Original Article

การศึกษากลไกเพิ่มการซึมผ่านผิวหนังของลิโมนีนบรรจุในไลโปโซมที่กักเก็บสารที่ชอบน้ำ

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บทคัดย่อ

บทนำ: สารเพิ่มการซึมผ่านที่บรรจุในถุงอนุภาคไลโปโซมได้มีการรายงานว่าเป็นตัวพาสำหรับนำส่งตัวยาผ่านผิวหนัง วัตถุประสงค์ของการศึกษานี้จึงเพื่อศึกษากลไกการเพิ่มการซึมผ่านของลิโมนีนที่บรรจุในไลโปโซม (LI) ต่อการนำส่งสารที่ชอบน้ำผ่านผิวหนัง **วิธีดำเนินการวิจัย:** โซเดียมฟลูออเรสซีน (NaFl) ถูกใช้เป็นโมเดลสารที่ชอบน้ำสำหรับบรรจุลงในถุงอนุภาคไลโปโซม จากนั้นทำการประเมินคุณสมบัติทางเคมีกายภาคของสูตรตำรับไลโปโซม และศึกษาการซึมผ่านผิวหนังภายนอกร่างกาย โดยใช้เซลล์ศึกษาการซึมผ่านของฟรานซ์ โดยกล้องจุลทรรศน์อิเล็กตรอนแบบส่องผ่าน (TEM) จะถูกนำมาใช้เพื่อดูภาพอนุภาคไลโปโซมที่ซึมผ่านเข้าสู่ของเหลวส่วนล่าง ส่วนเครื่องฟลูเรียร์ทรานส์ฟอร์มอินฟราเรดสเปคโทรมิเตอร์ (FTIR) นำมาใช้เพื่อวัดการเปลี่ยนแปลงของการจัดเรียงโครงสร้างของไขมันระหว่างเซลล์ของสตราตัมคอร์เนียม **ผลการวิจัย:** คุณสมบัติทางเคมีกายภาพของ LI แสดงขนาดอนุภาคเล็ก (40.59 นาโนเมตร) ความสามารถในการกักเก็บยาสูง และมีความยืดหยุ่นของผนังกั้นสูงกว่าไลโปโซมที่ไม่มีลิโมนีน โดยการซึมผ่านผิวหนังของ NaFl ที่กักเก็บอยู่ใน LI พบค่าสูงกว่าสูตรตำรับที่ NaFl ไม่ได้กักเก็บใน LI, NaFl ที่กักเก็บในไลโปโซมที่ไม่มีลิโมนีน, และสารละลายของ NaFl ตามลำดับ ซึ่งภาพจาก TEM แสดงถุงอนุภาคของ LI ที่สามารถซึมผ่านผิวหนังเข้าสู่ของเหลวส่วนล่างได้ ส่วนผล FTIR ของสตราตัมคอร์เนียมที่ได้รับ LI แสดงการจัดเรียงโครงสร้างของไขมันที่อยู่ระหว่างเซลล์ของตัวกั้นสตราตัมคอร์เนียมที่หลวมขึ้นอีกด้วย **สรุปผลการวิจัย:** การเพิ่มการซึมผ่านผิวหนังของลิโมนีนที่บรรจุในไลโปโซมอาจเป็นผลมาจากการที่ LI เป็นตัวพาที่นำส่งตัวยาที่ถูกกักเก็บผ่านผิวหนัง นอกจากนี้ ลิโมนีน (เป็นสารเพิ่มการซึมผ่าน) ส่งผลต่อการเปลี่ยนแปลงในส่วนของตัวกั้นของสตราตัมคอร์เนียมเป็นอย่างมาก นำไปสู่การเพิ่มการซึมผ่านผิวหนังของสารที่ชอบน้ำ

คำสำคัญ: สารเพิ่มการซึมผ่าน, ไลโปโซม, สารที่ชอบน้ำ, กลไกการซึมผ่านผิวหนัง

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Investigation of skin penetration enhancing mechanism of limonene-containing liposome entrapped hydrophilic compound

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Abstract

Introduction: Penetration enhancer-containing liposome vesicles have been reported as a carriers for transdermal delivery of entrapped drug. The aim of this study was to investigate the penetration enhancing mechanism of limonene-containing liposomes (LI) on the transdermal delivery of hydrophilic compound. **Methods:** NaFI was used as a model hydrophilic compound for loading into liposome vesicles. The physicochemical characteristics of liposome formulations were evaluated. The *in vitro* skin penetration study was performed by using Franz diffusion cells. Transmission electron microscopy (TEM) was used to observe the penetrated vesicles in the receiver medium. Fourier transform infrared spectroscopy (FTIR) was used to determine the modification of intercellular lipid organization of stratum corneum. **Results:** The physicochemical properties of LI showed smaller vesicle size (40.59 nm), higher entrapment efficiency, and higher membrane fluidity than liposomes without limonene. The skin penetration of NaFI-entrapped in LI was higher than non NaFI-entrapped in LI, NaFI-entrapped in liposomes without limonene, and NaFI solution, respectively. TEM image showed LI vesicles penetrated through skin into the receiver compartment. FTIR result of LI treated stratum corneum also exhibited high fluidity of intercellular lipid organization of stratum corneum barrier. **Conclusion:** The skin penetration enhancement of limonene-containing liposomes might result from LI as transdermal carriers to deliver the entrapped drug through skin. Moreover, limonene (as a penetration enhancer) provided the main modification in the stratum corneum barrier, leading to increase of skin penetration of hydrophilic compound.

Keywords: penetration enhancer, liposomes, hydrophilic compound, skin penetration mechanism
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Introduction

Penetration enhancer-containing vesicles have been used as carriers for dermal delivery of various drugs, such as minoxidil (Mura et al., 2009), diclofenac (Manconi et al., 2011), and temoporfin (Dragicevic-Curic et al., 2008). The advantage of these vesicles results from the combination of liposome potential as carriers and the ability of penetration enhancer to modify the order of stratum corneum packing, thus promoting skin delivery. Several lipophilic and hydrophilic penetration enhancers (i.e. labrasol, transcutol and cineole) were used to formulate penetration enhancer–containing vesicles (Mura et al., 2009). However, the mechanisms of penetration enhancements of each vesicle formulation have not been cleared yet.

The aim of this study was to investigate the penetration enhancing mechanisms of limonene-containing liposomes (LI) as a transdermal carrier of hydrophilic compound, Sodium fluorescein (NaFl). The physicochemical characteristics of formulations were evaluated. The *in vitro* skin penetration study of NaFl entrapped and non-entrapped in LI was performed by Franz diffusion cells. Transmission electron microscopy (TEM) was used to observe the vesicles in receiver compartment. Moreover, Fourier transform infrared spectroscopy (FTIR) was used to determine the modification of intercellular lipid organization.

Materials and Methods

Materials

Egg phosphatidylcholine (PC) and Na-salt N-(carbonyl-methoxypolyethylen glycol-2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (PEG2000-DSPE) were a gift from Lipoid GmbH, Ludwigshafen, Germany. Cholesterol (Chol) was purchased from Carlo Erba Reagent, Ronado, Italy. Tween 20 was purchased from Ajax Finechem, Auckland, New Zealand. NaFl and limonene were purchased from Sigma-Aldrich, MO, USA. Triton® X-100 was purchased from Amresco®, Solon, Ohio, USA. 1,6-Diphenyl-1,3,5-hexatriene (DPH) was purchased from Aldrich Chemical Co., Milwaukee, WI, USA. Trypsin EDTA was obtained from Gibco®, NY, USA.

Preparation of liposome formulations

The liposome formulations containing PC, Chol, and PEG2000-DSPE in the molar ratio of 10:2:0.12 mM were prepared using thin film hydration and sonication method. Briefly, a mixture of lipid components in chloroform/methanol (2:1, v/v) was evaporated using a N_2 gas and completely dry in a desiccator (6 h). Then, 0.2% (w/v) of NaFl in phosphate-buffered saline (PBS pH 7.4) was added into the lipid film to hydrate and disperse the liposome vesicles to obtain the final volume of 100 ml. D-limonene (dissolved in 2% (w/v) of Tween 20) was then added into the dispersion at 1% concentration. Probe-sonicated was performed under ice-bath for 30 min to reduce the size of the vesicles. An excess lipid composition was removed by using centrifugation at 15,000 rpm at 4 °C for 15 min.

For non NaFl-entrapped in LI, the blank LI was prepared in the same method of NaFl-entrapped in LI. After centrifugation, the same amount of NaFl was weighed and added into the blank LI vesicles by physical mixing.

Characterization of liposome formulations

Particle size was measured using a Dynamic Light Scattering particle size analyzer (Zetasizer Nano-ZS, Malvern Instrument, Worcestershire, UK) with a 4 mW He-Ne laser at a scattering angle of 173°.

For entrapment efficiency of NaFl, the 0.5 mL of liposome dispersion was placed in an ultrafiltration tube with a molecular weight cutoff of 3,000 Da (Microcon YM-3; Minipore, Billerica, Massachusetts, USA) and centrifuged at 4 °C at 10,000 xg for 60 minutes. The filtrate was discarded, and 0.25 mL of PBS was added to the retentate before further centrifugation at 4 °C at 10,000 xg for 40 minutes. The collected NaFl-loaded liposomes were then disrupted with 0.2 mL of 0.1% (w/v) Triton X-100 and centrifuged at 4 °C at 10,000 xg for 10 minutes. The NaFl content was determined by fluorescence analysis. The percent drug entrapment efficiency (%EE) was calculated with the following:

$$\%EE = (C/C_i) \times 100 \qquad \text{Eq.1}$$

where C is the concentration of NaFl in the liposomal formulation, and C_i is the initial concentration of NaFl added.

To evaluate the fluidity of liposome membrane, fluorescence anisotropy was used to monitor DPH molecule in the hydrophobic regions of the lipid bilayer. Liposome vesicles were incubated with 10 mM DPH stock solution (10 µl: 1000 µl of liposome dispersion) at room temperature for 2 h in the dark. The samples were measured the fluorescence anisotropy at an excitation wavelength of 350 nm and an emission wavelength 431 nm. (Hitachi F-450, Hitachi Co., Ltd., Tokyo, Japan). Temperature was controlled between 20 °C and 35 °C by a circulating water bath.

***In vitro* skin penetration study**

Abdominal porcine skin was taken from intrapartum stillborn animals from a farm in Nakhon Pathom. Subcutaneous fat was carefully removed using medical scissors and surgical blades. The skin thickness was approximately 0.6 - 0.7 mm.

The skin penetration of NaFl was performed using Franz diffusion cells. Briefly, approximately 2 mL of formulation was applied to the skin in the donor compartment. The receiver compartment of the cell was filled with 6 mL of PBS at 32 °C. Samples from receiver medium were taken at predetermined times (1, 2, 4, 6, 8, and 24 h) for analysis using fluorescence detection, and the same volume of PBS was added to the receiver compartment to maintain a constant volume. Each sample was analyzed in triplicate. The steady-state flux was determined from the slope of the linear portion of the plot of cumulative amount against time.

Fluorescence analysis

The NaFl concentration was analyzed using a fluorescence spectrophotometer (FusionTM Universal Microplate Analyzer, Packard Instrument Company, Inc., Downers Grove, Illinois, USA). The excitation wavelength was 485 nm, and the emission wavelength was 535 nm.

TEM study

The receiver medium at 4 h after skin penetration was diluted with an appropriate amount of distilled water, placed in a sonicator bath for 10 minutes, and then dropped onto a formvar-coated copper grid. The sample was observed using transmission electron microscopy (JEM 1230, JEOL Ltd, Tokyo, Japan) at 80 kV for particle size and shape measurements.

FTIR study

The stratum corneum layer of hairless mice skin was separated from the epidermis using

0.1% trypsin solution. The formulations were applied on the stratum corneum sheets at 32 °C for 4 h. The treated stratum corneum was rinsed with distilled water, dried, and investigated by FTIR spectrophotometer (FT/IR-4200; JASCO International Co., Ltd. Tokyo, Japan) between 3000 and 2800 cm^{-1}. The spectrum was collected in transmission mode. The temperature was collected between 20 °C and 100 °C (heating rate of 1 °C/min) to detect phase transitions.

Statistical analysis

All experimental measurements were collected in triplicate. The values are expressed as the mean and standard deviation (S.D.). Statistical significance was analyzed using one-way ANOVA followed by Turkey test. The significance level was set at $p < 0.05$.

Results

Physicochemical characterization

The particle size of LI was 40.59 ± 1.08 nm, which was significantly smaller than liposomes without limonene (78.92 ± 1.10 nm). Moreover, the %EE of LI was 30.70 ± 1.74 %, while liposomes without limonene was 21.24 ± 1.42 %. For non NaFI-entrapped in LI, the particle size was the same with NaFI-entrapped in LI, while the %EE was lower than other formulation (data not shown). For fluorescence anisotropy value, LI was lower value than liposomes without limonene at all temperatures, indicating that LI have a high membrane fluidity (Figure 1). Although tween 20 did not decrease the fluorescence anisotropy of DPH in liposome vesicles (data not shown) due to the localization near the polar head group of the liposome bilayer, the fluidity of the acyl chain near phospholipid bilayer might be increased.

The results suggested that limonene and tween 20 affected the liposomal vesicles. Tween 20 (as an edge activator) can interact with lipid bilayer, leading to reduced vesicle size and formation of pore in bilayer. Thus, the incorporation efficiency of hydrophilic drug in aqueous compartment of vesicles was increased (El Maghraby, Williums, and Berry, 2000, Jain et al., 2003). In addition, edge activators and terpenes can destabilize the lipid bilayers of vesicles and increase the flexibility of the membrane. Especially, d-limonene molecules were localized in the hydrophobic region of phospholipid acyl chain, leading to increased fluidity of phospholipid bilayer vesicles. Moreover, tween20 have been reported to en (Badran, Shazly, and El-Badry, 2012, Subongkot and Ngawirunpat, 2015).

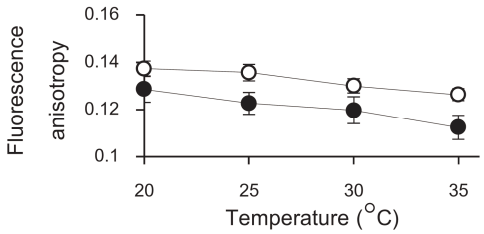

Figure 1 Fluorescence anisotropy of DPH added to liposomes without limonene (o) and LI (●) at the temperature between 20 °C and 35 °C. Results denote the mean ± S.D. (n = 3).

Skin penetration study

As shown in Figure 2, the skin penetrated flux of NaFl-entrapped in LI was significantly higher than non NaFl-entrapped in LI, NaFl-entrapped in liposomes without limonene, and NaFl solution, respectively. This result indicated that liposomes enhanced the transportation of NaFl across skin. However, conventional liposomes are of little or no value for transdermal drug delivery, because these vesicles are inability to deeply penetrate the alteration of the stratum corneum lipid structure (Touitou et al., 2000).

Deformable liposomes were used as a transdermal carriers to enhance the skin permeability. The high elasticity of these vesicles can enter the stratum corneum carring vesicle-bound drug through the skin (Cevc and Blume, 1992, Honeywell-Nguyen and Bouwstra, 2005). Moreover, the small size of the liposomes gave a higher skin penetration of NaFl than did larger ones (Rangsimawong et al., 2014). Therefore, higher NaFl entrapped in LI carriers exhibited the greatest effect to transportation of NaFl across the skin.

Additionally, limonene provided a main enhancing effect on the skin penetration of NaFl from liposome formulation. For liposomes with tween 20 that increased deformability of vesicle bilayer, thus resulting in increased skin permeability of NaFl. However, LI provide significantly higher NaFl permeated through the skin than liposome with tween 20 (data not shown). This suggested that terpene affected the stratum corneum barrier (Rangsimawong et al., 2014). Thus, limonene-containing vesicles led to a higher NaFl penetrate through the skin than those without limonene.

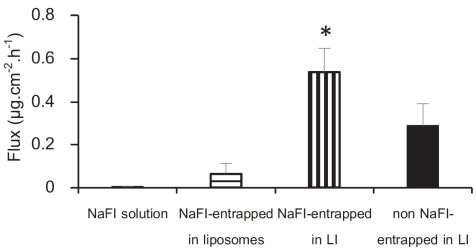

Figure 2 The skin penetrated flux of NaFl from different formulations through porcine skin. Each value represents the mean ± S.D. (n=3). *$p < 0.05$.

To confirm the intact vesicles of LI in the receiver compartment, TEM image was used to observe the receiver medium after skin penetration at 4 h as shown in Figure 3. The spherical in shape and nano-scale sized of vesicles were found in the receiver medium, which these vesicles seem to have the same morphology with LI vesicles in formulation. This suggested that the high deformability of LI vesicles might penetrate through skin into receiver compartment.

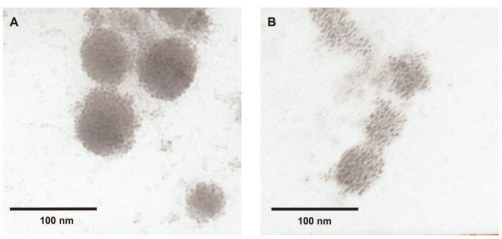

Figure 3 TEM images of LI vesicles in formulation (A) and LI vesicles in the receiver medium (B) after skin penetration at 4 h.

Stratum corneum modification

To investigate the greatest enhancing mechanism of LI on the modifying stratum corneum lipid organization, the asymmetric and symmetric stretching modes of methylene groups (near 2920 cm^{-1} and 2850 cm^{-1}, respectively) were evaluated as shown in Figure 4. The thermal phase transition of the lipid organization in stratum corneum treated with LI showed the blue shift between 20 °C to 100 °C of CH_2 asymmetric and symmetric stretching from 2918.74 cm^{-1} to 2925.97 cm^{-1} and 2849.73 cm^{-1} to 2855.10 cm^{-1}, respectively, indicating that high temperature induced the disordering of the stratum corneum lipid. As shown in Figure 4(B), the different CH_2 symmetric and asymmetric frequencies of LI treated stratum corneum and untreated stratum corneum were calculated. LI treated stratum corneum exhibited the shifting into higher value than untreated stratum corneum, which the highest difference was at 55 °C. This result suggested that LI increased lipid organization change. D-limonene have been reported to disrupt the stratum corneum lipid and create void in the upper epidermal region, leading to enhanced drug penetrated across the skin barrier (Badran, Shazly, and El-Badry, 2012). Therefore, limonene-containing liposomes induced the disorder of lipid lamellar structure of the barrier function, leading to enhancing the skin permeability of NaFI.

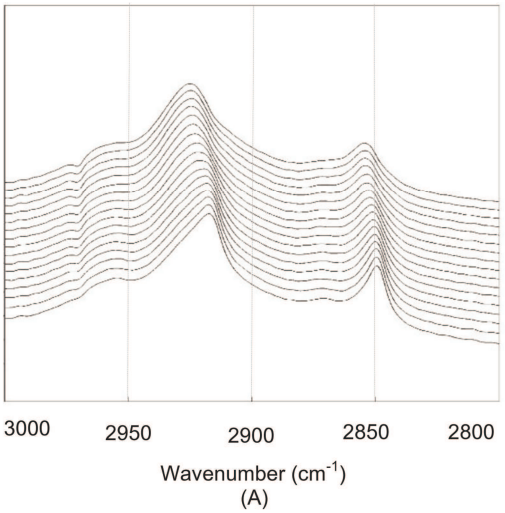

Figure 4 (A) FTIR CH2 asymmetric (near 2920 cm-1) and symmetric (near 2850 cm-1) spectra versus temperature of stratum corneum sheet treated with LI. The temperature was in the range of 20 °C to 100 °C (top). (B) The frequency change of the CH2 asymmetric (■) and symmetric (□) stretching of LI treated stratum corneum from untreated stratum corneum.

Discussions and Conclusion

In this study, limonene-containing liposomes enhanced the skin penetration of hydrophilic compound by using two possible mechanisms. Firstly, the high fluidity and small size of vesicle might act as carriers to deliver the entrapped drug penetrate into and through skin. Secondly, limonene might be the main penetration enhancing effect by modifying the stratum corneum lipid organization, leading to enhance the drug transportation across skin barrier.

Acknowledgments

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