

Enhancement of Skin Penetration of Hydrophilic and Lipophilic Compounds by pH-sensitive Liposomes

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Received, February 24, 2015; Revised, June 18, 2015; Accepted, June 24, 2015; Published, July 4, 2015.

Abstract - PURPOSE: Enhance skin penetration of hydrophilic and lipophilic compounds using liposomes that are responsible to the pH of the skin surface. **METHODS:** pH-sensitive liposomes were prepared by a thin layer and freeze-thaw method with dioleoyl phosphatidyl ethanolamine and cholesteryl hemisuccinate. Liposomal fusion with stratum corneum lipid liposomes was measured using fluorescence resonance energy transfer. Particle diameter and zeta potential of the liposomes after fusion were measured by dynamic light scattering and electrophoresis. **RESULTS:** Under neutral pH conditions, the diameter of the pH-sensitive liposomes was 130 nm and their zeta potential was -70 mV. In weakly acidic conditions, the diameter was larger than 3,000 nm and the zeta potential was -50 mV. In contrast, the particle diameter and the zeta potential of the non-pH-sensitive liposomes remained constant under various pH conditions. A skin penetration study was performed on hairless mice skin using vertical diffusion cells, showing that the fusion ability of pH-sensitive liposomes was higher than that of non-pH-sensitive liposomes. In the skin penetration study was carried out using hydrophilic (calcein) and lipophilic (*N*-(7-nitrobenz-2-oxa-1,3-diazol-4yl)-PE) (NBD-PE) model compounds which were applied to the skin with pH-sensitive liposomes as carrier. The fluorescent compounds contained within the pH-sensitive liposomes permeated the skin more effectively than those within non-pH-sensitive liposomes, and this ability was further enhanced with the lipophilic compound. **CONCLUSION:** These studies suggest that pH-sensitive liposomes have potential as an important tool for delivery of compounds into the skin.

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INTRODUCTION

The skin consists of the outer epidermis and the deeper dermis layer. From the inner layer to the outside, the epidermis consists of the stratum basale, the stratum spinosum, the stratum granulosum, and the stratum corneum (SC). The SC has an organized structure, in which keratinocytes act as "bricks" and intercellular lipids act as a "mortar-like material" (1). The stratum corneum acts as a barrier against many environmental agents, including drugs and active ingredients for cosmetics. Various studies have been conducted to find ways to improve the penetration of drugs and cosmetics (2-4). Generally, the sebum film that exists on the cutaneous surface has moisturizing and protective activities (5). Sebum and sweat coexist as a mixture and form a thin film that is weakly acidic (pH 4.5–6.0) and contains lactic acid in the fatty acids and sweat (6, 7).

Liposomes are lipid bilayer structures formed from phospholipids when the latter, taken from a biological membrane, are added to an aqueous

solution. Because hydrophobic and hydrophilic compounds can be held stably in either the lipid bilayer or an inner phase, liposomes are effective as drug carriers (8-10). Liposomes with various characteristics can be prepared by changing the type of lipids involved (11).

The use of liposomes as a transdermal drug carrier was reported by Mezei and Gulasekharan (12). Additionally, transfersome (13), niosome (14), ethosome (15) were recently reported as carriers that improve transdermal drug delivery. These liposomes increased membrane fluidity and flexibility by modification of the lipids. We proposed that liposomes functioning through a different mechanism than high membrane fluidity and flexibility may be useful as a transdermal drug carrier. We therefore considered a liposomal preparation aimed at a high penetration into the stratum corneum

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intercellular lipids. To enhance skin penetration of the drug, we used liposomes that have a high affinity for the stratum corneum rather than ultradeformable liposomes.

pH-Sensitive liposomes can fuse with the cell membrane in response to pH changes (16-19). Liposomal membranes are stable under neutral conditions but membrane fusion increases under acidic conditions. The sebaceous gland of the skin is weakly acidic so we hypothesize that increased delivery of compounds into the skin will be obtained with these types of liposome.

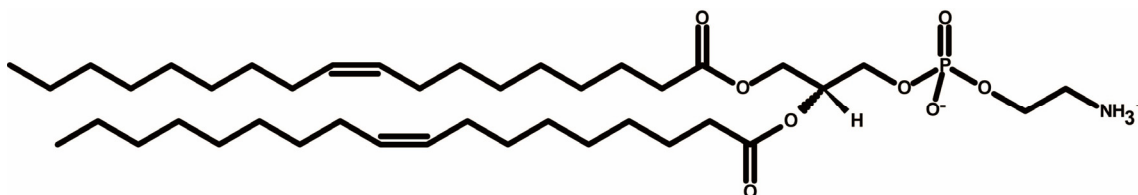
This manuscript presents the first study on the preparation and use of pH-sensitive liposomes for skin penetration. We measured the ability of pH-sensitive liposomes to fuse with the stratum corneum in vitro by fluorescence resonance energy transfer. Additionally, we evaluated the skin penetration enhancement of calcein as a hydrophilic model compound and (*N*-(7-nitrobenz-2-oxa-1,3-diazol-4yl)-PE) (NBD-PE) as a lipophilic model compound, entrapped in pH-sensitive liposomes that had been prepared using L- α -dioleoyl phosphatidyl ethanolamine (DOPE) and cholesteryl hemisuccinate (CHEMS), respectively. The results demonstrate the usefulness of pH-sensitive liposomes for skin penetration.

Materials and Methods

Materials

DOPE was purchased from NOF Corporation (Tokyo, Japan). CHEMS, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4yl)-PE (NBD-PE) and *N*-(lissamine Rhodamine B-sulfonyl)-PE (Rh-PE) were purchased from Avanti Polar Lipid (Alabaster, AL, USA). Ceramide types II and III (CER NS and NP) were purchased from Matreya (Pleasant Gap, PA, USA). Cholesterol (CHOL), Palmitic acid (PA), and cholesterol sulfate (CS) were purchased from Sigma Aldrich (St. Louis, MO, USA). Calcein (sodium salt) and Tween-80 were purchased from TCI (Tokyo, Japan). Triton X-100 was purchased from Alfa Aesar (Ward Hill, MA, USA). Phosphate buffered saline (PBS) was purchased from Takara Bio Inc. (Otsu, Shiga, Japan). Chloroform and methanol were purchased from Wako Pure Chemical Industries (Osaka, Japan). The chemical structures of DOPE and CHEMS are shown in Figure 1.

DOPE



CHEMS

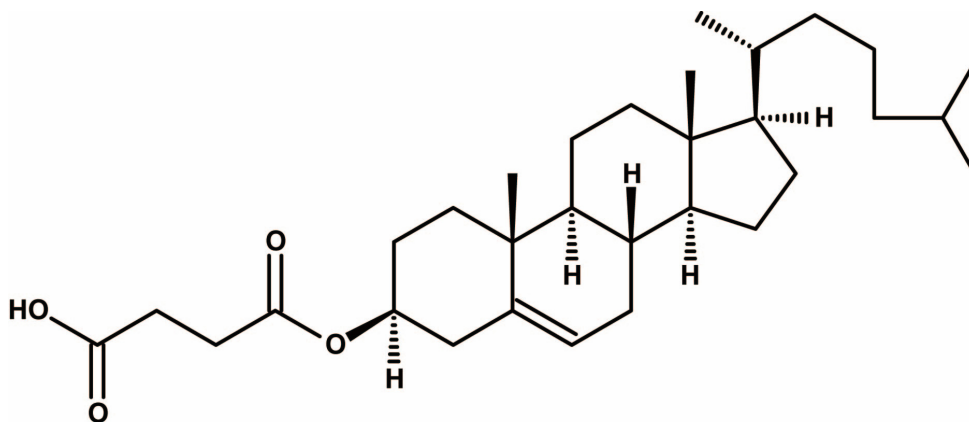


Figure 1. Dioleoyl phosphatidyl ethanolamine (DOPE) and cholesterol hemisuccinate (CHEMS).

Preparation of pH-sensitive and non-pH-sensitive liposomes

pH-sensitive liposomes were composed of DOPE:CHEMS at a molar ratio of 6:4 and non-pH-sensitive liposomes were composed of DOPE:CHOL at a molar ratio of 6:4. The lipids in chloroform were dried in a rotary evaporator and the lipid film was then hydrated with PBS. The liposomes were subjected to freeze-thawing for five cycles using liquid nitrogen, and extruded through a polycarbonate membrane filter with a 100-nm pore size using an extruder (Lipex Biomembranes Inc., Vancouver, British Columbia, Canada).

Calcein entrapped liposomes were hydrated in 10 mM calcein and the pH was adjusted to pH 7.4 with sodium hydroxide. Free calcein was removed by ultracentrifugation (Himac CS 120GX II, HITACHI, Tokyo, Japan) at 113,000×g, at 4°C for 15 min. NBD-PE containing liposomes were hydrated with PBS.

Preparation of SCLL (stratum corneum lipid liposomes)

Stratum corneum lipid liposomes (SCLL) were composed of CER II:CER III:CHOL:PA:CS = 20:20:25:25:10 (w/w %) (20). CER II, CER III, CHOL, and PA were dissolved in chloroform. CS was dissolved in chloroform:methanol (2:1). The lipids in either chloroform or chloroform:methanol (2:1) were dried in a rotary evaporator and the lipid film was then hydrated with PBS (pH 7.4). The liposomes were subjected to freeze-thawing for five cycles using liquid nitrogen and extruded through a polycarbonate membrane filter with a 100-nm pore size using the extruder, as above.

Zeta potential and particle diameter measurements

The zeta potential and particle diameter for pH-sensitive liposomes, non-pH-sensitive liposomes, and for SCLL at pH 5.0, 6.0, and 7.4, respectively, were determined using a Zetasizer Nano-ZS (ZEN3600, Malvern Instruments, Malvern, Worcestershire, UK).

Fluorescence resonance energy transfer measurements

Measurement of the ability of liposomes to fuse to SCLL was performed using fluorescence resonance energy transfer (FRET) (20, 21). Labeled SCLL were prepared with Rh-PE and NBD-PE at a final concentration of 1 mol%. Labeled SCLL (60 μL) and unlabeled liposomes (160 μL) were mixed and the

fluorescence of the donor NBD-PE (ex: 470 nm, em: 535 nm) was followed for 15 min on a SpectraMax M2^c micro plate reader (Molecular Devices, Sunnyvale, CA, USA). At the end of each measurement, the vesicles were disrupted with 20 μL of 10% (w/v) TritonX-100. Lipid mixing as a percentage of infinite probe dilution was determined using the following equation: liposome fusion ability (%) = $(F_t - F_0) / (F_{max} - F_0) \times 100\%$, where F_t is the fluorescent intensity of NBD-PE during the assay, F_0 is the initial value of NBD-PE fluorescence, and F_{max} is the maximum fluorescence possible, given by infinite probe dilution in the presence of Triton X-100.

Animals

HR-1 male hairless mice (body weight, 16-20g) were purchased from Japan SLC Inc. (Hamamatsu, Shizuoka, Japan). They were housed in temperature-controlled rooms (25±2°C) with a 12-h light-dark cycle (07:00-19:00 h), and they were allowed free access to food (M.F. Oriental) and tap water for 1-week. All animal experiments and maintenance were performed under conditions approved by the animal research committee of Josai University.

In vitro penetration study

Hairless mice skin was excised and excess the subcutaneous fat was carefully eliminated. The excised skin was mounted on a vertical diffusion cells (effective penetration area 1.77 cm²). The receiver volume was 5 mL of phosphate buffered saline (PBS) or PBS containing 5% Tween-80 at pH 7.4. at 32 °C.

In vitro skin penetration studies using calcein-containing liposomes

Calcein-containing pH-sensitive liposomes and non-pH-sensitive liposomes were used as donor solutions. The receiver solution was 5.0 mL of PBS. The control was PBS containing calcein. The penetration experiments were performed for 24 h. The skins were then removed from the diffusion cells and washed briefly with purified water. Each skin sample was cut into several pieces and then homogenized in 10 mL methanol. The calcein content in skin was assayed at ex:495 nm, em: 515 nm by a Shimadzu RF-5300 spectrofluorophotometer (Nakagyo-ku, Kyoto, Japan).

In vitro penetration studies using NBD-PE-containing liposomes

NBD-PE contained in either pH-sensitive-liposomes

or non-pH-sensitive liposomes were used as donor solutions. The receiver solution was 5.0 mL of PBS containing 5% Tween-80. Because NBD-PE did not dissolve in water, the control NBD-PE solution was not examined. The penetration experiments were performed for 24 h, after which the skins were removed from the diffusion cells and washed briefly with purified water. Each skin sample was cut into several pieces and then homogenized in 10 mL methanol. The NBD-PE content in the skin was assayed at ex: 470 nm, em: 535 nm by spectrofluorophotometer.

RESULTS

Changes in particle diameter and zeta potential of pH-sensitive and non-pH-sensitive liposomes

Immediately after sizing, the particle diameters of the pH-sensitive and non-pH-sensitive liposomes at pH 7.4 were 130.0 and 134.3 nm, respectively and their zeta potentials were -70.5 and -68.6 mV, respectively. The particle diameters and zeta potentials of the liposomes did not change over the 24 hours (Fig. 2A and B).

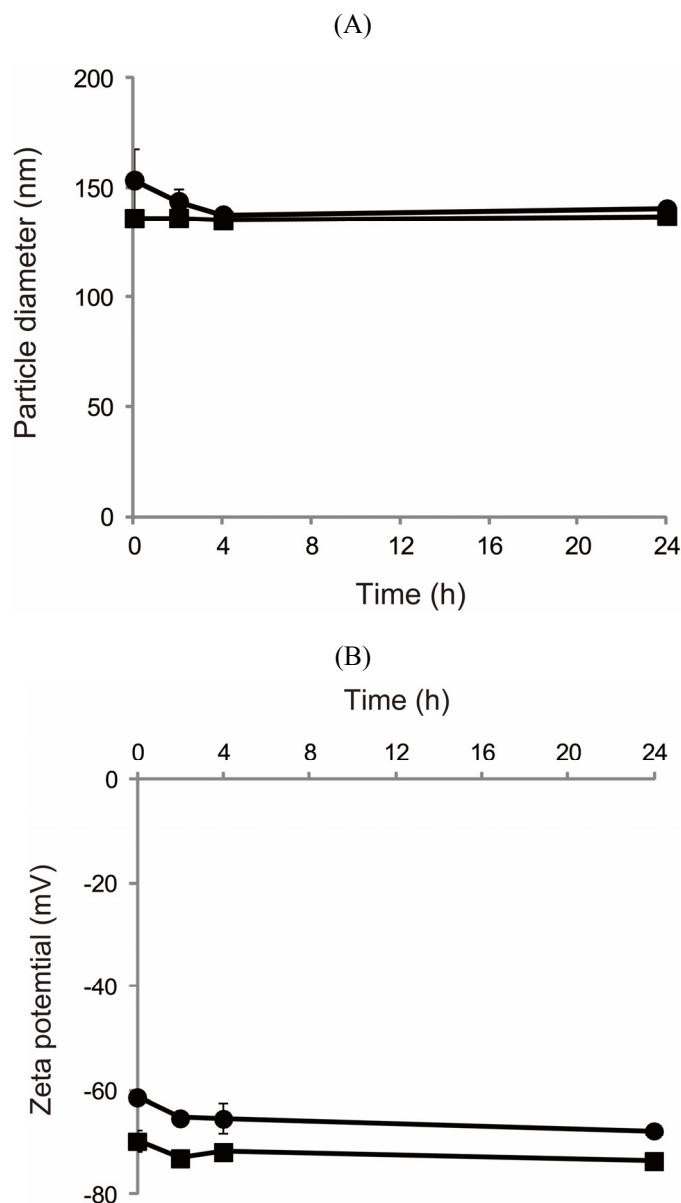


Figure 2. Changes in particle diameter (A) and zeta potential (B) of pH-sensitive (squares) and non-pH-sensitive (circles) liposome at 32°C; mean \pm SD, n=3.

Changes in the particle diameter and zeta potential of the pH-sensitive and non-pH-sensitive liposomes under various pH conditions.

The particle diameters of SCLL and non-pH-sensitive liposomes (DOPE/CHOL) were unchanged in phosphate buffer at pH 5.0, 6.0, and 7.4 (Fig. 3A). Moreover, the diameters of the pH-sensitive liposomes (DOPE/CHEMS) were

unchanged at pH 6.0 and 7.4. However, the particle diameter of pH-sensitive liposomes increased only at pH 5.0. The zeta potentials of the pH-sensitive and non-pH-sensitive liposomes were unchanged at pH 6.0 and 7.4. In contrast, the zeta potential approached neutrality only for the pH-sensitive liposomes at pH 5.0 (Fig. 3B).

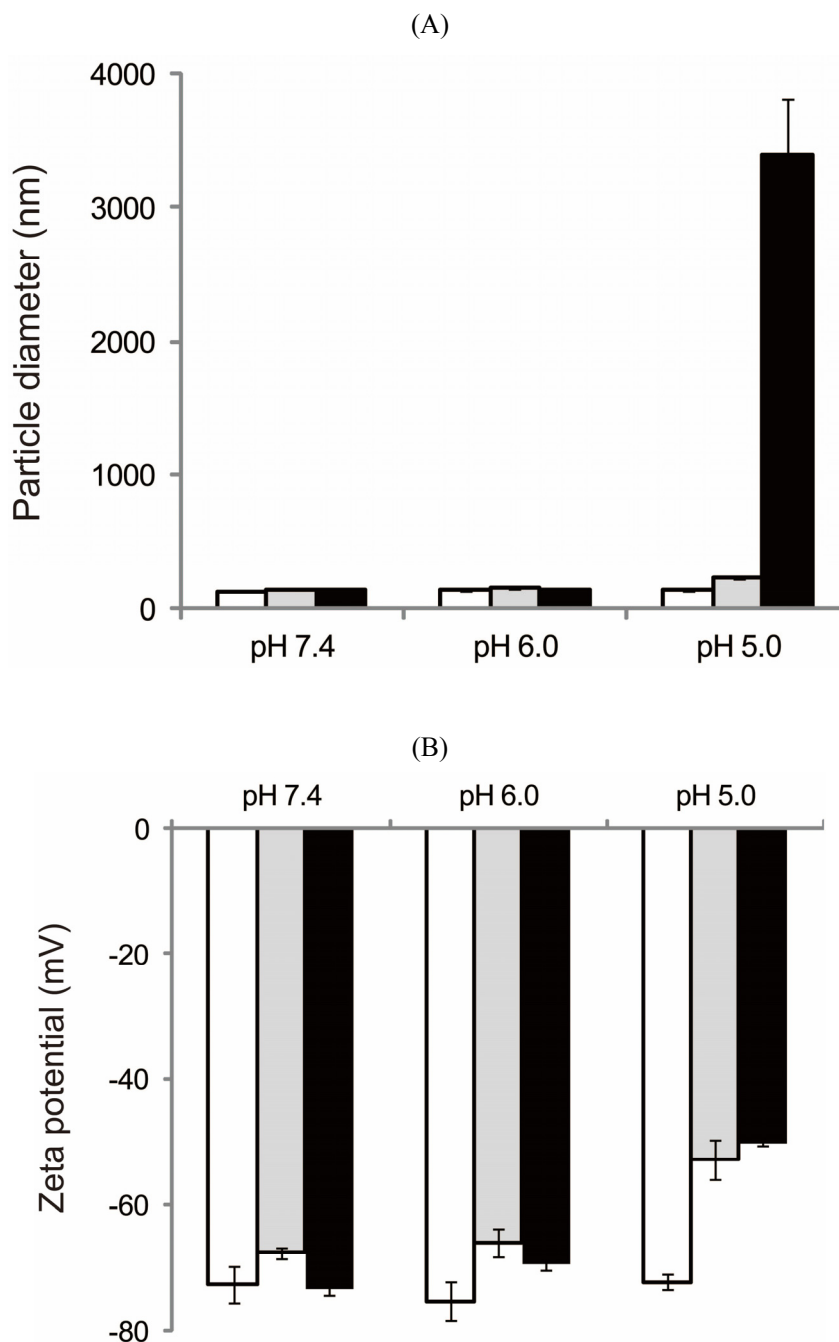


Figure 3. Particle diameter (A) and zeta potential (B) of SCLL (open bars) . non-pH-sensitive liposome (DOPE/CHOL, gray bars) and pH-sensitive liposome (DOPE/CHEMS, filled bars); mean±SD, n=3.

Evaluation of *in vitro* fusion ability of pH-sensitive and non-pH-sensitive liposomes with SCLL at pH 5.0

The abilities of pH-sensitive liposomes and non-pH-sensitive liposomes to fuse with SCLL are shown in Figure 4 as 48.4% and 8.7% at 15 min, respectively. These differences were significant ($p < 0.001$) and demonstrate the potentially important activity of pH-sensitive liposomes on drug delivery into the skin.

In vitro skin penetration

Hydrophilic compound delivery to the skin by pH-sensitive and pH non-pH-sensitive liposomes

The calcein concentration delivered to the skin by calcein encapsulated in pH-sensitive and non-pH-sensitive liposomes did not differ from the control but that delivered by pH-sensitive liposomes was significantly higher ($p < 0.05$, Fig. 5), again showing the potential of the pH-sensitive liposomes for drug delivery into the skin.

Lipophilic compound concentration delivery to the skin by pH-sensitive and non-pH-sensitive liposomes.

The NBD-PE concentration delivered to the skin by the pH-sensitive liposomes was significantly higher than that delivered by non-pH-sensitive liposomes ($p < 0.01$, Fig. 6). The NBD-PE concentrations delivered to the skin by the pH-sensitive and the non-pH-sensitive liposomes were 35.81 and 1.79 pmol/cm², respectively.

DISCUSSION

It was reported recently that ultra-deformable liposomes enhance skin penetration of drugs. Generally, flexible liposomes incorporate surfactants within their membranes and this is a general method for imparting fluidity to liposomal membranes. Instead of using elastic liposomes, we attempted to enhance skin penetration using liposomes with a high affinity for the stratum corneum. This study examined the physical properties and skin penetration activities of hydrophilic and lipophilic drugs entrapped in CHEMS- and DOPE-based pH-sensitive liposomes.

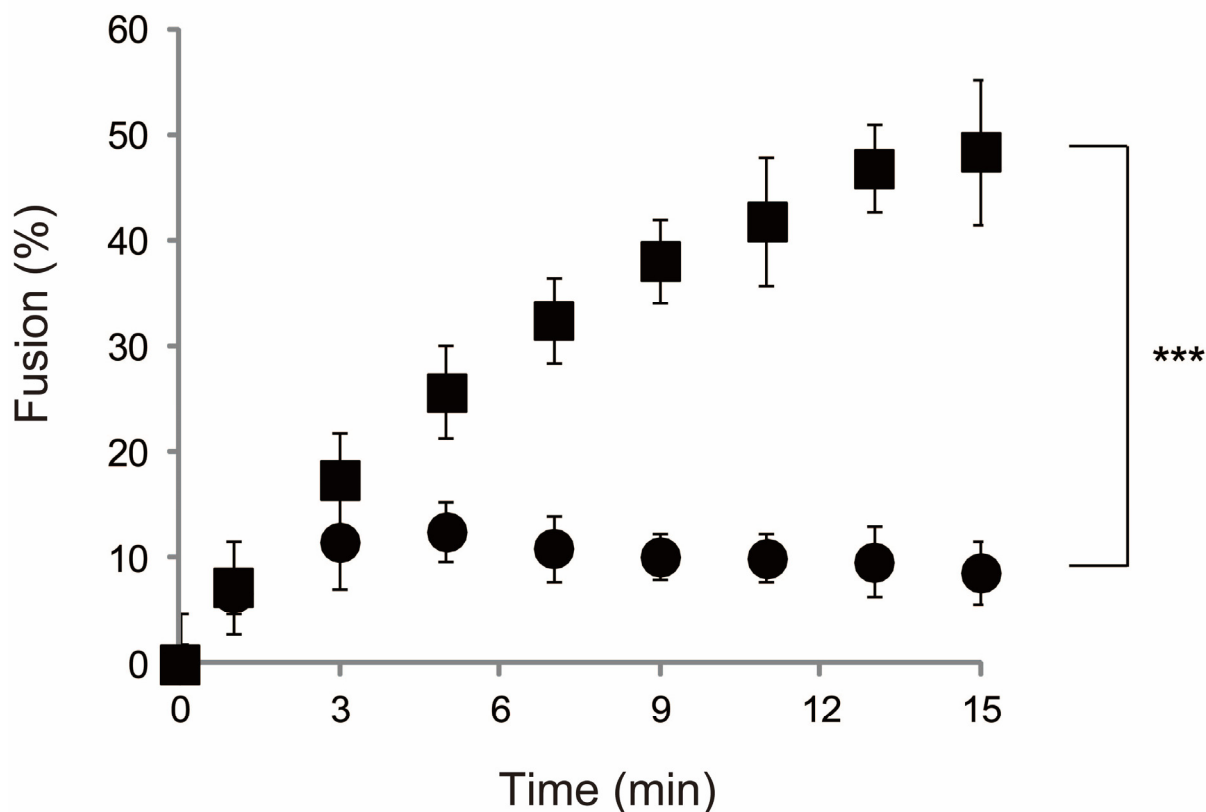


Figure 4. Ability of pH-sensitive (filled square) and non-pH-sensitive liposomes (filled circle) to fuse with stratum corneum liposomes at pH 5.0.

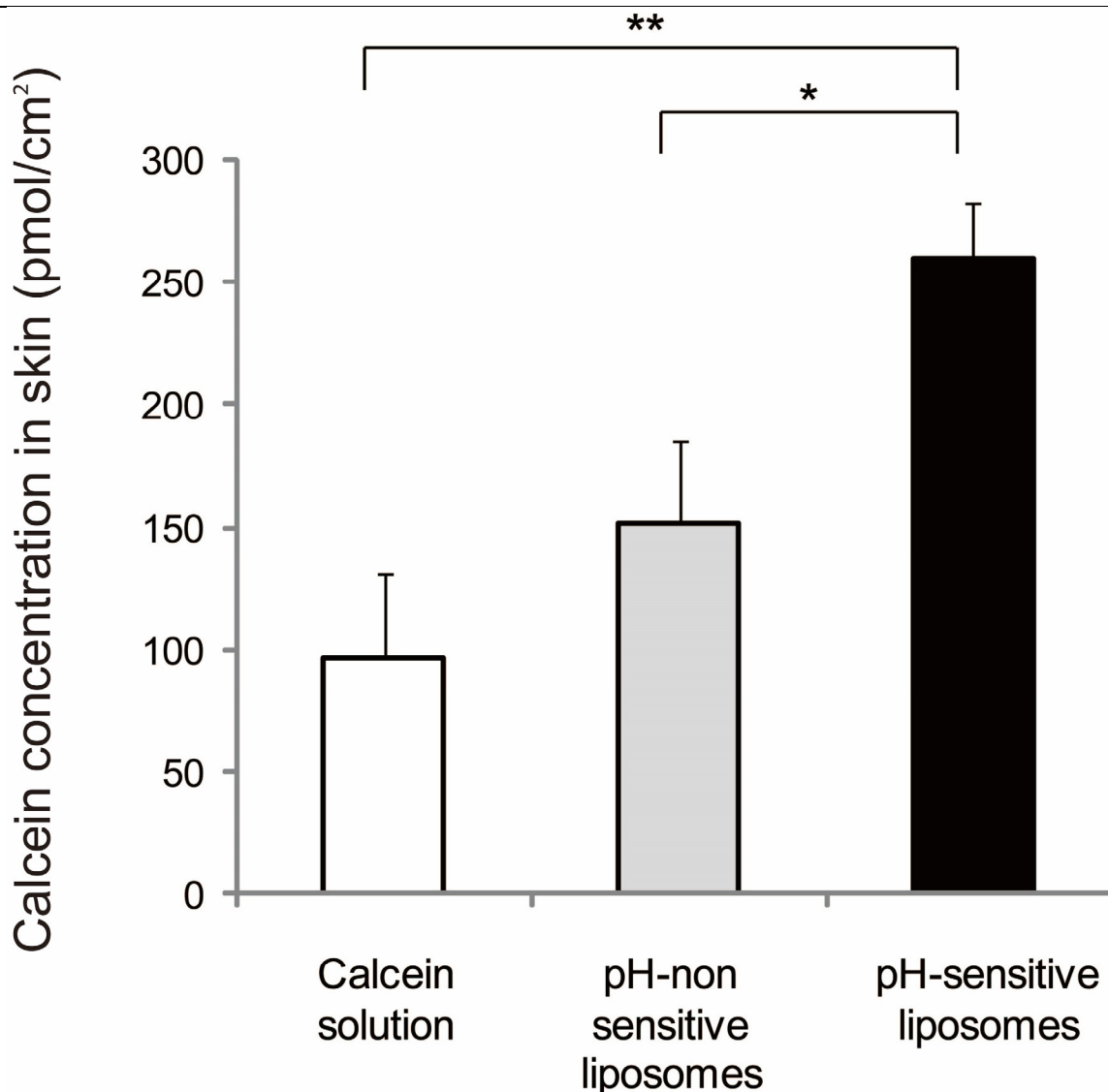


Figure 5. Skin permeation of calcein encapsulated in pH-sensitive (filled bar) and non-pH-sensitive liposomes (gray bar), and by calcein solution alone (open bar). The data represent the average and standard deviation (n=4), respectively. *, p<0.05, **, p<0.01, Tukey's multiple comparison test.

In terms of physical properties, the particle diameters and zeta-potentials of pH-sensitive (DOPE/CHEMS) liposomes and non-pH-sensitive liposomes were constant at pH 6.0 and 7.4. In contrast, the particle diameters changed significantly at pH 5.0, and the zeta potentials were lower for both types of liposomes. The increase in liposome particle diameters is thought to be caused either by fusion of liposomes to one another or by the instability of the liposomes themselves. CHEMS becomes partially protonated under acidic conditions. Therefore, we suggest that the negative charge is lost under acidic conditions and DOPE becomes a reversed micelle

structure because of the loss of repulsion between the polar groups. Therefore, the electric charge reverses, and it is likely that the liposomal zeta potential was inclined towards neutral.

In our study, FRET was used to measure the fusion ability (20, 21). FRET involves excitation energy being transferred directly by electronic resonance between two adjoining fluorescent molecules. When NBD-PE (excitation: 470 nm, emission: 535 nm) and Rh-PE (excitation: 575 nm, emission: 589 nm) coexist in liposomes, this allows most of the fluorescence of NBD-PE to be quenched by the excitation energy transfer.

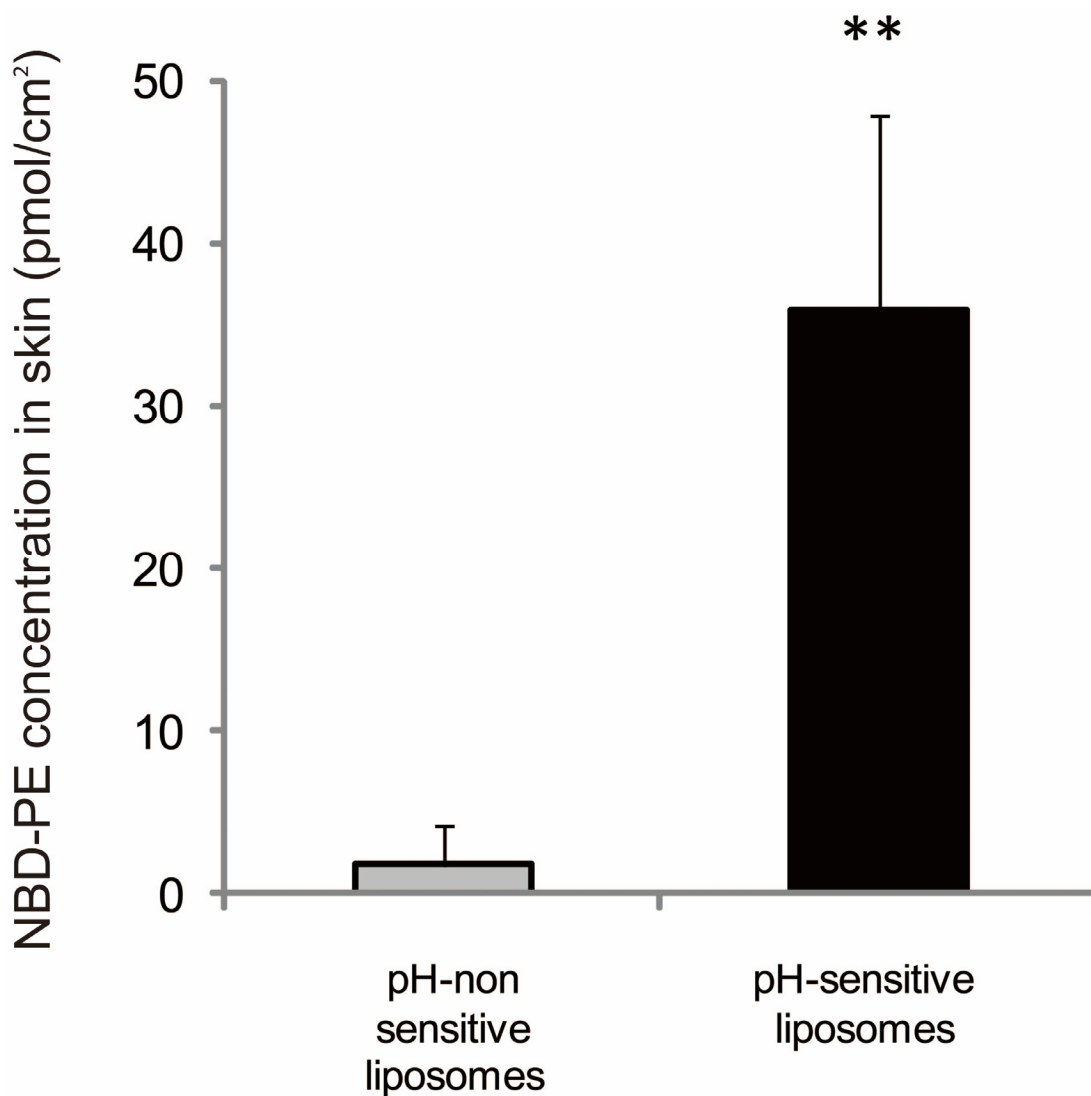


Figure 6. Skin permeation of NBD-PE encapsulated in pH-sensitive (filled bar) and non-pH-sensitive liposomes (gray bar). The data represent the average and standard deviation (n=4), respectively. ***, p<0.001 compared with the non-pH-sensitive liposomes (Student's statistical test).

When two fluorescence compounds are contained in liposomes that fuse with non-labeled liposomes, the concentration of fluorescent probes decreases. Therefore, the fluorescence quenching of NBD-PE can be recovered and the membrane fusion ability can be evaluated by measuring this. pH-sensitive liposomes showed a high fusion activity for SCLL compared with non-pH-sensitive liposomes at pH 5.0. Because pH-sensitive liposomes fuse easily with SCLL under acidic conditions, it was assumed that the skin will also show a high fusion ability. Therefore, an *in vitro* skin penetration study was performed.

Calcein was entrapped as a hydrophilic model drug in pH-sensitive and non-pH-sensitive liposomes. The *in vitro* skin concentration resulting from calcein entrapped in pH-sensitive liposomes was significantly higher than that from non-pH-sensitive liposomes. The *in vitro* skin concentration of NBD-PE as a hydrophilic model compound entrapped in pH-sensitive liposomes was significantly higher than that of non-pH-sensitive liposomes. Under acidic conditions, DOPE is thought to have a reversed micelle structure (22, 23) and to be able to fuse with the membrane. Subsequently, release of the entrapped compound can occur, resulting in enhanced skin penetration.

In conclusion, pH-sensitive liposomes are a unique and important approach to increasing skin penetration and delivery of hydrophilic and lipophilic compounds or drugs.

Declaration of interest: The authors report no financial conflicts of interest. The authors alone were responsible for the content and writing of this paper.

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