Confocal laser scanning microscopy study using lipophilic fluorescent probe DiI incorporated in liposomes for investigating the efficacy of a new device for substance deposition into deeper layers of the skin : Dermaroller®

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Introduction

Skin is a complex layered composite of several tissues that function cooperatively. The skin is the largest organ of our body (up to 16% of body weight), forming a barrier between our physical body and the outside environment. However, this barrier remains open and permeable to the environment to allow an exchange of heat, air and fluids.

The skin consists of three layers namely the epidermis, the dermis and the subcutaneous layer. The epidermis is the outer layer covering the whole outside of the body, which contains numerous nerve endings which make the skin into one large sense organ, detecting warmth, cold, light, taste and touch. The outer layer of the epidermis, called stratum corneum, is constantly being shed as thin scales and replaced with new layers from below. The stratum corneum is the rate-limiting barrier to percutaneous absorption and serves as a protective barrier. It impedes the evaporation of water from the tissue beneath it and acts as a barrier to water and foreign substances with which the skin comes in contact. The stratum corneum is composed of dense layers of the dead, flattened cells filled with fibrous protein keratin that derive from the epidermis beneath. In its absence, as when the skin is denuded by some disease process or even after stripping off by repeated applications of cellophane tape, the absorption of drugs across the skin is increased.

Many factors govern the rate at which drugs and cosmetics penetrate the skin from topical application formulations. These factors mainly includes the size of the molecule, the lipophilicity of the molecule, type of formulation, presence of penetration enhances and physical state of the stratum corneum. The rate of percutaneous penetration varies according to the anatomic site under consideration. Penetration from the sites where the thickness of the stratum corneum is thin and from moist flexural areas is greater than from skin with thick stratum corneum and extensor sites.

There has been several methods reported in literature for percutaneous penetration enhancement and it quantification. These include diffusion experiments (1-3), visualization by electron microscopy (4-5) and micro dialysis (6) etc. Micro dialysis and diffusion experiments provide information about the amount and the rate of penetration of the model compound but do not give any information about the effect of the model drug on cells and lipid organisation. The visualization by electron microscopy provides detailed information about the structure of the cells and lipid organization in the skin, but lacks in providing information on the penetration pathways and penetration depth of the model compound. The other techniques used include fluoro-micrography (7-9) and confocal laser scanning micrography (CLSM, 10). Fluoromicrographs of skin treated with fluorescently labeled liposomes demonstrated that the fluorescent marker remained in the stratum corneum (7,8) or penetrated deeper in the epidermis mainly along the hair shaft (9). A disadvantage of the fluoromicrograph technique is that the tissue needs to be (cryo)fixed, which may change skin lipid organization or may result in redistribution of the marker (11). CLSM provide information about the localization and the permeation pathway of a fluorescent model compound in the tissue. The major advantage of CLSM is that the distribution of the fluorescent model compound in the sample can be visualized without cryofixing or embedding the tissue. However, in the case of penetration studies with liposomes, CLSM does not provide information about the permeation of the entire liposome, but only about the penetration of the fluorescent label (12).

van Kuijk-Meuwissen ME et al. (12,13) showed that the label applied non-occlusively in flexible liposomes penetrated deeper into the skin than after occlusive

application. Kirjavainen M et al. (14) reported that the fluorescence from liposome compositions containing DOPE (dioleylphosphatidyl ethanolamine) was able to penetrate deeper into the stratum corneum than that from liposomes without DOPE and the pretreatment of skin with unlabeled liposomes containing DOPE or lyso-phosphatidyl choline (lyso-PC) enhanced the subsequent penetration of the fluorescent markers, N-Rh-PE and sulforhodamine B into the skin, suggesting possible enhancer activity. Boderke et al. (15) used CLSM to show that the amino peptidase activity was evenly distributed throughout the viable part of the epidermis, with enhanced fluorescence in the upper layers of the stratum granulosum, while dermis and stratum corneum showed considerably less amino peptidase activity. Zellmer et al. (16) used CLSM to demonstrate that vesicles, made of native human stratum corneum lipids rapidly mixed with PS liposomes, weakly with hSCL liposomes and did not mix with PC liposomes. Vardaxis et al. (17) employed CLSM to examine the structure of porcine skin and concluded that it provides valuable additional morphological information of material examined by conventional microscopy for wound healing studies. Zellmer et al. (18) reported that neither the vesicles nor the fluorophore N-(lissamine rhodamine B sulfonyl)diacylphophatidylethanolamine (Rho-PE) penetrates in detectable amounts into the human skin. Turner and Guy (19) showed that iontophoresis of calcein into hairless mouse skin enhanced delivery, particularly via follicular structures, to significant depths into the barrier. Simonetti et al.(20) visualized diffusion pathways across the stratum corneum of native and *in-vitro* reconstructed epidermis by using CLSM.

In the present study, we have visualized the potential of dermarollers for delivering the lipophilic fluorescent compound DiI (1,1'-Dioctadecyl-3,3,3',3'-trtramethylindocarbo -cyanine perchlorate) across human abdominal skin using confocal laser scanning microscopy. Three different dermarollers with different needle geometries were used for visualizing their potential of improving the penetration of the DiI.

Material and Methods

Materials

DiI (1,1'-Dioctadecyl-3,3,3',3'-trtramethylindocarbocyanine perchlorate) was purchased from Aldrich Chem. Co. USA. Lipid for producing liposomes was obtained from Nattermann Phospholipid GmbH (Cologne, Germany). Water used was demineralized by means of a Milli-Q plant (Millipore, Darmstadt, Germany). All other chemicals were of the highest available purity degree (Merck, Darmstadt, Germany). The mini-extruder (Liposofast) for the production of liposomes was purchased from Avestin (Ottawa, Canada).

Equipment

Three different Dermarollers were delivered from Horst Liebl ETS (67860 Friesenheim, France):

a) Model C 8 0,13-15°, for the deposition of dermatics and cosmetic active agents beyond the stratum corneum. This dermaroller has 192 needles with a length of 0.13 ± 0.02 mm, 24 needles per row with a lateral distance of 2.5 mm in eight rows.

B) Model M 8 1,5-15° dermaroller for deposition of dermatics and cosmetic active agents into deeper skin layers of approx. 1 mm: 192 needles with a length of 1.5 ± 0.02 mm, 24 needles per row with a lateral distance of 2.5 mm in eight rows.

C) Model M 8 1,5-30° for deposition of dermatics and cosmetic active agents in deeper skin layers of 1.5 mm: 96 needles with a length of 1.5 ± 0.02 mm, 12 needles per row with a lateral distance of 2.5 mm in eight rows.

All Dermarollers have a diameter of 20 mm and a width of 21.5 mm. They run on an axis which is connected to a guiding fork. The forks are screwed together with a handle.

The Franz diffusion cells (Gauer Glas, Püttlingen, Germany) (max. 6 at the same time operated) had a nominal surface of 3.14 cm^2 and a receiver compartment of 12 ml volume. The Franz cells were kept at 37° ± 1° by means of water bath (Julabo, Germany). The cryotome cuts were carried out with a Vogel Cryotome AS 620 (Anglia-Scientific, U.K.).

Liposome production and size measurement

Multilamellar vesicles were produced by means of a conventional method (21). These multilamellar vesicles were extruded through polycarbonate membrane pores with 50 nm diameters by means of the Avestin-Miniextrusion device in order to get liposomes of the desired size. The diameter of the liposomes was determined by a Zetasizer IV instrument (Malvern Instrument, Herrenberg, Germany).

Formulation

Formulations contained a liposomal concentration of 100 mg/ml lipid. The concentration of DiI in liposomes was 50 µg/ml liposomal formulation.

Skin preparation and perforation by dermarollers

Excised human skin from female patients, who had undergone abdominal plastic surgery, was used. Immediately after excision the subcutaneous fatty tissue was removed using a scalpel. The skin was wrapped in aluminum foil and stored in polyethylene bags at -25°C until use. For CLSM experiments, the skin was taken out from the deep freezer, it was kept at room temperature for 5 minutes. The surface of the skin was washed with ringer solution. The liposomal formulation was than applied and the dermaroller was than rolled 12 times with applying pressure with the hand with different sites. The skin was than placed on Franz diffusion cell and incubated for 3 hrs. In this study skin of one donor was used for all the experiments.

Franz diffusion cell

On the Franz diffusion cell, the skin sections were mounted with nominal surface areas of 3.14 cm^2 and receiver compartments with 12 ml capacities. The epidermal side of the skin was exposed to ambient conditions while the dermal side was bathed by a phosphate buffer saline pH 7.4. The receptor fluid was mixed with a magnetic stirring bar at 500 rpm. Buffer was kept at 37 ± 1 °C by a water jacket controlled by a water bath. Care was exercised to remove any bubbles between the under surface of the skin and the solution in the receiver compartment. All experiments were carried out with non-

occluded donor compartments. After 3 hrs the experiments were stopped and the diffusion set-up was dismantled. The surface of the skin was wiped off with the help of moist cotton wool and immediately frozen in liquid nitrogen.

Dosage regime and incubation times

The dose applied was 20 µl of liposomes per cm^2 of skin surface non-occlusively. The drug preparation was applied to the skin for 3 hrs on Franz diffusion cell.

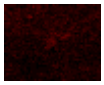
Skin Cryosectioning

The skin sections of 7 µm thickness, perpendicular to the skin were cut by the help of cryomicrotome. These cross-sections were investigated with a laser scanning confocal imaging system MRC-1024 (Bio-Rad Laboratories, Muenchen, Germany), equipped with an argon ions laser (American Laser Corp., Salt Lake City, USA) and a Zeiss axiovert 100 microscope using a lens with a magnification of 2.5 (Carl Zeiss, Oberkochen, Germany).

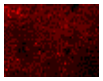
The following parameters were used for the confocal laser scanning microscope: magnification 2.5, laser power 3 %, scan modus slow, gain 1000, measurement Kalman and method red/green fluorescence (filter system A1 and A2 - 488 and 514 nm).

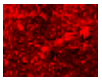
Scoring

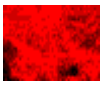
The fluorescence intensities of the CLSM images were semi-quantitatively blind-scored by 4 educated individuals and classified as follows : - no fluorescence; +/- very weak fluorescence; + weak fluorescence; + + medium fluorescence; + + + bright fluorescence


- no fluorescence


+/- very weak fluorescence


+ weak fluorescence


+ + medium fluorescence


+ + + bright fluorescence

Results and discussion

Figure 1 represents CLSM images of the skin cross-sections perpendicular to the skin surface. Table 1 shows the blind scoring data from CLSM pictures. The images are taken after 3 hrs of the non-occlusive application of the liposomes containing DiI as lipophilic label.

In all the images including control and different dermarollers, very high fluorescence was observed in the stratum corneum, which is obvious as the fluorescence label DiI is highly lipophilic and will be accumulated in the stratum corneum.

The control formulation showed a higher deposition of the fluorescent label in stratum corneum, followed by weak fluorescence in viable epidermis. In the deeper layers of the skin, we observed only a very weak fluorescence.

The dermaroller *C8 0,13-15°* was designed to improve the deposition of drug in *stratum corneu*m. The application of this *dermaroller* resulted in a bright fluorescence of stratum corneum, followed by medium fluorescence in viable epidermis and weak fluorescence in the deeper skin layers. As compared to the control, this dermaroller have showed significantly enhanced fluorescence both in epidermis and in deeper skin layers. It can be concluded from Fig. 1 b, that this dermaroller may have penetrated the layers of the stratum corneum and perhaps made some holes into the stratum corneum, through which the liposomes had passed. This supports the notion, that the needle length was long enough to make pores inside the *stratum corneu*m, but was not able to make pores through the whole *stratum corneum* due to the length of the needles and the flexibility of the *stratum corneu*m. However, as expected, this dermaroller did not show the higher deposition of the fluorescence label in the deeper skin layers. These finding support our earlier skin penetration studies carried out with retinol as lipophilic model drug in liposomes with dremarollers.

The *dermaroller M8-1,5-15°*, which has the same number of needles but a much larger needle length (1,5 mm in comparison to 0,13 mm in the case of C8-0,13-15°), was designed to deliver the drug in deeper layers of the skin by perforating the whole *stratum corneu*m. This dermaroller showed bright fluorescence in stratum corneum, epidermis and dermis (Fig. 1 C). As expected, this dermaroller showed the highest deposition of fluorescent substance in deeper skin layers. However, there seems to exist a continuous region beneath the stratum corneum, where there was weak to medium fluorescence. A possible explanation of this findings may be that the dermaroller was able to push the liposomes into the deeper layers during perforating the stratum corneum at the time of application. Therefore, the DiI liposomes which had been reached in the deeper layers at the time of the dermaroller application, were able to diffuse further in the dermis to a great extent. However, the liposomes which stayed after the dermaroller application on the surface, showed the maximum fluorescence in the stratum corneum and were able to penetrate to a small extent though the holes made in stratum corneum by dermaroller into the deeper skin layers. The lateral diffusion of the fluorescent label in between the holes was very less and this might be the reason for the less fluorescence presence in the continuous region beneath the stratum conneum.

In the case of the *dermaroller M8-1,5-30°,* which possesses only half the number of needles as the other dermarollers, bright fluorescence was observed in stratum corneum followed by medium fluorescence in epidermis and bright fluorescence in deeper skin layers. As compared to the control, this dermaroller has shown statistically enhanced fluorescence in both epidermis and deeper skin layers. It can be concluded from Fig. 1 d, that there was area of weak fluorescence beneath the stratum corneum. This area of weak fluorescence under the stratum corneum was less in the case of dermaroller M-8 1,5-15° as compared to this dermaroller. The reason for this large area of weak fluorescence is due to the number of needles and the angle position of the needles. However, the depth and intensity of the fluorescent label was maximum with this dermaroller.

This study has shown that the fluorescent model compound can be visualized in deeper layers of the skin by making cross-sections perpendicular to the skin surface and than taking the CLSM images . The method used in this study does not require fixation and embedding of the samples, and thereby reducing the redistribution of the label or tissue damage.

Conclusions

The tested Dermaroller were able to deposit lipophilic fluorescent label DiI into the *stratum corneum, epidermis* and deeper skin layers.

The experimental findings, visualised on the presented confocal pictures, support the intentions of the development protocols for the dermarollers. The Dermaroller C8-0,13-15° is particularly qualified to deposit substances into the *stratum corneu*m, whereas the Dermaroller M8-1,5-15° promotes very well the deposition of model compounds into deeper layers of the skin. The third Dermaroller M8-1,5-30° showed the best results for depositing model substances in deeper skin layers.

The examined dermarollers are pieces of equipment with a simple but effective operation, which shows surprisingly high deposition effects for compounds formulated in into the *stratum corneum* and into deeper skin layers.

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Types of dermaroller	Stratum corneum	Viable epidermis	Dermis
Control	+ + +	+	+/-
1,3-15°	+ + +	+ +	+
1,5-15°	+ + +	+ + +	+ + +
1,5-30°	+ + +	+ +	+ + +

Table 1. Summarized blind scoring data from CLSM images. CLSM images were taken from cross-sections of human skin that was non-occlusively incubated with DiI labelled liposomes for 3 hrs. The images were scored on the depth and fluorescence intensities of DiI penetration in stratum corneum, viable epidermis and dermis. The fluorescence intensities of the CLSM images were semi-quantitatively blind-scored by 4 individuals and classified as : - no fluorescence; +/- very weak fluorescence; + weak fluorescence; + + medium fluorescence; + + + bright fluorescence

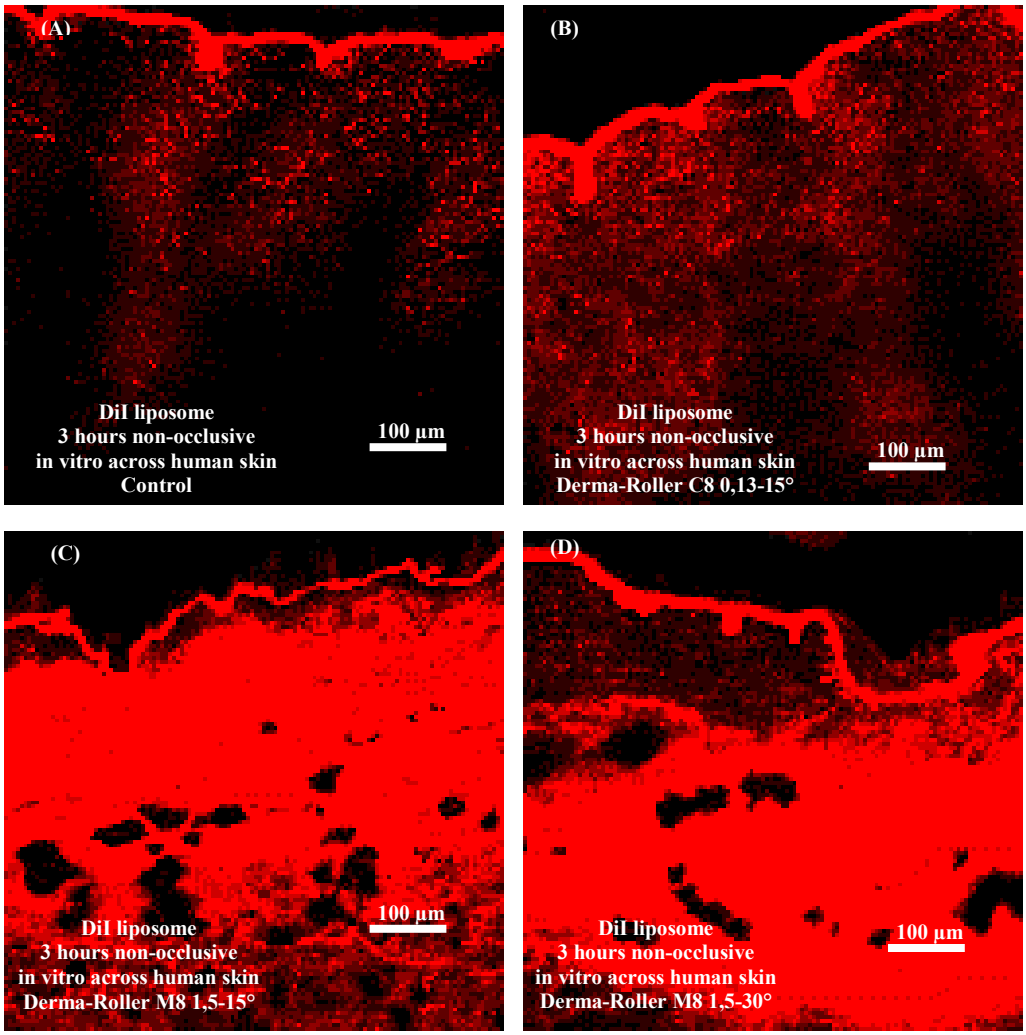

Fig. 1 CLSM images of a cross-section of human abdominal skin incubated on Franz diffusion cell with liposomes containing lipophilic label DiI. The liposomes were applied non-occlusively for 3 hrs.