Effects of Application Method on Skin Penetration of Carboxyfluorescein Incorporated in Liposomes

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We investigated the skin penetration of liposomes under two different application conditions; occluded and large application amount (1 ml/cm^2), and open and small application amount (10 μl/cm^2). Liposomes containing fluorescence-labeled phospholipids or carboxyfluorescein (CF) were used. In application under occluded conditions, phospholipids showed no penetration, even in the stratum corneum (SC). CF penetration in the skin after application of liposome was no different that after application of CF solution. In contrast, phospholipids penetrated the skin, particularly the SC and hair follicles, under open conditions. CF in liposome showed enhanced penetration in the SC and epidermis, but not in the dermis. On observation of the drying process, CF recrystallized from solution, but this did not occur with CF incorporated into liposome. It is possible that crystallization of CF is prevented by encapsulation in liposome, or that penetration occurs more readily with liposome.

Key words liposome; skin penetration; finite; infinite; carboxyfluorescein

Liposomes are lipid vesicles, and their potential for transdermal delivery of drugs has been investigated by many researchers.[1,2] However, the enhancement effects and mechanisms of action differ among the reports. Liposomes do not represent a mono-phase formulation; thus, the conditions of the formulation change due to penetration into the skin or evaporation of volatile components. These changes depend on the amount of liposome applied and occlusion conditions. Many studies have employed non-occluded conditions, but the application amount was varied. The amounts of formulation per square centimeter have ranged from 30 μl[3,4] to 1.2 ml.[5] These differences may be related to variations in the observed effects of liposomes, but there have been few reports aiming to clarify the effects of dose on liposome penetration.

In this study, we investigated the skin penetration of liposomes under two different application conditions; occluded and large application amount (1 ml/cm^2) (occluded condition), and open and small application amount (10 μl/cm^2) (open condition) to clarify the effect of liposome condition applied on the skin. Under occluded condition, the formulation is not changed when the sample is applied in excess amounts and evaporation is prevented. The shape of liposomes should remain in the donor phase. On the other hand, the amount of topically applied formulation is usually small and the application site is usually non-occluded. In the case of liposomes, there is a possibility of destruction or fusion, and this would affect the penetration of encapsulated drugs. We used carboxyfluorescein (CF) as a model hydrophilic drug.

Experimental

Materials The liposomes were unilamella and contained 5% lipid (4% hydrogenated soybean phospholipids and 1% cholesterol), 3% ethanol, 0.015% citric acid tri-sodium salt and purified water. Liposome A contained 0.45 ng/ml dipalmitoylphosphatidylethanolamine labeled with nitrobenzoxadiazole (DPPE-NBD). Liposome B prepared with CF, dialyzed before skin permeation study to remove CF not incorporated into liposomes. CF incorporated in the liposome was 24—25 μg/ml, ratio of incorporation was 48—50%. In the case of Liposome C, the same volume of liposome, in which lipid content was 10%, and 50 μg/ml CF solution were mixed, so that CF was not incorporated into liposomes.

Average size of liposome A, B and C determined using a dynamic light scattering spectrophotometer (DLS-8000HL, Otsuka Electronics, Osaka, Japan) were 136, 122 and 143 nm, respectively.

Skin Penetration Studies Skin penetration studies were carried out under two conditions; occluded and open condition.

Yucatan micropig (YMP) skin sets frozen at −80 °C were purchased from Charles River, Japan, Inc. (Kanagawa, Japan). Skin was thawed at room temperature for approximately 30 min, followed by removal of the adhering fat layer using scissors and a grater, and cutting into appropriate sizes. Skin penetration was measured in a modified Franz-type diffusion cell apparatus (effective area, 1.1 cm^2; receptor, 16 ml isotonic phosphate buffered solution (PBS, pH 7.1) with 0.001% of kanamycin maintained at 37 °C mixed with a star-head magnet at 600 rpm).

In the case of occluded conditions, skin was mounted on the cell directly, a 1.0-ml aliquot of test formulation was poured into the donor phase, and the donor phase was occluded. At predetermined times, 200-μl aliquots were withdrawn from the receptor compartment. The same volume of fresh solution was added to the receptor compartment after withdrawal in order to maintain constant volume. At 24 h after application, skin was removed from cell, washed with purified water, wiped and used for further determination.

In the case of open conditions, test formulation was spread on the skin at 10 μl/cm^2, and skin was mounted on the cell. The donor phase was not occluded. At 24 h after application, skin was removed from the cell and used for further determination without washing.

Confocal Laser Scanning Microscopy (CLSM) Skin pieces were divided into three. One piece was stripped 10 times with adhesive tape (Scotch™ 665-3-18m 3M) mounted on slide glasses. The other pieces of skin were frozen at −80 °C after embedding in optimal cutting temperature (OTC) compound. Frozen skin was cross-sectioned at 20 μm, and observed by CLSM (FLUOVIEW FV-300, OLYNPUS, Tokyo, Japan) a using 488 Ar laser.

Determination of CF in Skin Skin was stripped 10 times with adhesive tape (Scotch CC1820-Bx-J, 3M), which was then soaked in a methanol and PBS (1 : 2) mixture. The CF collected from the 1st and 2nd strips of tape was considered to be 'on the surface', and that from 3rd—10th strips was considered to be from the 'stratum corneum (SC).' The skin was then separated into the epidermis and dermis by the heat separation method.[6] Methanol and PBS (1 : 2) mixture was added to each part, the epidermis and dermis were homogenized, centrifuged at 3000 rpm for 5 min, and the supernatant was filtered with a membrane filter (0.45 μm for epidermis and 0.20 μm for dermis). CF concentrations in obtained solutions were determined using a fluorospectrometer (Fluoroskan Ascent FL, Labsystem) with at 485 nm excitation and 538 nm emission.

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Results and Discussion

We studied the penetration of liposomes alone by Liposome A, as well as the penetration enhancement of hydrophilic drugs by using Liposome B.

Figure 1 shows CLSM images of the 5th strip of SC and a cross-section of YMP skin at 24 h after application of liposome A. No fluorescence was observed in the SC after application under occluded conditions. In the cross-section, there was slight fluorescence in the outer layer of SC, and there was a reduced possibility of penetration of liposome phospholipids under occluded conditions.

In the case of open condition, fluorescence was observed in the 5th strip of SC. On the cross-section, there was fluorescence on and in the SC. The liposomes on the skin were fused, and they had diameters of *ca.* 2 μm. Some follicles also showed fluorescence in deep areas along the hair; however, some showed no fluorescence. This might be because liposomes applied to the skin aggregate during the drying process, and are drawn into hair follicles by capillary action. Although there was no evidence of the intact liposomes themselves penetrating the skin, labeled phospholipids penetrated into the skin under open conditions.

Penetration of CF encapsulated in liposomes was compared with that of aqueous CF solution. Liposome size and free fraction of CF (about 100 ng) were almost the same before and after application. Figure 2 shows the amount of CF in the skin. There were no differences among the CF solution and Liposome B with regard to concentration in the SC, epidermis and dermis under occluded condition. CF was detected at 0—2 ng in the receptor phase at 24 h after application, regardless of formulation. The penetration ratio relative to the amount applied was less than 0.2%. Thus, liposomes showed no enhancement effect on penetration of CF under occluded conditions.

Under open conditions, liposomes were applied at 10 μl/cm^2, which included a total of 250 ng of CF. Recovery of CF, including at the skin surface, was about 80%. CF amount in SC after application of liposome B was about 33% of the dose, and was significantly higher than after application of solution. The amount in epidermis was also significantly higher. There were no differences in dermis concentration among the formulations.

Despite the low amount of CF applied when compared with occluded conditions, CF amounts in SC and in epidermis were higher. In contrast, the amount in dermis was lower. It is possible that the CF partition coefficient improved because of the partition of phospholipids in the SC and epidermis under open conditions.

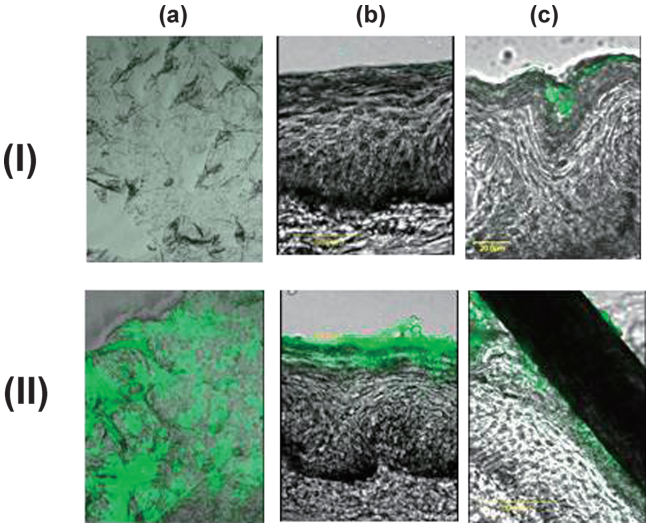

Fig. 1. Fluorescence of NBD-DPPE Observed by CSLM in 5th Strip of Stratum Corneum (a), Cross-Section of Skin (b), and around Hair Follicle (c) at 24 h after Application of Liposome A under Occluded (I) or Open (II) Conditions

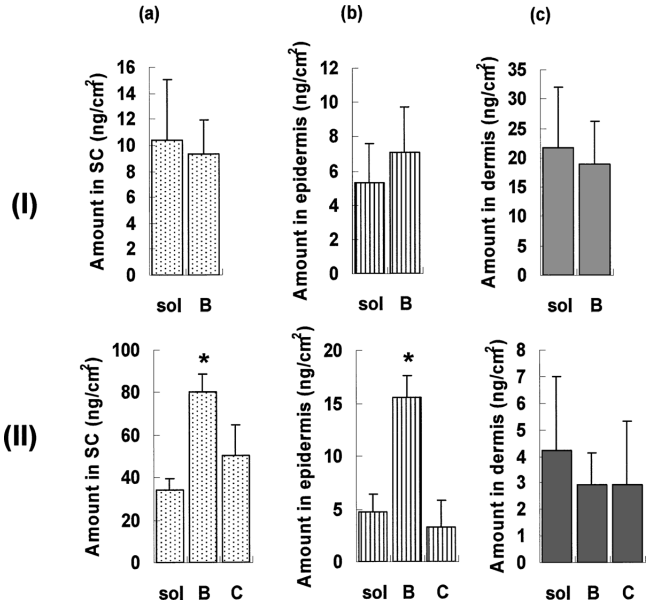

Fig. 2. Amount of CF in SC (a), Epidermis (b) and Dermis (c) at 24 h after Application of CF Solution (sol) or Liposomes under Occluded (I) or Open (II) Conditions

Columns and bars represent means±S.D. of at least 3 experiments. * Significantly different ($p<0.05$) from control (solution) by Dunnett's test.

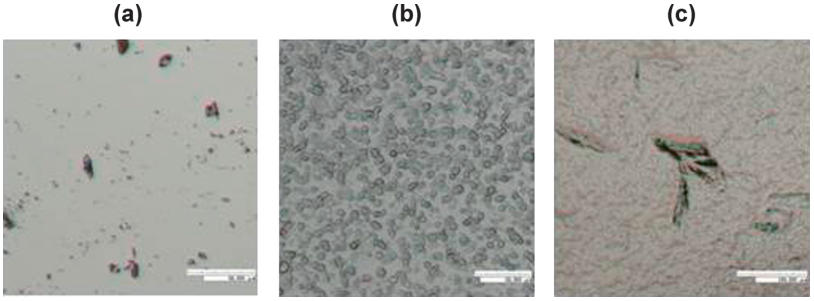

Fig. 3. Microscope Images Obtained after Drying CF Solution (a), Liposome B (b) and C (c)

The effects of encapsulation were then confirmed using the same composition but different CF positions; inner-phase of liposome (Liposome B) or outer-phase of liposome (Liposome C). There were no differences in the amounts of CF in the SC, epidermis and dermis between solution and Liposome C. This suggests that encapsulation is an important factor in enhancing the skin penetration of CF.

It suggests that liposomes themselves penetrated the SC or epidermis in their original form, as only CF encapsulated in liposomes showed enhanced penetration. However, it is also possible that formulation changes due to the evaporation of water played a role.

Figure 3 shows microscope images obtained after drying CF solution, liposome B and C. CF crystals were observed in the case of CF solution and liposome C, but not in the case of liposome B. The recrystallization of CF after evaporation of water in the cases of solution and liposome C caused large amounts of CF to remain on the skin surface. Encapsulation of CF in liposomes prevented the recrystallization of CF. These results suggest that one of the reasons penetration of encapsulated CF was enhanced was the lack of recrystallization of CF on the skin surface.

We compared the penetration of CF in two application methods. Under occluded conditions, liposome phospholipids showed no penetration. In addition, CF penetration from liposomes was no different from that after application of CF solution. In contrast, phospholipids penetrated into the skin, particularly the SC and hair follicles, under open conditions. This suggests that some force affects penetration during the drying process.[7] CF in liposomes showed enhanced penetration into the SC and epidermis, but not in the dermis. The improved penetration of CF may be partially explained by the prevention of CF crystallization after encapsulation in liposomes and the possibility of penetration by intact liposomes.

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