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Enhancement of the transdermal delivery of catechins by liposomes incorporating anionic surfactants and ethanol

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Abstract

The aim of this study was to develop and evaluate liposomal formulations encapsulating tea catechins, which possess antioxidant and chemopreventive activities. Liposomes were characterized for size, zeta potential, and entrapment efficiency. Both in vitro and in vivo skin permeation were examined using nude mouse skin as a model. The results suggested that the liposomal composition plays an important role in affecting the efficiency of transdermal catechin delivery. Incorporation of anionic surfactants such as deoxycholic acid (DA) and dicetyl phosphate (DP) in the liposomes in the presence of 15% ethanol increased the (+)-catechin permeation by five to seven-fold as compared to the control. The flexibility of bilayers is suggested as an important factor governing the enhancing effect of liposomes. Intercellular spaces within the stratum corneum but not shunt routes are the major pathways for catechin delivery from liposomes. (+)-Catechin and (−)-epicatechin are isomers which showed similar encapsulation efficiencies and skin permeation in liposomes. (−)-Epigallocatechin-3-gallate showed the highest encapsulation rate and in vivo skin deposition level in liposomes among all catechins tested. The stability and in vitro tranepidermal water loss test indicated the safety of the practical use of liposomes developed in this study.

Keywords: Catechins; Transdermal delivery; Liposomes; Ethanol

1. Introduction

Most of the polyphenols present in green tea are flavanols, commonly known as catechins. These polyphenols are antioxidants and have been shown to function as chemopreventive and anticarcinogenic agents (Katiyar et al., 2000). The oral bioavailability of catechins is low at less than 5% (Baba et al., 2001; Catterall et al., 2003). The half-life of catechins is short due to strong systemic clearance (Cai et al., 2002). Hence a transdermal system would provide an alternative way to resolve these problems. Green tea extract is also used for topical purpose such as photoprotection to UV, skin cancer prevention, and skin care (Katiyar et al., 2000). Batchelder et al. (2004) have developed the transdermal patch for tea extract. The permeation of the major catechins is determined and they find a sufficient amount of catechins can penetrate across pig skin. This result encourages the skin routes for catechin delivery.

The main barrier of the skin is located within the stratum corneum (SC). One of the possibilities for increasing skin permeation of drugs is the use of vesicular systems (Honeywell-Nguyen et al., 2002). Classic liposomes are of little value as carriers for transdermal drug delivery because they do not deeply penetrate the skin. Only specially designed vesicles are shown to be able to allow transdermal delivery (Touitou et al., 2000). Cevc et al., 1997; Cevc and Blume, 1992 introduced the first elastic liposomes with high deformability referred to as transfersomes, consisting of phosphatidylcholine (PC) with sodium cholate or sodium deoxycholate. El Maghraby et al., 2000,

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2001a developed so-called ultradeformable liposomes, which are considered to be elastic to permeate into the skin. Ethosomes developed by Touitou's group are vesicles composed of PC and a high proportion of ethanol generally >30% (Dayan and Touitou, 2000; Touitou et al., 2000).

The aim of this present study was to develop a new liposomal system with elastic properties to enhance the skin permeation of catechins. The influence of liposomes on the transdermal permeation of three catechins, (+)-catechin, (−)-epicatechin, and (−)-epigallocatechin-3-gallate (EGCG), was systematically investigated. The liposomes were applied to various skin membranes, including intact nude mouse skin, SC-stripped skin, delipidized skin, and furry mouse skin, to explore the mechanisms of drug permeation. The stability of liposomes and their possible disturbance to the skin were also examined.

2. Materials and methods

2.1. Materials

(+)-Catechin, (−)-epicatechin, EGCG, cholesterol (CH), deoxycholic acid (DA), and dicetyl phosphate (DP), were obtained from Sigma Chemical (St. Louis, MO, USA). Egg phosphatidylcholine (EPC, 99%) was purchased from Nippon Oil (Tokyo, Japan). Polyethylene glycol with MWs of 200 and 1000 (PEG200 and PEG1000) was purchased from Kanto Chemical (Tokyo, Japan). A cellulose membrane (CelluSep® T2, with a MW cut-off of 6000–8000) was provided by Membrane Filtration Products (Seguin, TX, USA).

2.2. Preparation of liposomes

EPC (4%, w/v), CH (1%, w/v), and other additives with determined quantities were dissolved in a 10-ml volume of a chloroform:methanol (2:1) solution. The organic solvent was evaporated in a rotary evaporator at 40 °C, and solvent traces were removed by maintaining the lipid film under a vacuum overnight. The films were hydrated with 15% ethanol in water containing 17.2 mM drug using a probe-type sonicator (VCX 600, Sonics and Materials, USA) at 25 W for 30 min. In some experiments, the liposomes were extruded through a LipoFast® extruder (Avestin, Canada) using a polycarbonate membranes with a pore size of 200 nm. The additives and contents of various formulations utilized in this study are listed in Table 1.

2.3. Vesicle size and zeta potential

The mean particle size and zeta potential of the liposomes were measured by a laser scattering method (Nano ZS® 90, Malvern, UK). Liposomal suspensions were diluted 100-fold with double-distilled water before the measurement. The determination was repeated three times per sample for three samples. The size and zeta potential of the liposomes were also monitored after storage at 40 °C for 35 days.

2.4. Drug encapsulation percentage

The encapsulation efficiency of catechins entrapped by liposomes was determined by ultracentrifugation. Immediately after the end of preparing the liposomes, the product was centrifuged at 48,000 × *g* and 4 °C for 30 min in a Beckman Optima MAX® ultracentrifuge (Beckman Coulter, USA) in order to separate the incorporated drug from the free form. The supernatant and precipitate were analyzed by HPLC to determine the encapsulation percentage. The entrapment capacity of liposomes was calculated as follows: $[(T - C)/T] \times 100$, where T is the total amount of drug that is detected both in the supernatant and sediment, and C is the amount of drug detected only in the supernatant (Touitou et al., 2000).

2.5. In vitro release and skin permeation

Drug release and skin permeation were determined using a Franz diffusion cell. A cellulose membrane or female nude mouse skin (Balb/c-nu, 6–8 week old) was mounted between the donor and receptor compartments. The SC-stripped skin and delipid skin were obtained as described earlier (Sung et al., 2003). The donor medium consisted of 1 ml of a liposomal vehicle without separation of the non-entrapped drug. The top of the donor was covered with parafilm. The receptor

Table 1
The composition and characterization of (+)-catechin, (−)-epicatechin, and EGCG liposomes by vesicle size, zeta potential, and drug encapsulation

Code	Composition[a]	Drug	Size (nm)	Zeta potential (mV)	Encapsulation (%)
Basic	EPC + CH = 4:1	(+)-Catechin	131.1 ± 0.3	−0.9 ± 0.4	39.5 ± 4.8
DA	EPC + CH + DA = 4:1:0.25	(+)-Catechin	378.2 ± 10.9	−26.2 ± 0.9	53.4 ± 0.6
DP	EPC + CH + DP = 4:1:0.25	(+)-Catechin	357.2 ± 20.4	−11.2 ± 0.4	58.8 ± 3.3
PEG200	EPC + CH + PEG200 = 4:1:0.25	(+)-Catechin	168.5 ± 14.2	−5.5 ± 0.2	44.4 ± 2.2
PEG1000	EPC + CH + PEG1000 = 4:1:1.25	(+)-Catechin	314.7 ± 45.5	−5.3 ± 1.5	38.7 ± 3.2
Extrude	EPC + CH + DA = 4:1:0.25 extrude	(+)-Catechin	215.8 ± 21.3	−36.1 ± 1.7	57.0 ± 6.6
Without CH	EPC + DA = 4:0.25	(+)-Catechin	121.3 ± 4.6	−20.4 ± 1.0	65.5 ± 3.6
DA	EPC + CH + DA = 4:1:0.25	(−)-Epicatechin	378.2 ± 10.9	−26.2 ± 0.9	62.7 ± 7.8
DP	EPC + CH + DA = 4:1:0.25	EGCG	378.2 ± 10.9	−26.2 ± 0.9	93.0 ± 0.1

EPC, egg phosphatidylcholine; CH, cholesterol; DA, deoxycholic acid; DP, dicetyl phosphate. Each value represents the mean ± S.D. ($n = 3$).
[a] The ratio of liposome composition is weight ratio (%).

medium consisted of 10 ml of pH 7.4 citrate-phosphate buffer. The sink condition could be maintained throughout the experimental duration. The available diffusion area was 1.54 cm^2. The stirring rate of receptor and temperature were kept at 600 rpm and 37 °C. At appropriate intervals, 300-μl aliquots of the receptor medium were withdrawn and replaced with fresh buffer.

The amount of drugs retained in the skin was detected at the end of the experiments (12 h). The skin was wiped 10 times using a cotton cloth immersed in methanol. A sample of skin was weighed, cut with scissors, positioned in a glass homogenizer containing 1 ml 0.1 M HCl, and ground for 5 min. The resulting solution was centrifuged for 10 min at 10,000 rpm and then filtered through a membrane with a pore size of 0.45 μm. Samples were analyzed by HPLC. An HPLC system consisting of a Hitachi L-7110 pump, a Hitachi L-7200 sample processor, and a Hitachi L-7480 fluorescence detector. A 25-cm-long, 4-mm inner diameter stainless RP-18 column (Merck, Darmstadt, Germany) was used. The mobile phase for catechins was 10:90 acetonitrile:2.7% acetic acid/water at a flow rate of 1.2 ml/min. The fluorescence detector was set at 280 nm for excitation and 320 nm for emission (Fang et al., 2005). The detection limit of (+)-catechin, (−)-epicatechin and EGCG was 10, 10, and 40 μg/ml, respectively.

2.6. In vivo topical application

A glass cylinder with an available area of 0.79 cm^2 was placed on the back skin of a female nude mouse (Balb/c-nu, 6–8 week) with glue. A 0.2-ml liposomal suspension was added to each cylinder. The application time of the vehicle in the cylinder was 12 h. After excising the skin on which the solution was applied, the drug amount within the skin was extracted as described in the in vitro experiments.

2.7. Surface tension measurements

Measurements were carried out at room temperature by the Wilhemy plate method (Jumaa and Müller, 1998). Surface tension was determined by the using a thin platinum plate attached to a transducer amplifier (Kyowa CBVP-A3, Saitama, Japan).

2.8. In vitro transepidermal water loss (TEWL) examination

A Franz diffusion cell was used for the in vitro examination of the TEWL of the skin after treatment with liposomes. After application of liposomes without the drug for 5 h, the donor compartment was removed, and a Tewameter® 300 evaporimeter probe (Courage and Khazaka, Germany) was positioned on the skin surface. TEWL values were automatically calculated and are expressed in g/m^2/h.

2.9. Statistical analysis

The statistical analysis of differences between different formulations was performed using unpaired *t*-test. A 0.05 level of probability ($p < 0.05$) was taken as the level of significance. The analysis of variance (ANOVA) test was used also.

3. Results

3.1. Physicochemical characteristics of liposomes

The sizes and zeta potentials of the prepared liposomes are shown in Table 1. The liposomes made from EPC and CH in the presence of ethanol (basic liposomes) had a relatively small size of 133.1 nm. The incorporation of anionic surfactants such as DA and DP significantly ($p < 0.05$) increased the sizes. This may be due to that the extraneous amount of the surfactants increased the whole volume of lipid bilayers. On the other hand, the incorporation of PEG 200 only increased the liposomal size in a minimal level. The higher MW of PEG1000 at the same molar concentration produced a greater size compared to the lower-MW one. DA liposomes were selected to extrude across a membrane with a 200-nm pore size. An average size near the pore size after extruding liposomes was determined (215.8 nm) (Table 1). When removing the CH from liposomes, the vesicle size greatly reduced ($p < 0.05$) to 121.3 nm. This result was consistent with the previous investigation, which indicates a tendency to decrease the size of liposomes with negative charges on the surface with decreasing CH concentration (Touitou et al., 1994).

There were almost no surface charges on the basic liposomes (Table 1). The addition of anionic surfactants resulted in the increase of negative charges on the vesicle surface. The zeta potential of DA-containing formulation was much higher ($p < 0.05$) than that of DP-containing formulation, indicating that DA strongly interacted with EPC in the bilayers. As compared to anionic surfactants, PEG slightly increased the charges to −5 mV. It is noticeable that the process of extrusion significantly increased ($p < 0.05$) the zeta potential.

3.2. Encapsulation of catechins in liposomes

The structure of (+)-catechin is the basic skeleton for all catechins. Hence the entrapment efficiency of (+)-catechin into liposomes was first examined. As shown in Table 1, (+)-catechin encapsulation is higher for liposomes with DA or DP than the others. No difference ($p > 0.05$) was found in encapsulation before or after extrusion. The entrapment of liposomes without CH was 65.5%, which was the highest among all formulations tested.

Liposomes with DA were utilized to further examine the entrapment efficiency of (−)-epicatechin and EGCG. (+)-Catechin and (−)-epicatechin are isomers to each other. The encapsulations of both compounds in liposomes were comparable ($p > 0.05$). EGCG showed the greatest encapsulation of near 100% (Table 1).

3.3. Release and skin permeation of (+)-catechin from liposomes

To develop liposomes for transdermal delivery, it is important to optimize the ability of drug release from the vesicles.

Table 2
The release rate of (+)-catechin, (−)-epicatechin, and EGCG from various liposomes across cellulose membrane

Code	Drug	Release rate (nmol/cm^2/h)
Solution[a]	(+)-Catechin	515.34 ± 25.33
Basic	(+)-Catechin	254.38 ± 56.12
DA	(+)-Catechin	277.71 ± 24.13
DP	(+)-Catechin	275.29 ± 9.88
PEG200	(+)-Catechin	244.81 ± 20.60
PEG1000	(+)-Catechin	229.03 ± 7.64
Extrude	(+)-Catechin	288.15 ± 21.82
Without CH	(+)-Catechin	356.50 ± 22.13
Solution	(−)-Epicatechin	519.35 ± 65.4
DA	(−)-Epicatechin	252.59 ± 14.41
Solution	EGCG	41.97 ± 7.76
DA	EGCG	0

Each value represents the mean ± S.D. ($n = 4$).
[a] The vehicle of solution means drug dissolved in a hydroalcoholic solution (15% ethanol).

(+)-Catechin was used as a model drug in the release study. Plotting the amount of drug released from each formulation as a function of time, a linear relationship was obtained following a pseudo-zero order kinetic. As shown in Table 2, (+)-catechin release from the hydroalcoholic solution (15% ethanol in water) was significantly higher ($p < 0.05$) than those determined from all liposomes. Discrepancies among the release rates of liposomes composed of various additives were not large. The release of (+)-catechin was not changed by extruding liposomes. The liposomes without CH were more efficient than those with CH in facilitating (+)-catechin release (Table 2).

The cumulative amounts of drug in the receptor during 12 h were plotted after administration of various liposomal formulations as shown in Fig. 1. (+)-Catechin flux across the skin was significantly lower ($p < 0.05$) than that released from the same formulations. Liposomes with EPC and CH in 15% ethanol (basic liposomes) significantly increased ($p < 0.05$) in vitro drug permeation compared to the control. This flux was also higher than that of liposomes without ethanol (Fig. 2). Liposomes incorporated DA or DP could further enhance the (+)-catechin flux (Fig. 1). The enhancement of liposomes with PEG (F4 and F5) on (+)-catechin permeation did not reach the levels of other liposomes. As shown in Fig. 2, the extrusion procedure resulted in a two-fold increase in drug permeation compared to the unextruded liposomes. The exclusion of CH significantly increased the ($p < 0.05$) (+)-catechin flux. The receptor medium was examined by laser scattering at the end of in vitro topical application. No particle size could be determined in the receptor, indicating there was no intact vesicles permeated across the skin.

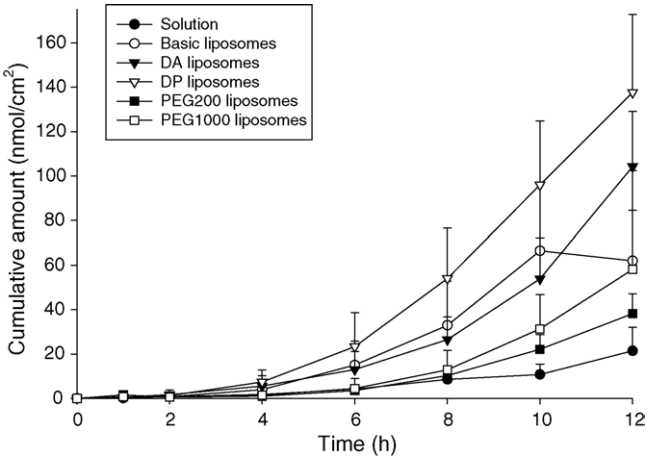

Fig. 1. In vitro cumulative amount-time profiles of transdermal (+)-catechin delivery from liposomal formulations with various additives in the presence of ethanol. All data are presented as the mean ± S.D. of four experiments.

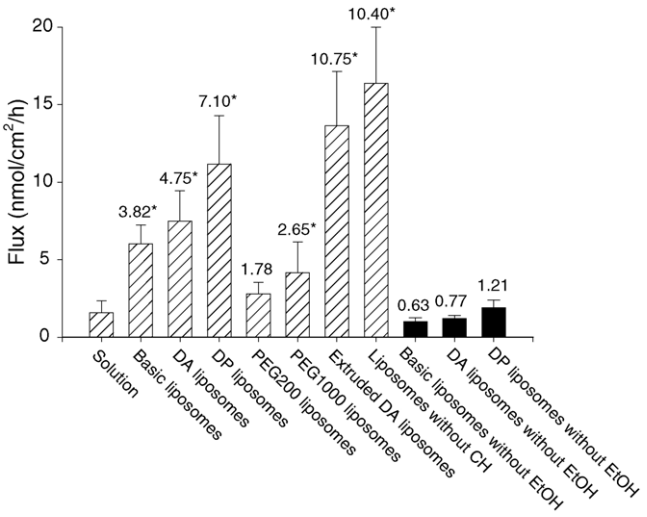

Fig. 2. (+)-Catechin fluxes from various liposomal formulations measured from in vitro cumulative amount-time profiles. All data are presented as the mean ± S.D. of four experiments.

3.4. Mechanisms involved in skin permeation of (+)-catechin from liposomes

The delivery efficiency of liposomes with anionic surfactants increased after the open application compared with the occlusive application ($p < 0.05$) (Fig. 3). To investigate the possibility of enhancing potential by phospholipids, the skin was pretreated by solutions of 4% EPC for 5 h in Franz cells, following administrating (+)-catechin in control solution. The pretreatment method avoided a cosolvent effect on the thermodynamic activity of drugs or enhancers. As shown in Fig. 4, pretreatment of water increases drug permeation as compared to the group without any pretreatment although no significant difference ($p > 0.05$) was determined. Both pretreatments of 15% ethanol and 4% EPC in 15% ethanol did not produce any enhancement of (+)-catechin permeation compared with pretreatment of water ($p > 0.05$). EPC (4%) was directly added to solution to further examine the (+)-catechin permeation across skin. As illustrated in Fig. 4, the presence of EPC aggregates retarded ($p < 0.05$) the skin permeation of (+)-catechin.

Table 3 shows that the (+)-catechin flux across SC-stripped skin from the hydroalcoholic solution was 343-fold higher than that across intact skin. On the other hand, drug permeation by DA liposomes across SC-stripped skin was only 25-fold higher than that across intact skin. The flux permeated across delipid skin by

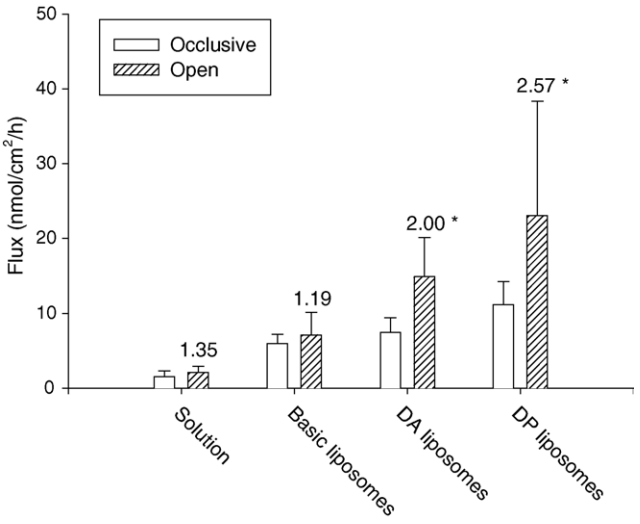

Fig. 3. (+)-Catechin fluxes from various liposomal formulations by an occlusive or open application on the top of the donor compartment. All data are presented as the mean ± S.D. of four experiments.

applying liposomes was similar to that across SC-stripped skin. The enhancement when applying liposomes across furry mouse skin showed a similar trend to that across nude mouse skin.

3.5. Skin permeation of tea catechins from liposomes

We carried out a further investigation of the delivery of three catechins by DA-containing liposomes. According to the data in Table 2, differences in steric conformations between (+)-catechin and (−)-epicatechin did not influence the release from either the hydroalcoholic solution or liposomes. EGCG showed a relatively lower release from the hydroalcoholic solution. This result was consistent with the previous study that EGCG shows the slowest release among all catechins (Batchelder et al., 2004). No EGCG was detected in the receptor compartment following the application of liposomes. Since EGCG may strongly interact within the bilayers due to its high level of encapsulation, it was difficult for EGCG to escape from the vesicles. Another possibility for the low skin permeation of EGCG was the larger molecular size (MW = 458.4 Da) as compared to the other catechins. Finnin and Morgan (1999) have indicated that the molecules with MW higher than 500 Da are difficult to penetrate across skin because of the barrier properties of SC.

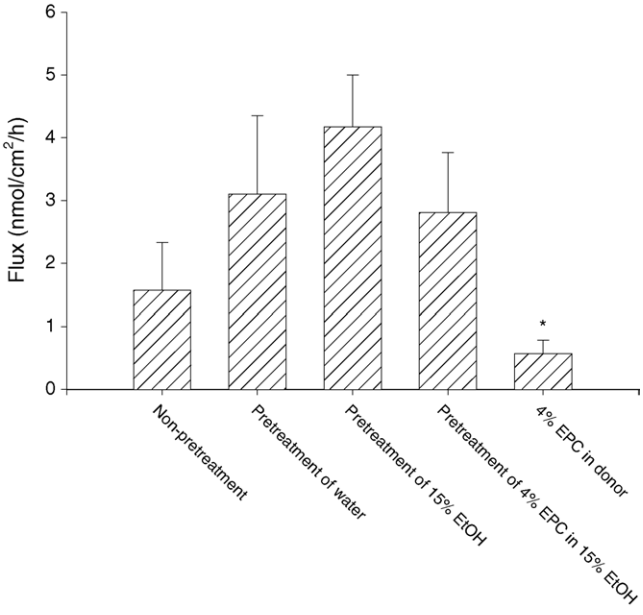

Fig. 4. (+)-Catechin fluxes across skin pretreated with various vehicles or by the direct addition of 4% EPC in the donor compartment. All data are presented as the mean ± S.D. of four experiments.

Table 3
In vitro permeation data of (+)-catechin from liposomes across various skin barriers

Code	Skin type	Flux (nmol/cm²/h)	Ratio[a]
Solution[b]	Intact skin	1.57 ± 0.76	–
	SC-stripped skin	542.96 ± 138.05	–
	Delipid skin	336.31 ± 68.20	–
	Balb/c skin	2.47 ± 1.31	–
DA[c]	Intact skin	7.48 ± 1.97	4.75
	SC-stripped skin	188.68 ± 43.63	0.35
	Delipid skin	176.89 ± 42.16	0.53
	Balb/c skin	10.56 ± 2.83	4.27

Each value represents the mean ± S.D. ($n = 4$).
[a] Ratio = (+)-catechin flux from DA liposomes/(+)-catechin flux from hydroalcoholic solution (control group).
[b] The vehicle of solution means drug dissolved in a hydroalcoholic solution (15% ethanol).
[c] DA means the liposomes composed of EPC + CH + DA = 4:1:0.25.

Liposomes can improve the permeation of all catechins as shown in Table 4. The enhancement ratios were comparable for both (+)-catechin and (−)-epicatechin (4.75-fold versus 5.39-fold). The incorporation of liposomes could raise the EGCG flux from 0 to 0.17 nmol/cm²/h. The skin deposition of (+)-catechin from the control group was significantly higher ($p < 0.05$) than that of (−)-epicatechin. However, an opposite result was found in the liposomal formulations. EGCG showed the highest skin deposition among all catechins after encapsulation with DA liposomes.

3.6. Stability of liposomes

The vesicle size increased when 15% ethanol was present in the vehicle (day 0) as shown in Table 5. The size of liposomes without ethanol had greatly increased to 9.56-fold after 35 days. On the other hand, the presence of ethanol moderately decreased the size after storage. The liposomes without ethanol produced a larger increment in the zeta potential than those with ethanol after 35 days.

3.7. In vitro TEWL examination

The TEWL values of skin treated by 15% ethanol and F2 were 8.73 ± 0.75 and 9.87 ± 1.69 g/m²/h, respectively, which were comparable to the non-treated group (12.00 ± 2.31 g/m²/h, $p > 0.05$).

Table 4
In vitro and in vivo permeation data of (+)-catechin, (−)-epicatechin, and EGCG from aqueous solutions and liposomes

Code	Drug	Flux (nmol/cm^2/h)	In vitro skin deposition (nmol/g)	In vivo skin deposition (nmol/gx10^1)
Solution[a]	(+)-Catechin	1.57 ± 0.76	1.38 ± 0.57	2.43 ± 1.23
DA[b]	(+)-Catechin	7.48 ± 1.97	0.31 ± 0.18	0.76 ± 0.38
Solution	(−)-Epicatechin	2.27 ± 0.72	0.50 ± 0.15	0.24 ± 0.03
DA	(−)-Epicatechin	12.26 ± 6.98	0.80 ± 0.30	0.21 ± 0.03
Solution	EGCG	0	1.68 ± 0.36	2.37 ± 0.21
DA	EGCG	0.17 ± 0.04	1.78 ± 0.19	3.60 ± 0.25

Each value represents the mean ± S.D. (n = 4).
[a] The vehicle of solution means drug dissolved in a hydroalcoholic solution (15% ethanol).
[b] DA means the liposomes composed of EPC + CH + DA = 4:1:0.25.

Table 5
Vesicle size and zeta potential of DA liposomes with or without 15% EtOH before and after incubation at 40 °C for 35 days

Property	Day	DA liposomes[a]	DA liposomes without 15% EtOH
Size (nm)	0	378.2 ± 10.9	157.4 ± 2.9
	35	216.2 ± 3.8	1506.3 ± 290.5
Zeta potential (mV)	0	−26.2 ± 0.9	−18.3 ± 1.1
	35	−43.2 ± 1.1	−61.4 ± 2.7

Each value represents the mean ± S.D. (n = 3).
[a] DA means the liposomes composed of EPC + CH + DA = 4:1:0.25.

4. Discussion

(+)-Catechin encapsulation was higher for liposomes with DA or DP than the others. Drug leakage often involves the aggregation of liposomal membranes (Fang et al., 1997). The high surface potentials of DA and DP liposomes tended to increase the interbilayer distance owing to electrostatic repulsion (Yamaguchi, 1996). The entrapment of liposomes without CH was the highest among all formulations tested. The inclusion of CH in liposomal bilayers results in a rigid structure and slight leakage of contents from vesicle (Nagayasu et al., 1999). The encapsulation of (+)-catechin and (−)-epicatechin indicates that the probability of isomers, which have the same MW but different steric conformation, incorporated into the aqueous core inside vesicles was similar. EGCG contains a galloyl group, which is absent from the other catechins. The n-octanol/water partition coefficient indicates the greater lipophilicity of EGCG (16.0 ± 0.2) (Hashimoto et al., 1999). Hence it is possible that EGCG was strongly located within the bilayers thus increasing the entrapment.

Incorporation of liposomes to (+)-catechin significantly reduced the release rate as compared to the hydroalcoholic solution. This may indicate that (+)-catechin did not diffuse free when entrapping in the vesicles. The (+)-catechin release from the hydroalcoholic solution at different doses of 0.1–0.5% (w/v) was examined. A linear relationship between the dose and release rate was determined, revealing an equation of the release rate of 7.25 + 306.01 × drug dose (r = 0.97). Since the liposomal suspension contained both entrapped and non-entrapped drug molecules, the true release of (+)-catechin encapsulated in liposomes was calibrated by excluding the percentage of free drug. Except for basic liposomes showed a calibrated release rate of +15.48 μg/cm^2/h, all other liposome formulations exhibited negative values of drug release in the entrapped form. This suggests the slower release of non-entrapped molecules in the presence of vesicles. A possible reason may be the reduction of thermodynamic activity in the presence of liposomal vesicles. This mechanism can be partly confirmed by the skin permeation of (+)-catechin in the presence of 4% EPC (Fig. 4). EPC aggregates significantly decreased (+)-catechin flux, which may indicate the decrease of thermodynamic activity. Another possibility could be that the distance traveled by the free drug would be increased by the interference of liposomal vesicles.

The release of (+)-catechin was not changed by extruding the liposomes, indicating the same rigidity of bilayers before and after extrusion. The liposomes without CH showed higher (+)-catechin release than the other formulations (Table 2). CH can decrease the fluidity of the bilayers to above the phase transition temperature of EPC (ca. −7 °C) (Yu and Liao, 1996), thus reducing the drug released from the liposomes.

The basic liposomes significantly increased drug permeation as compared to the control and the liposomes without ethanol. The interaction between skin and liposomes themselves may be an important contributory element for this improvement. Ethanol has been shown to influence EPC membranes by increasing the environmental heterogeneity of EPC (Kirjavainen et al., 1999). Thus it seems that ethanol loosens the structure of the EPC bilayers. Elastic vesicles clearly demonstrate superior characteristics of interactions with the skin compared to rigid vesicles, since rigid vesicles do not penetrate the skin very far (Touitou et al., 2000; Honeywell-Nguyen et al., 2002).

The liposomes incorporated DA or DP further enhanced the (+)-catechin flux, probably due to the increased flexibility of the liposomal membranes by the anionic surfactants to carry the drugs into deeper strata (Ogiso et al., 1997; Fang et al., 2005). As noted in Fig. 2, the presence of DA is not the only

reason for increasing the drug permeation, since no enhancement was observed for DA-containing liposomes without ethanol. This may demonstrate that DA exhibits its benefits toward drug permeation in the presence of ethanol. Another reason for facilitating (+)-catechin permeation by the presence of DA or DP may be the negatively charged surface of the liposomes. Since SC possesses negative charges, the negatively charged liposomes would suffer the weak electrostatic repulsion in the intercellular domains, thus ensures the rapid penetration of vesicles (Ogiso et al., 2001).

When the vesicle size of the liposomes is smaller, there is a chance that a number of vesicles can penetrate into deeper layers of the skin (Verma et al., 2003). Our results confirmed the mechanism that vesicles after extrusion can interact in a fixed area of the SC increased. This suggests that the skin permeation may prefer to the liposomes with smaller size. However, we may not expand this trend beyond this present study since the size range of different liposomes used is limited. Further study is needed to explore this mechanism. The selection of optimal formulations and additives is important as well. CH is important as a membrane stabilizer for liposomes to add strength to the bilayers. The exclusion of CH from the bilayers may result in a flexible structure (Nagayasu et al., 1999), thus increasing drug permeation from vesicles.

Phospholipids have a tendency to avoid dry surroundings (xerophobia). When liposomes are applied onto the skin surface non-occlusively, they will partially dehydrate by evaporation. In doing so they penetrate into the more strongly hydrated and deeper skin strata by a transdermal hydration gradient (Van der Bergh et al., 1999; El Maghraby et al., 2001b). As depicted in Fig. 3, the delivery efficiency increased after the open application compared with the occlusive application for liposomes with anionic surfactants. An increment was not observed in liposomes with rather-rigid structures (basic liposomes). These results provide support for the hydration gradient theory.

Some phospholipids can disrupt SC lipids and facilitate transdermal absorption of drugs (Yokomizo, 1996; Kirjavainen et al., 1999). The presence of a certain amount of ethanol is sometimes necessary for the enhancing effect of phospholipids (Valjakka-Koskela et al., 1998; Kirjavainen et al., 1999). These results in Fig. 4 excluded the possibility of skin disruption by EPC as the enhancing mechanism of liposomes. The presence of 4% EPC in the donor without further manipulation decreased (+)-catechin permeation. It is plausible that EPC is not able to penetrate into the skin and thus it forms an extra barrier onto the skin surface.

The permeation of (+)-catechin across SC-stripped skin was similar to that across the cellulose membrane, indicating that the SC is the principal barrier for (+)-catechin. As shown in Table 3, the lower drug flux across delipid skin than SC-stripped skin ($p < 0.05$) from hydroalcoholic solution may suggest both trans- and inter-cellular routes contribute to the (+)-catechin permeation pathways. The comparable fluxes between SC-stripped skin and delipid skin suggests that the enhancing effect of liposomes on (+)-catechin permeation was mainly via the intercellular lipid matrix. In addition to the intercellular lipid bilayers, it was indicated that the follicular pathway contributes to liposomal delivery into deeper skin strata (Ogiso et al., 1997; El Maghraby et al., 2001b). Furry Balb/c mouse skin was used as a permeation barrier since it has a typical and greater number of hair follicles. The enhancement ratios of liposomes over the hydroalcoholic solution of drug permeating across nude mouse skin and furry mouse skin were comparable (4.75-fold versus 4.27-fold). The result indicates a negligible effect of follicular routes on liposome permeation in this case.

The incorporation of liposomes raised the in vitro skin permeation of the three catechins. EGCG was used as an example to compare its permeation in liposomes to the patch form (Batchelder et al., 2004). The permeability coefficient k_p (flux/applied dose) is used for comparison since the EGCG amount used is different between two studies. The k_p of EGCG is 9.88×10^{-6} and 1.76×10^{-6} cm/h from DA liposomes and patch, respectively. This may suggest an enhancing efficiency by liposomes. The in vitro skin uptake of EGCG from liposomes was greater than those of the other catechins. In order to verify the effect of liposomes on skin reservoirs of drugs, the in vivo topical application was also undertaken. The same as for the in vitro data, liposomes reduced (+)-catechin deposition within the skin (Table 4). The discrepancy of the skin reservoir between (+)-catechin and (−)-epicatechin was greater in the in vivo status. This suggests evidence of the selective absorption of (−)-epicatechin over (+)-catechin. The same results have been observed with other biomembranes, i.e., of these two isomers, that (+)-catechin shows more efficient absorption in the gastrointestinal tract (Zhu et al., 2002; Catterall et al., 2003). Since (+)-catechin and (−)-epicatechin showed similar encapsulation and release rates in liposomes, the skin uptake of these isomers from liposomes should be comparable. The lower deposition of (+)-catechin from the liposomal suspension may have been due to the non-entrapped molecules. The vesicles in the vehicle may retard the partitioning of free (+)-catechin from the formulation to the skin. EGCG liposomes showed the highest in vitro and in vivo deposition rates within the skin among the three drugs. Phospholipids in liposomes can mix with the SC lipids, thus creating a lipid-enriched environment (Ogiso et al., 1997). This lipid depot may be preferred to the lipophilic EGCG, resulting in enhanced skin uptake by liposomes.

In the stability test by characterization of the size and zeta potential, it was found that the addition of 15% ethanol to the liposomes increased the vesicle size (day 0). This was probably due to a decrease in interfacial tension (24.7 dyne/cm for DA liposomes versus 31.5 dyne/cm for liposomes without ethanol) or the induction of interdigitation (Kirjavainen et al., 1999). Both formulations showed increased surface potential after storage, which may have been due to the oxidation of EPC, with the liposomes without ethanol producing a larger increment. The instability of vesicle size was detected when no ethanol was present in the formulation. On the other hand, the presence of ethanol moderately decreased the size after storage. This is possible since the destruction of lipid vesicles is not uncommon during storage (Yamaguchi, 1996).

TEWL is performed to assess damage of the SC, and a good correlation between chemical damage to the skin barrier and an increase in TEWL is demonstrated (Zhao and Singh, 2000). The

results in this study suggest that liposomes were insufficient to produce macroscopic disruption of the SC.

5. Conclusions

The liposomal composition is the most important factor affecting the efficiency of transdermal delivery of encapsulated catechins. From the data presented in this study, the advantages of liposomes developed in this study are they provide enhancing efficiency on catechin delivery, limited skin disruption, and good stability. The key for enhancing effect of liposomes may be due to the flexibility of the bilayers. Skin pretreatments with both EPC and EPC directly dispersed in vehicles were demonstrated to be valueless for enhancing drug permeation. It was suggested that liposomes must be applied concurrently with drugs to achieve a positive effect. (+)-Catechin and (−)-epicatechin entrapped in liposomes showed similar encapsulation levels, release rates, and skin permeation. However, liposomes significantly reduced the skin deposition of (+)-catechin but not (−)-epicatechin. EGCG showed the highest level in encapsulation and skin deposition among the three catechins, which may be because of its lipophilic characteristic.

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