnol (2.5 mg/kg), and midazolam (2 mg/kg). The hairless rats were then sacrificed immediately by injection of pentobarbital sodium (40 mg/kg).

Table 1
Abbreviations, numbers of carbon atoms and double bonds in the alkyl chain of the phospholipids used in the present study.

Full name	Abbreviation	Carbon length: double bond
1,2-dilauroyl-*sn*-glycero-3-phosphocholine	DLPC	12:0
1,2-Dilauroyl-*sn*-glycero-3-phosphoglycerol, sodium salt	DLPG	12:0
1,2-dimyristoyl-*sn*-glycero-3-phosphocholine	DMPC	14:0
1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol, sodium salt	DMPG	14:0
1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine	DPPC	16:0
1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol, sodium salt	DPPG	16:0
1,2-distearoyl-*sn*-glycero-3-phosphocholine	DSPC	18:0
1,2-distearoyl-*sn*-glycero-3-phosphoglycerol, sodium salt	DSPG	18:0
1,2-dioleoyl-*sn*-glycero-3-phosphocholine	DOPC	18:1
1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol, sodium salt	DOPG	18:1

2.3. Preparation of liposomes

Liposomes were prepared using phospholipids and cholesterol in a ratio of 4:1 w/w. The compositions were dissolved in chloroform:methanol (2:1 v/v) in a round-bottomed flask, and the solvent was evaporated to form a thin film using a rotary evaporator under reduced pressure. The obtained film was purged with nitrogen gas and kept overnight to remove traces of organic solvent. The flask was then immersed in a water bath at 90 °C for annealing of the thin film for 30 min, and then 100 mM caffeine in phosphate-buffered saline (PBS) pH 7.4 solution was added to adjust the phospholipid concentration to 3% (w/v). The thin film was hydrated for 30 min and the resulting liposomes containing caffeine were then sonicated using a probe sonicator (VCX-750, Sonics & Materials Inc., Newtown, CT, USA) for 30 s. Next, four freeze-thaw cycles were performed by immersing the flask in liquid nitrogen and in a 90 °C water bath for 3 min each. The obtained liposomes were further extruded using a mini-extruder (Avanti Polar Lipids, Inc., Alabaster, AL, USA) assembled with a membrane filter (with pore sizes of 400, 200, and 100 nm, Whatman® track-etched membranes, GE Healthcare Japan, Tokyo, Japan). All final liposome formulations containing caffeine were kept at 25 °C and freshly used for skin permeation experiment within a day after preparation. In the final formulations, caffeine must be contained both inside and outside of the liposomes. These liposome formulations were used to evaluate caffeine permeation through the skin.

Caffeine-free liposomes (blank liposomes) was also prepared using the same procedure but without addition of caffeine.

2.4. Characterizations of liposomes

2.4.1. Particle size and zeta potential

The particle size and zeta potential of liposomes were measured after 100-fold dilution with PBS using a Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK). The size measurements were performed at 25 °C and a scattering angle of 90°. Individual zeta potential measurement was repeated for at least 10 readings from each liposome sample.

2.4.2. Caffeine distribution in liposome formulations

The entrapment efficiency (*EE*) of caffeine in each liposome sample was determined using an ultracentrifuge technique to evaluate the caffeine distribution either inside or outside of the liposome formulations. The final liposome suspension (400 μL) was placed in a centrifuge tube and centrifuged using a micro-ultracentrifuge (Himac CS120GXII, Hitachi Koki Co., Ltd., Tokyo, Japan) at 289,000 × *g*, 4 °C for 20 min to separate the liposome pellet (entrapped drug, E_{drug}) from the supernatant (unentrapped drug, U_{drug}). The supernatant was collected, and the free caffeine content was determined after 10-fold dilution with ethanol followed by 10-fold with PBS. In addition, the entrapped drug content inside the liposomes was determined by dispersing the packed liposome pellet with 400 μL PBS and further disrupting with 10-fold

dilution with ethanol followed by 10-fold dilution with PBS. Caffeine contents were analyzed by high-performance liquid chromatography (HPLC) and the % *EE* was calculated according to the following equation.

$$\%\ \text{Entrapment efficiency (EE)} = \left(\frac{E_{drug}}{E_{drug} + U_{drug}}\right) \times 100 \quad (1)$$

Thus, caffeine content outside of the liposomes was represented by 100 – *EE*.

2.4.3. Differential scanning calorimetry

The phase transition temperature of liposomes (T_m) was determined using a differential scanning calorimeter (DSC) (Thermo plus EVO/DSC8230, Rigaku Corporation, Akishima, Tokyo, Japan). About 5 mg of liposome pellets obtained from the ultracentrifugation process, as described in section 2.4.2, were placed in an aluminum pan. An empty pan was used as a reference. The DSC heating scan was performed at a heating rate of 5.0 °C/min in a 15–80 °C range.

2.5. In vitro skin permeation experiment

2.5.1. Determination of the cumulative amount of caffeine permeated through the skin over 12 h from different phospholipid dispersions

First, the skin penetration-enhancing effect of different types of phospholipids were determined using a diffusion cell array system as shown in Fig. 1 (Ikeda Scientific Co., Ltd., Tokyo, Japan). This system was comprised of 12 wells wherein the donor compartments were above the receiver chambers with the excised skin sandwiched between them. This system can simultanously determine 12 sets of permeation data at a single run. However, the time course of the cumulative amount of caffeine that permeated through skin could not be determined using this system, which differs from a Franz-type diffusion cell because of the lack of a sampling port. Effective permeation area and receiver volume for each well were 0.785 cm^2 and 1.36 mL, respectively. The study was performed using excised abdominal skin from hairless rat after removing subcutaneous fat. The skin was excised and cut into two pieces of 3 × 4 cm size from the middle line of the rat abdomen and set on the diffusion cell array system. At first, 1.0 and 1.36 mL PBS were added in each donor and receiver chamber, respectively, for 1 h for skin hydration. After this, PBS was removed from the donor compartment and replaced with 200 μL of 3% (w/v) phospholipid dispersions containing caffeine in PBS at a concentration of 100 mM. The permeation experiment was performed at 32 °C using a thermo-shaker at the rotation speed of 200 rpm and the receiver solution was stirred using a stir ball for 12 h. At the end of the permeation experiment, the receiver solution was collected to determine the cumulative amount of caffeine that permeated per unit area of skin (Q_{12})

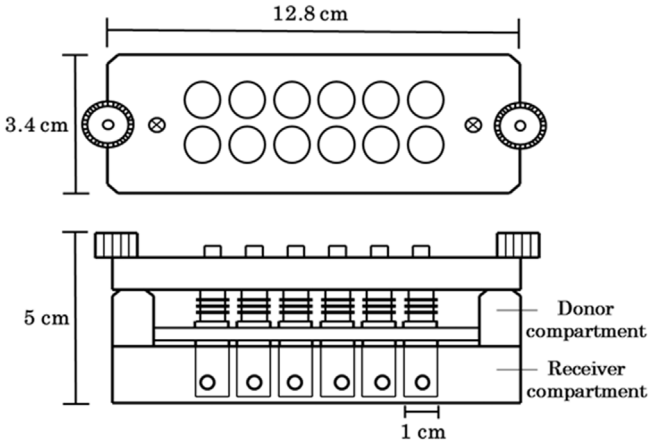

Fig. 1. Schematic representation of currently designed diffusion cell array system.

using an HPLC. Caffeine solution (100 mM) in PBS was used as a control, and the skin penetration-enhancement ratio (*ER*) of each sample was calculated using the following equation;

$$ER = \left(\frac{Q_{12,\ sample}}{Q_{12,\ control}}\right) \quad (2)$$

where $Q_{12,\ sample}$ and $Q_{12,\ control}$ are the cumulative amount of caffeine permeated per unit area of skin over 12 h from different phospholipid dispersions and the control solution, respectively.

2.5.2. Determination of the time course of skin permeation of caffeine from different liposome formulations

Excised abdominal skin from hairless rat was mounted in a vertical-type Franz diffusion cell (effective permeation area: 1.77 cm^2, receiver cell volume: 6.0 mL) with the SC side facing the donor cell and the dermal side facing the receiver cell. The receiver and donor cells were filled with 6.0 and 1.0 mL of PBS, respectively, for 1 h for skin hydration. Then, 400 μL of liposomes containing caffeine at a concentration of 100 mM were replaced in the donor compartment to determine its skin permeation at 32 °C over 8 h, while the receiver solution was agitated at 500 rpm using a magnetic stirrer. At predetermined times, 0.5 mL aliquots were collected, and the same volume of PBS was added to keep the volume constant. The amount of caffeine that permeated through the skin was determined using an HPLC.

2.5.3. Determination of the effect of pretreatment with caffeine-free phospholipid dispersions and liposomes on caffeine permeation through skin

Selected caffeine-free liposomes (400 μL) prepared using phospholipids (DPPG, DLPC, or DSPG) were applied onto the SC surface of skin for 1 h after a hydration period with PBS. Phospholipid dispersions (3% DPPG, DLPC, or DSPG) without caffeine were also applied to the skin for comparison. Then, the liposomes or phospholipid dispersions without caffeine were removed from the skin surface by washing with 1.0 mL fresh PBS 10 times. Caffeine solution (100 mM, 400 μL) was then applied to the skin. The skin permeation experiment was conducted using a vertical-type Franz diffusion cell for 8 h, as described in section 2.5.2.

2.5.4. Determination of the skin permeation of caffeine from the physical mixture of blank liposomes and caffeine solution (caffeine-spiked liposomes)

Caffeine-spiked liposomes containing 3% phospholipid and 100 mM caffeine were prepared by mixing the same volume of double-concentrated blank liposomes (the preparation method was similar to the blank liposomes as above) and 200 mM caffeine. The resultant caffeine-spiked liposomes (400 μL) were used for the skin permeation experiment with caffeine using a vertical-type Franz diffusion cell.

2.5.5. Determination of the skin permeation of caffeine from caffeine entrapped in liposomes

Caffeine entrapped in DPPG, DLPC, or DSPG liposomes was obtained by ultracentrifugation separation, as described in section 2.4.2. In the preparation process, free caffeine was fully removed and the remaining caffeine-entrapping liposomes pellet was dispersed with PBS (400 μL). The skin permeation experiment using this caffeine-entrapping liposome formulation was performed and compared with the same concentration of free caffeine solution using a vertical-type Franz diffusion cell.

2.6. Determination of caffeine concentration

The concentration of caffeine was determined using an HPLC system (Prominence, Shimadzu Corporation, Kyoto, Japan) equipped with a UV detector (SPD-M20A, Shimadzu Corporation). The sample solutions were mixed with the same volume of methanol and then centrifuged at 21,500 ×*g* and 4 °C for 5 min. The supernatant (20 μL) was injected

Table 2
Physicochemical properties of liposomes prepared in the present study.

Main component		T_m (°C)	*EE* (%)	Particle diameter (nm)	Polydispersity Index	Zeta potential (mV)
DLPC	(12:0)	49.8	15.44 ± 0.74	161.2 ± 0.3	0.211 ± 0.023	0.2 ± 0.5
DLPG	(12:0)	–	23.98 ± 0.42	109.3 ± 0.3	0.088 ± 0.016	−41.2 ± 3.0
DMPC	(14:0)	49.0	18.68 ± 0.54	165.3 ± 2.0	0.111 ± 0.079	−1.3 ± 1.6
DMPG	(14:0)	65.3	27.95 ± 1.10	130.2 ± 0.8	0.059 ± 0.011	−40.3 ± 3.5
DPPC	(16:0)	47.6	17.46 ± 0.63	169.9 ± 2.4	0.250 ± 0.011	−1.3 ± 0.7
DPPG	(16:0)	41.5	13.35 ± 0.03	152.1 ± 0.9	0.164 ± 0.016	−44.3 ± 2.5
DSPC	(18:0)	51.7	12.93 ± 0.15	184.6 ± 3.4	0.214 ± 0.005	−0.5 ± 1.2
DSPG	(18:0)	62.8	21.14 ± 0.27	162.1 ± 0.9	0.096 ± 0.007	−45.1 ± 1.0
DOPC	(18:1)	65.8	49.08 ± 1.27	143.3 ± 1.0	0.070 ± 0.007	−6.6 ± 1.2
DOPG	(18:1)	54.5	45.38 ± 0.11	110.7 ± 0.5	0.116 ± 0.122	−41.6 ± 1.7

directly into the HPLC system. Chromatographic separation was performed at 40 °C using an Inertsil ODS-3 (5 μm diameter) column (4.6 mm I.D. × 150 mm, GL Sciences Inc., Tokyo, Japan). The mobile phase was 0.1% phosphoric acid:methanol (7:3 v/v) and the flow rate was 1.0 mL/min. Detection was performed at UV 280 nm.

2.7. Statistical analysis

Data were expressed as the mean ± S.E. or S.D. The differences among the obtained data were analyzed using unpaired *t*-test. The differences were considered to be significant when $p < 0.05$.

3. Results

3.1. Characteristics of liposomes

Table 2 summarizes the physicochemical properties of liposomes prepared in this study. All liposome formulations had a small diameter in the range of 110–185 nm. Phosphatidylcholine-containing (DLPC, DMPC, DPPC, DSPC, and DOPC) liposomes showed larger particle sizes than phosphatidylglycerol-containing (DLPG, DMPG, DPPG, DSPG, and DOPG) liposomes. The zeta potential of these phosphatidylcholine-containing liposomes showed roughly neutral charges, whereas phosphatidylglycerol-containing liposomes had negative surface charges less than -40 mV. The *EE* was less than 50% and T_m was within a range 41–66°C for all liposome formulations.

3.2. Skin penetration-enhancing effect of phospholipid dispersions

Fig. 2 shows the obtained *ER* of hairless rat skin permeation of caffeine in the presence of 3% phospholipid dispersion in PBS against that of free caffeine solution in PBS (without phospholipids). A diffusion cell array system (Fig. 1) was used in this experiment to determine Q_{12}, which was used as an index for skin permeation of caffeine, because data over 12 h was more reliable to the shorter periods like 8 h. Both DSPC and DPPG dispersions significantly enhanced the skin permeation of caffeine (*ER* was 1.93 and 1.57, respectively), whereas DOPG dispersion showed significantly lower skin permeation than the control (PBS) (*ER* = 0.38). The other phospholipid dispersions showed almost the same permeation of caffeine as the control (PBS).

3.3. Skin permeation of caffeine from liposomes

Then, liposomes prepared from different phospholipids were determined for their skin penetration-enhancing effect on caffeine. Franz diffusion cell sets were used in this experiment. The experimental period using Franz cells was set at 8 h, because the reliablity was proven from the time course data. Fig. 3 shows the time course of the cumulative amount of caffeine that permeated through skin from different kinds of liposomes over 8 h. Typical lag time and following steady-state permeation profiles were observed for all liposome

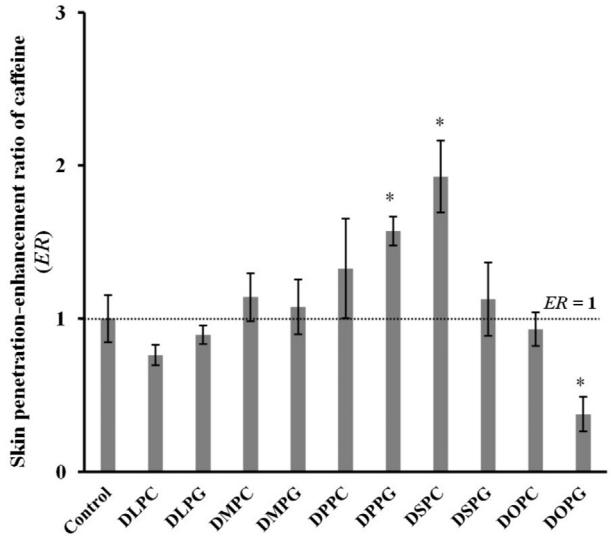

Fig. 2. *ER* of skin permeation of caffeine from different phospholipid dispersions. Each value represents the mean ± S.E. (n = 3–4). *: $p < 0.05$ significantly different from control (free caffeine solution in PBS).

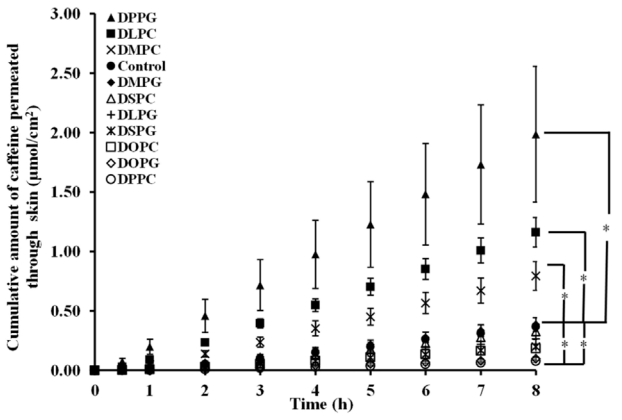

Fig. 3. Time course of the cumulative amount of caffeine that permeated through skin from different liposome formulations. Each value represents the mean ± S.E. (n = 3–5). *: p < 0.05 significantly different from control (free caffeine solution in PBS).

preparations prepared in this experiment. Only DPPG, DLPC, and DMPC liposomes significantly promoted caffeine permeation compared with the control (caffeine solution in PBS) (*ER* was 5.43, 3.17, and 2.17, respectively), whereas DPPC and DOPG liposomes significantly decreased caffeine permeation (*ER* was 0.21 and 0.27, respectively). No or little change in skin penetration was found with other phospholipid liposomes.

Then, the skin penetration-enhancing effect with phospholipid

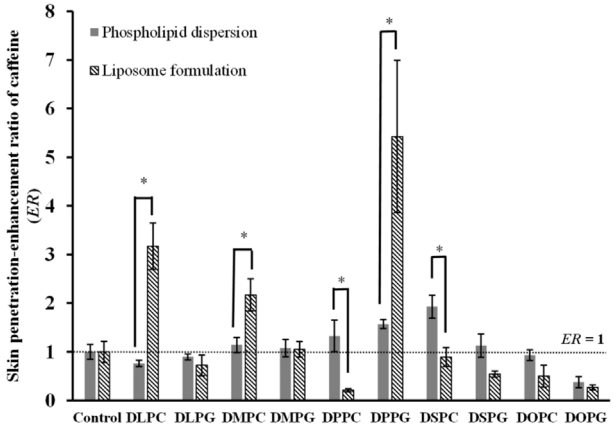

Fig. 4. Comparison of *ER* of skin permeation of caffeine from phospholipid dispersions and liposomes. Each value represents the mean ± S.E. (n = 3–5). *: $p < 0.05$ significantly different for liposomes from their phospholipid dispersion.

dispersions and liposome preparations were summarized to compare them, as shown in Fig. 4. Of note, DLPC, DMPC, and DPPG liposomes showed significantly higher *ER* values compared with their phospholipid dispersions, whereas DPPC and DSPC liposomes showed significantly lower *ER* values compared with their phospholipid dispersions.

Although DSPC phospholipid provided the highest *ER* in its dispersion form, the skin permeation of caffeine from DSPC liposomes was substantially decreased. DPPG showed an enhancement effect with both phospholipid dispersions and liposomes. However, its *ER* value was highly increased by modification of the liposome formulation. In the case of other phospholipids, such as DLPG, DMPG, DSPG, DOPC, and DOPG, their liposomes showed no significant difference in *ER* values from those of their dispersions.

Especially in case of DPPG, liposome formulations showed high skin penetration-enhancing effects, although the mechanism has not yet been clarified. Then, the following experiments were designed.

3.4. Effect of pretreatment with caffeine-free phospholipid dispersions and liposomes on the skin permeation of caffeine

DPPG and DLPC liposomes exhibited the highest skin penetration-enhancement effects among all the liposomes tested, but DSPG liposomes exhibited low skin permeation of caffeine. Then, these three phospholipids were selected and a 1-h pretreatment experiment using caffeine-free phospholipid dispersions or liposomes was done before skin permeation measurements from the caffeine solution to clarify the possible skin penetration-enhancing effect of liposomes. Fig. 5 shows the results. The pretreatment with phospholipid dispersions enhanced caffeine permeation for DLPC (*ER* = 1.35) and DPPG (*ER* = 2.47), but decreased it for DSPG (*ER* = 0.48). A pretreatment experiment was also performed using caffeine free-liposomes. Of note, the 1-h pretreatment with caffeine free-liposomes showed different results: a lower *ER* value was observed compared with the effect of pretreatment with phospholipid dispersions. The ER values for caffeine-free DLPC, DPPG, and blank DSPG liposomes were 0.87, 1.50, and 0.46, respectively.

3.5. Effect of physical mixture of blank liposomes and caffeine solution (caffeine-spiked liposomes) on the skin permeation of caffeine

In order to evaluate the contribution of caffeine contents in the inside and outside of liposomes on its skin permeation, a physical mixture of blank liposomes and caffeine was applied on excised skin to measure the skin permeation of caffeine. The results are shown in Fig. 6. Caffeine-spiked DLPC and DSPG liposomes exhibited lower skin permeation of caffeine (*ER* values 0.58 and 0.44, respectively). On the other hand, only the caffeine-spiked DPPG liposomes significantly enhanced the skin permeation of caffeine (*ER* = 2.65) compared with the control caffeine solution.

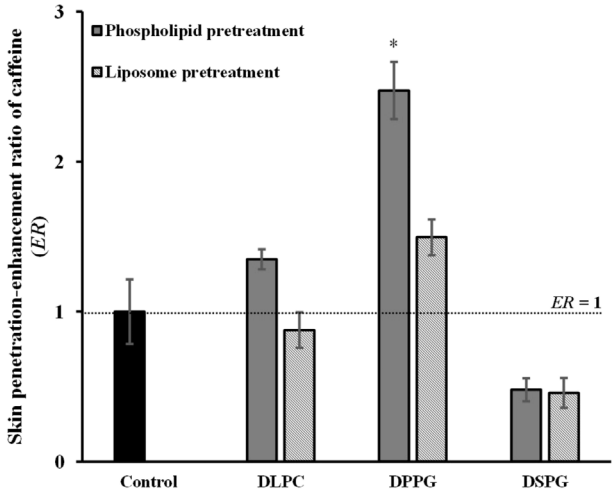

Fig. 5. Effect of 1-h pretreatment with caffeine-free phospholipid dispersions and liposomes on the *ER* of skin permeation of caffeine. Each value represents the mean ± S.E. (n = 3–5).
*: $p < 0.05$ significantly different from the control (no pretreatment; free caffeine solution in PBS).

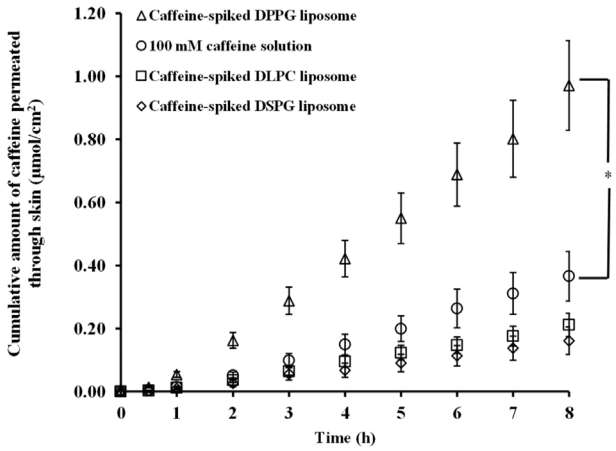

Fig. 6. Time course of the cumulative amount of caffeine that permeated through skin from physical mixture of blank liposomes and caffeine solution (caffeine-spiked DPPG, DLPC, and DSPG liposomes). Each value represents the mean ± S.E. (n = 3–5). *: $p < 0.05$ significantly different from the control (free caffeine solution in PBS).

3.6. Effect of caffeine-entrapping liposomes on the skin permeation of caffeine

Next, caffeine entrapped in liposomes was evaluated for the skin permeation of caffeine. Fig. 7 shows the results. Caffeine-entrapping DPPG liposomes showed the highest caffeine permeation (*ER* = 4.39), whereas caffeine-entrapping DLPC liposomes enhanced about 1.65-fold compared with the caffeine solution, which contained the same concentration as in liposome formulations. No penetration-enhancing effect was observed for caffeine-entrapping DSPG liposomes.

4. Discussion

Although several studies have reported the potential of liposomes as a topical/transdermal drug delivery system compared with conventional formulations [3,14,15], there seems to be a general lack of understanding among researchers regarding the formulation factors of

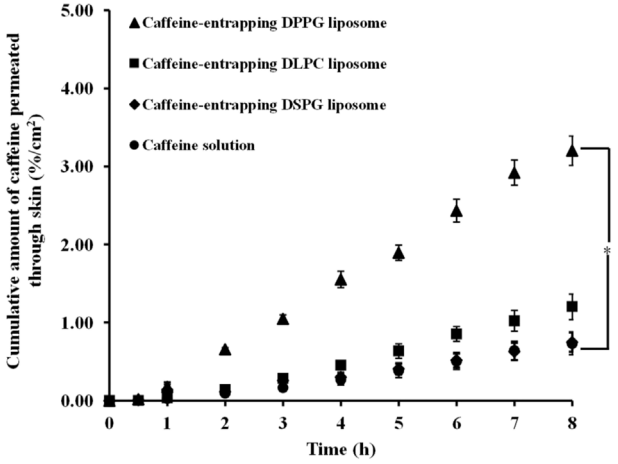

Fig. 7. Time course of the normalized cumulative amount of caffeine that permeated through skin from caffeine-entrapping DPPG, DLPC, and DSPG liposomes. Each value represents the mean ± S.E. (n = 3–5). *: $p < 0.05$ significantly different from the control (free caffeine solution in PBS). Y-axis was calculated with dividing the cumulative amount of caffeine that permeated through skin by the total amount of applied drug.

liposomes to provide high skin penetration-enhancing effects. Therefore, the present study focused mainly on designing strategies for liposomes by investigating the effect of the phospholipid composition of liposomes on the enhanced skin permeation of drugs.

First, the skin penetration-enhancing effect of different phospholipids used for liposome preparations was evaluated using caffeine as a model penetrant, because the selection of phospholipids will be very important to design suitable liposomes formulations. Among 10 kinds of phospholipids (5 phosphatidylcholines and 5 phosphatidylglycerols) examined, DPPG and DSPC dispersions significantly improved the skin permeation of caffeine, whereas the DOPG dispersion markedly decreased skin permeation. In addition, the other phospholipids had no or little skin penetration-enhancing effects against the control group (Fig. 2).

A few studies have reported the skin penetration-enhancing effect of phospholipids [16–18]. Unfortunately, propylene glycol was used to dissolve the phospholipids in previous studies, which differed from the present study (we used PBS). The present results showed that all phospholipids provided only mild penetration-enhancing effects or decreased the skin permeation of caffeine. Valjakka-Koskela et al. [19] reported that phospholipids inhibited the skin permeation of naproxen from aqueous gel, but only gels containing ethanol or propylene glycol as a co-solvent increased the skin permeation of the drug. In addition, Yokomizo and Sagitani [18] reported that the penetration-enhancing effect of phospholipids was affected by their solubility in solvents. Thus, organic solvents such as propylene glycol may overestimate the effect of phospholipids on drug permeation [20].

Although the size of liposomes prepared in the present study was equally small (110–180 nm), but T_m, *EE*, and zeta potential were dramatically affected by changes in the kinds of phospholipids (Table 2). The reasons for these differences in the skin penetration-enhancing effects depending on the phospholipids present are still unknown with these parameters.

The reason why only DPPG, DLPC, and DMPC liposomes showed higher permeation than other liposomes (Fig. 3) can be explained as follows: (1) Some liposomes might have a rigid structure that could form an extra lipid barrier on the skin surface, which retards the skin permeation of caffeine, and this extent must differ depending on the phospholipids, or (2) some liposomes might release caffeine in a slower rate than other phospholipid-based liposomes, probably due to the interaction between the drug and phospholipids [21].

The *ERs* of the phospholipid dispersions were different from those of their liposome formulations (Fig. 4), suggesting that liposomes containing caffeine could have different mechanisms to increase skin permeation than phospholipid dispersions.

Understanding of different physiochemical properties of phospholipids and liposomes may be necessary to clarify the skin penetration-enhancing mechanism of phospholipids and liposomes. DLPC, DPPG, and DSPG were selected, because DLPC showed a high *ER* only in the dispersion form and DPPG showed a high effect in both forms, whereas DSPG had no effect in both forms. For the above purposes, the skin pretreatment approach and following skin permeation experiments with caffeine were carried out using caffeine-free phospholipids dispersions and liposomes (Fig. 5). In addition, the effect of a physical mixture of blank liposomes and caffeine solution (caffeine-spiked liposomes) and caffeine-entrapping liposomes (caffeine presented only in liposomes) were determined (Figs. 6 and 7, respectively). Pretreatment with DLPC and DPPG dispersions enhanced the skin permeation of caffeine (Fig. 5), indicating that these phospholipids had skin penetration-enhancing effects, because they might rearrange and fuse with an ordered structure of intercellular lipids (such as ceramides) to reduce the SC barrier function made by disruption of well-packed intercellular lipids and the creation of a permeation pathway for drugs [6,22,23]. Of note, pretreatment of caffeine-free liposomes provided lower *ER* values than their corresponding phospholipid dispersions (Fig. 5). This could be due to the presence of such vesicles having less fluidity to disrupt the rigid structure of the SC than its dispersion forms. Obviously, only pretreatment with caffeine-free DPPG liposomes increased the skin permeation of caffeine.

For the physical mixture of blank liposomes and caffeine, caffeine-spiked DPPG liposomes could deliver the drug through skin with the highest *ER* (Fig. 6). Caffeine-free DPPG liposomes might disrupt the SC structure, allowing the free caffeine being mixed outside the liposome vesicles to diffuse through the skin barrier. As a consequence, the caffeine-entrapping DPPG liposomes also provided the highest *ER* compared with caffeine-entrapping DLPC and DSPG liposomes (Fig. 7).

The highest skin penetration-enhancing effect observed from DPPG liposomes was due to the synergistic of different actions; the skin penetration-enhancing effect of DPPG dispersions and the skin penetration-enhancing effect of caffeine-free DPPG liposomes as well as the penetration-enhancing ability of caffeine both outside (caffeine-spiked liposome) and inside (caffeine-entrapped liposome) of liposomal vesicles. Similar to the previous report [24], the penetration of a non-entrapped and entrapped hydrophilic fluorescence probe, carboxyfluorescein, in liposomes through human skin were increased compared with the control solution. The fluorescence may penetrate along with intact liposomes or associated with liposomal fragment.

The skin penetration-enhancing effect of DLPC liposomes was observed after pretreatment only with its phospholipid dispersions. Furthermore, only caffeine-entrapped DLPC liposomes showed enhance caffeine permeation. Thus, the overall skin penetration-enhancing effects obtained from DLPC liposomes were lower compared to DPPG liposomes. On the other hand, no skin penetration-enhancing effect was observed for DSPG liposomes in all cases resulting in low caffeine permeation.

Lipophilic tails of fatty acids and phospholipids are known to increase the skin permeation of drugs. Kim et al. [25] investigated the effect of carbon-chain length of saturated fatty acids on the skin penetration-enhancing effect and revealed a parabolic correlation between the penetration-enhancing effect and their carbon-chain length of the saturated fatty acids. These results suggested that fatty acids with a certain-chain length possess an optimal partition coefficient or solubility parameter. As the carbon-chain length of lipophilic tails in phospholipid increased from C12 (DLPC) to C18 (DSPC) in the present experiment, the skin permeation was increased in the dispersion form. Because interaction between liposome membranes and SC intercellular lipids has not yet been investigated, the reason for the highest skin permeation from DLPC liposomes is still unclear.

The modes of action of liposomes to enhance the skin permeation of

drugs observed in the present study were summarized as follows; (1) the phospholipid molecule could disrupt the SC lipid barrier and enhance drug permeation, (2) the free liposome vesicles themselves could also disrupt the SC lipid, and (3) the drug could associate with the outside of or encapsulate inside of liposome vesicles and then liposomes carry the drug to pass through the skin. However, different compositions of liposomes resulted in different degrees of those effects. Further studies should be carried out to understand the molecular mechanisms of each liposomes composition on their skin penetration-enhancing effect.

5. Conclusion

Our findings demonstrated that the composition of liposomes is an important factor to improve their performance. Understanding the effect of such factors in liposomes could enable researchers to develop effective liposome formulations with high skin permeation of drugs.

Conflicts of interest

The authors declare no conflict of interest.

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