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Interactions between liposomes and human skin in vitro, a confocal laser scanning microscopy study

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Abstract

One major problem in (*trans*)dermal drug delivery is the low penetration rate of drugs through the barrier of the skin. Encapsulation of a drug in lipid vesicles is one strategy to increase the penetration rate of a drug across the skin. In this study, the interactions between fluorescent-labelled liposomes and skin are visualized by confocal laser scanning microscopy (CLSM). Bilayer labelled gel-state and liquid-state liposomes (conventional or with flexible bilayers) were non-occlusively applied on human skin in vitro. The penetration pathway and penetration depth of the lipophilic fluorescent label into the skin were visualized. From the CLSM images, it was clear that the label applied in micelles and gel-state liposomes did not penetrate as deep into the skin as the label applied in liquid-state vesicles. Among the liquid-state vesicles, the suspensions with the flexible bilayers showed the highest fluorescence intensity in the dermis. Thus, the thermodynamic state of the bilayer and, to a smaller extent, the flexibility of the bilayer influence, strongly the penetration depth of the label into the skin. The label applied non-occlusively in flexible liposomes penetrated deeper into the skin than after occlusive application. © 1998 Elsevier Science B.V.

Keywords: Liposome; Skin; Confocal laser scanning microscopy; (Human)

1. Introduction

Human skin consists of an outer avascular stratified squamous epidermis and an inner vascular dermis, which mainly consists of connective tissue and contains nerves, hair follicles, sebaceous and sweat glands. Basal layer cells of the epidermis differentiate as they move towards the dermis and extrude lipids. The cells cornify and loose their cell organelles and get embedded in lipid lamellar sheets. This highly organized structure becomes the outermost layer of the skin and is also the main barrier of the skin, the stratum corneum.

Abbreviations: CLSM, confocal laser scanning microscopy; DLPC, 1,2-dilauroyl-*sn*-glycero-3-phosphatidylcholine; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine; Fl-DHPE, *N*-(5-fluoresceinthiocarbamoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine; Fl-C18, *N*-octadecyl-*N′*-(5-(fluoresceinyl))thiourea; $C_{12}EO_7$, heptaoxyethylenelaurylether; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethane-sulphonic acid; CHEMS, 5-cholesten-3β-ol 3-hemisuccinate; SC, stratum corneum; E, epidermis; D, dermis

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One of the most encountered disadvantages in (*trans*)dermal drug delivery is the low penetration rate of most substances through the skin.

Various strategies have been developed to overcome the skin barrier and to increase drug transport across the skin. One method to increase the penetration rate of a drug across the skin, is encapsulation of the drug in lipid vesicles. Mezei and Gulasekharam [1,2] were the first to report that liposomal triamcinolone acetonide facilitated the accumulation of the drug within the epidermis and dermis. Although both the experimental set-up and the interpretation of the results of this study have been criticized [3–5], the feasibility of an enhanced transdermal drug transport by means of liposomes was recognised to be valid; therefore, numerous studies were carried out, in which vesicles were used to increase drug transport through the skin [5]. Methods for studying the skin–vesicle interactions are, e.g., diffusion experiments [6–8], visualization by electron microscopy [9–11], and fluoro-micrography [12–14]. Fluoromicrographs of skin treated with fluorescent-labelled liposomes demonstrated that the fluorescent marker remained in the stratum corneum [12,13] or penetrated deeper in the epidermis predominantly along the hair-shafts [14]. 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 label [15]. The major advantage of confocal laser scanning microscopy (CLSM) is that the tissue can be optically sectioned, and that the distribution of the fluorescent probe in the sample can be visualized by images parallel to the surface of the sample, without cryofixing or embedding the tissue.

Until now, a few studies have been carried out using CLSM to investigate the interactions between vesicles and skin. It should be noted that CLSM does not provide information about the permeation of the entire liposome, but only about the penetration of the fluorescent label. Zellmer et al. [16] applied non-occlusively liposomes with a lipophilic fluorescent probe on human mamma skin. After 18 h of application, the skin was examined by CLSM, and the label could only be detected on the surface of the skin. In another study, Cevc et al. [17] and Schätzlein and Cevc [18] used fluorescent-labelled transfersomes™ and claimed that the fluorescent marker could be visualized by CLSM, until an apparent depth of 25 μm in murine skin.

In the present study, the interactions between various types of liposomes and human skin were investigated, in vitro. A comparison was made (i) between gel-state and liquid-state liposomes and (ii) between several liquid-state suspensions, which varied in the flexibility of their bilayer (by incorporation of a surfactant). Micelles were used as a control formulation. A lipophilic fluorescent dye was incorporated in the bilayer of the vesicles, and the penetration pathway and penetration depth of the phospholipid bound label into the skin was examined by CLSM, after various application periods. The treated skin was visualized perpendicular to the skin surface, using CLSM in combination with the cross-section device, as described by Van Kuijk-Meuwissen et al. [19]. This mechanical cross-section method has the advantage to visualize fluorescence in the stratum corneum, viable epidermis and dermis in a single image.

2. Materials and methods

2.1. Chemicals

1,2-Dilauroyl-*sn*-glycero-3-phosphatidylcholine (DLPC) and 1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine (DSPC) were gifts from Nattermann Phospholipids (Cologne, Germany). Heptaoxyethylenelaurylether ($C_{12}EO_7$) was purchased from Servo (Delden, The Netherlands). *N*-(5-fluoresceinthio-carbamoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanol-amine (Fl-DHPE) and *N*-octadecyl-*N′*-(5-(fluoresceinyl))thiourea (Fl-C_{18}) were

Table 1
The composition of the formulations used in this study

Formulation	Composition (% molar ratio)	Mean size (nm)
$C_{12}EO_7$ (micelles)	100	24
DLPC:$C_{12}EO_7$	60:40	180
DLPC:$C_{12}EO_7$	82:18	108
DLPC:CHEMS	82:18	178
DSPC:CHEMS	82:18	228

The polydispersity indices of the formulations ranged between 0.25 and 0.35.

both from Molecular Probes (Eugene, OR, USA). *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonicacid) (HEPES) and cholesteryl-hemisuccinate (CHEMS) were obtained from Sigma (Hilversum, The Netherlands) and sodium chloride was purchased from Merck (Darmstadt, Germany).

2.2. Preparation of liposomes

The formulations used in this study are presented in Table 1. Lipids and/or surfactant were dissolved in dichloromethane: methanol (3:1) and the lipophilic fluorescent marker (Fl-DHPE) was added to achieve

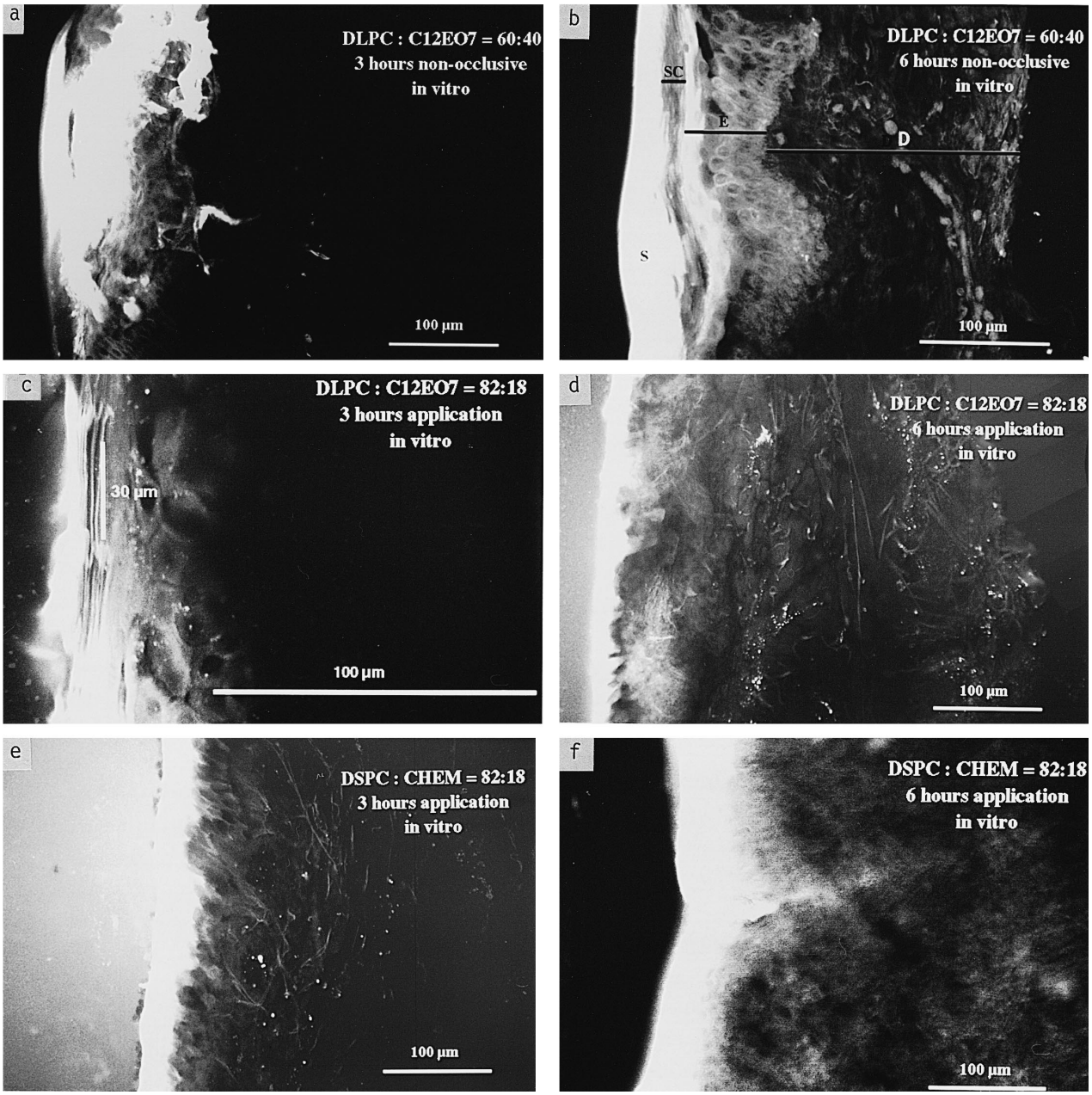

Fig. 1. CLSM images of a cross-section of human skin treated with fluorescent-labelled liposomes. DLPC:$C_{12}EO_7$ (60:40), DLPC:$C_{12}EO_7$ (82:18) or DSPC:CHEMS (82:18) liposomes were non-occlusively applied to the skin for 3 or 6 h. In Fig. 1b, stratum corneum (SC), viable epidermis (E), dermis (D) and remaining suspension (S) are indicated.

a final (molar) concentration of 2.75%. The solvent was evaporated in a vacuum centrifuge (Speed Vac Concentrator, Savant, Dunee, Soest, NL). The lipid film was hydrated with HEPES buffer at pH = 7.4 (20 mM HEPES, 135 mM NaCl) in a sonication bath (Transonic 460/H, Elma, Singen, Germany) at room temperature, except the DSPC:CHEMS liposomes, which were prepared above the transition temperature (> 56°C). The final lipid concentration of the liposomes was 50 mg/ml. The size and polydispersity were determined by dynamic light scattering (Malvern 4700C, Malvern Instruments, Malvern, UK). The measurements were performed at 27°C at an angle of 90° between laser and detector. The mean sizes and polydispersity indices are summarized in Table 1.

Mixing of $C_{12}EO_7$ with Fl-DHPE resulted in the formation of vesicles, instead of micelles; therefore, Fl-C_{18} was used as a single chain fluorescent marker in the micelles.

2.3. Application of liposomes on human skin, in vitro

Human abdomen skin was obtained after cosmetic surgery. After removal of the subcutaneous fat, the skin was dermatomed to a thickness of 200–250 μm (Padgett Dermatome, Kansas City, USA). The skin was placed in Franz-type diffusion cells. The acceptor compartment was filled, till 3 mm below the dermal surface of the skin, with HEPES buffer and kept at 32°C. In this way, overhydration of the skin could be avoided; therefore, the watergradient across the skin will not be changed. Liposomes were applied non-occlusively on the stratum corneum side of the skin (33 μl/cm^2), except for one experiment in which DLPC:$C_{12}EO_7$ (60:40) liposomes were applied occlusively, by covering the donor compartment with a stopper. After 1, 3, 6, and 16 h of application, the skin surface was rinsed 3 times with HEPES buffer and was blotted dry with a tissue. The cleaned skin was cross-sectioned and immediately examined by CLSM (see below).

2.4. Confocal laser scanning microscopy (CLSM)

A mechanical cross-section was made from the liposome-treated skin, with a special designed cross-section device [19]. The cross-sectioned skin was mounted in a sample holder in such way that the freshly obtained cutting surface was positioned against the cover glass. To avoid interference by fluorescence from damaged cells, the mechanical cross-section of the skin was examined by CLSM, 10 μm below the cutting surface.

The CLSM used was a BioRad MRC 600 confocal unit (BioRad, Hertfordshire, UK) equipped with a Krypton–Argon laser and mounted on a Nikon Optiphot (Nikon, Tokyo, Japan). For excitation of the probe the 488-nm laser line was used. The fluorescein–DHPE or the fluorescein–C_{18} was detected using the BioRad Blue High-Sensitivity (BHS) filterblock, which passes emitted light with a wavelength longer than 515 nm. Confocal images were

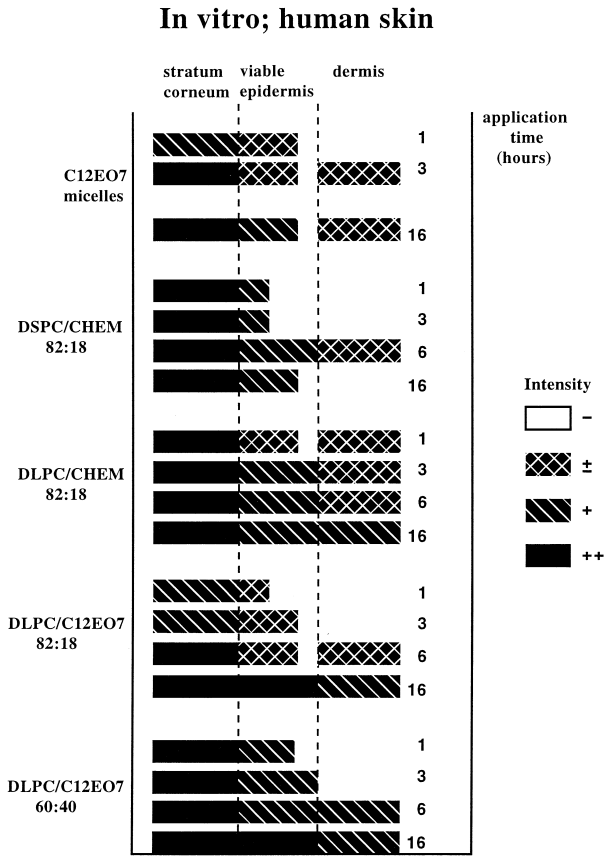

Fig. 2. Summarized blind scoring data from CLSM pictures. CLSM images were taken from cross-sections of human skin, which was non-occlusively incubated with fluorescent-labelled liposomes or micelles for 1, 3, 6 and 16 h. The images were scored on depth of label penetration and fluorescence intensities in stratum corneum, viable epidermis and dermis: − no fluorescence; ± weak fluorescence; + fluorescence; + + bright fluorescence.

obtained using a Zeiss Plan Neofluor 25×/0.8 multi-immersion objective, on its oil position. The images were corrected for autofluorescence of the skin, with the blacklevel setting. All the pictures were averages of 10 scans.

2.5. Scoring

For each liposome suspension, skin from at least two skin donors was used.

The fluorescence intensities of the CLSM pictures were semi-quantitatively blind-scored by 3 individuals and classified as follows: − no fluorescence, ± weak fluorescence, + medium fluorescence and ++ bright fluorescence. At the same time, the number of cell layers of the viable epidermis that contained fluorescence was counted and classified in one of these three categories: 1–2 cell layers, 1–4 cell layers, complete epidermis. The results of the semi-quantitatively blind scoring are summarized in Fig. 3.

3. Results

3.1. Comparison between the various suspensions

In Fig. 1, representative images are shown of skin incubated for 3 or 6 h with either DLPC:$C_{12}EO_7$ (60:40), DLPC:$C_{12}EO_7$ (82:18) or DSPC:CHEMS liposomes.

The fluorescent label can be detected in the stratum corneum (SC), viable epidermis (E) and dermis (D). Most images have an intense white band on the left side corresponding to the skin surface. On the right side, the area is less bright, correlating to the dermal side of the skin. In some pictures, a striped structure can be observed, within the white band.

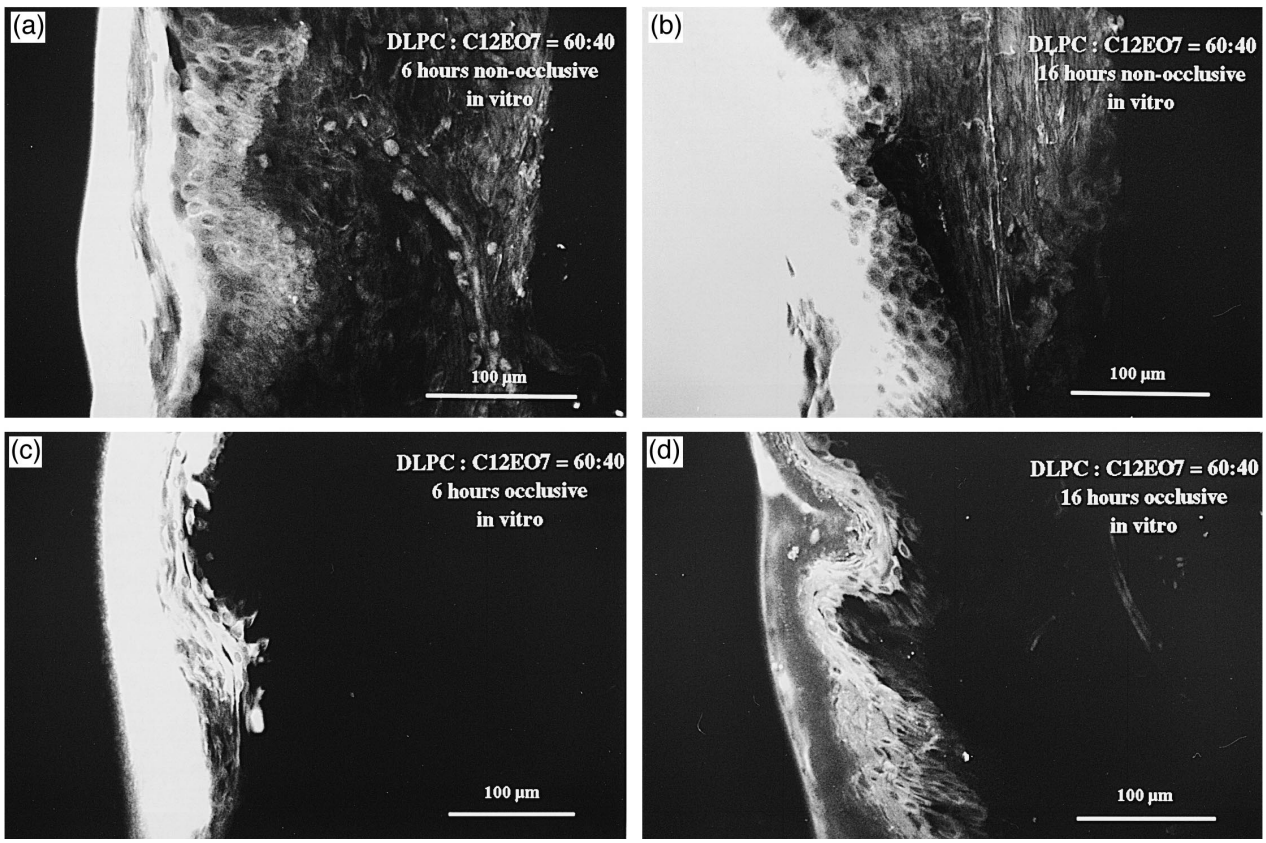

Fig. 3. CLSM images of a cross-section of human skin incubated with fluorescent-labelled DLPC:C12EO7 (60:40) liposomes. The liposomes were applied non-occlusively or occlusively for 6 or 16 h.

This relates to the flattened stratum corneum cells (corneocytes) that are embedded in lipid lamellae. In Fig. 1c, the individual corneocytes (30 μm) can be observed; the fluorescent label is present around these flattened cells. In this image also, the viable epidermis cells are visualized. In these cells, the fluorescence is present in the cell interior, except for the dark round areas in the centre of the cells, corresponding to the nuclei.

In Fig. 2, the results of the blind scoring are summarized for each formulation and application period. The various liposomes and micelles can be compared with respect to the penetration depth of the label and the intensity of the fluorescence in the various skin layers.

3.2. Occlusive vs. non-occlusive application

DLPC:$C_{12}EO_7$ (60:40) liposomes were applied occlusively or non-occlusively on human skin. The fluorescence distribution in the skin after 6 and 16 h is presented in Fig. 3. While after occlusive application the fluorescent label was restricted to the viable epidermis, non-occlusive administration revealed the label in the dermis, already after 6 h. In Fig. 4, the results of the blind scoring are summarized for each application period.

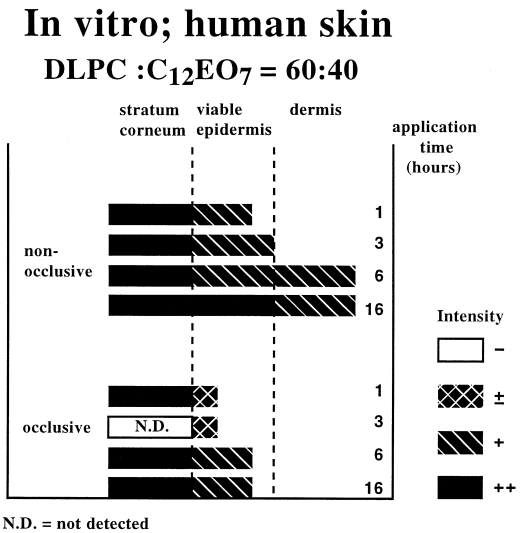

Fig. 4. Summarized blind scoring data from CLSM pictures. CLSM images were taken from cross-sections of human skin that was occlusively or non-occlusively incubated with fluorescent-labelled DLPC:$C_{12}EO_7$ (60:40) liposomes for 1, 3, 6 and 16 h. The images were scored on depth of label penetration and fluorescence intensities in stratum corneum, viable epidermis and dermis: − no fluorescence; ± weak fluorescence; + fluorescence; + + bright fluorescence.

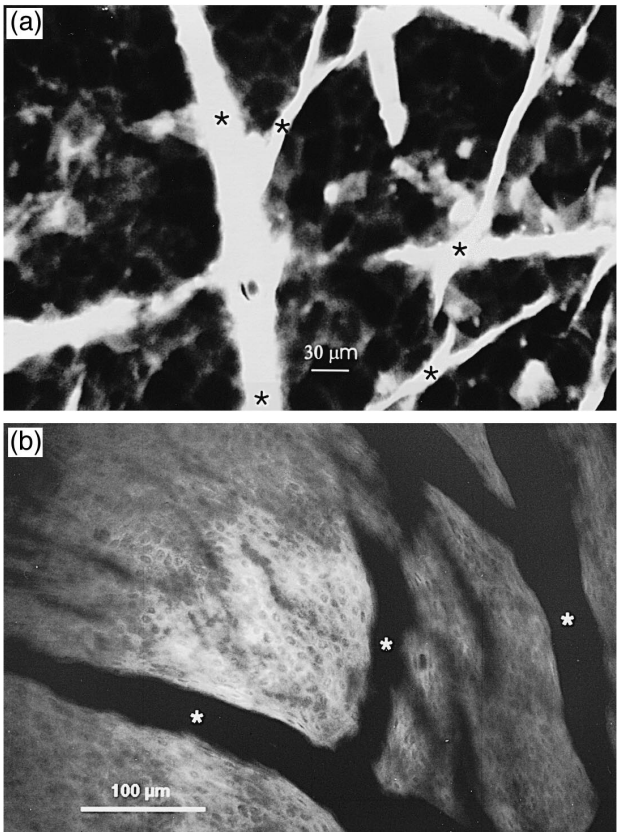

Fig. 5. Optical CLSM sections parallel to the skin surface depict skin wrinkles (see *). DLPC:C12EO7 (82:18) liposomes were non-occlusively applied on the stratum corneum side (a), or on the dermal side (b) of human skin. The hexagon with a diameter of 30 μm presents a corneocyte.

3.3. Skin wrinkles (dermatoglyphics)

Skin treated with DLPC:$C_{12}EO_7$ (82:18) for 6 h non-occlusively on the stratum corneum side, was also examined by CLSM parallel to the skin surface. Fig. 5a presents such a parallel image, in which very bright lines (see * in Fig. 5a) and hexagonal-shaped structures (30 μm), which correspond to the shape of a corneocytes, can be observed.

Fig. 5b depicts a parallel image of skin on which the same type of vesicles was applied non-occlu-

sively on the dermal side of the skin for 6 h. This image is made on the level of the viable epidermis and shows fluorescence around these small cells. In addition, black wide lines (see * in Fig. 5b) can be observed, and they have a similar contour as the white lines shown in Fig. 5a. These lines most probably correspond to skin wrinkles.

4. Discussion

4.1. Comparison between gel-state and liquid-state liposomes

At skin temperature (32°C), DSPC:CHEMS form gel-state liposomes and consequently have very rigid bilayers, while DLPC:CHEMS liposomes are in the liquid-state. The latter were prepared from phospholipids with a shorter chain length than the phospholipids in the gel-state vesicles. Therefore, these liposomes were used to study the effect of the thermodynamic state of the bilayers on the penetration of the label into the skin.

The label incorporated in the gel-state DSPC:CHEMS liposomes did not penetrate as deep into the skin as when it was applied in the liquid-state DLPC:CHEMS liposomes. Only after 6 h application of the gel-state vesicles a small amount of the dye could be detected in the dermis, while during all other application periods, the label did not penetrate further than the viable epidermis.

From these observations, it can be concluded that the penetration of the label is strongly influenced by the thermodynamic state of the liposomal bilayer.

4.2. Comparison between the various liquid-state liposomes

In two liquid-state liposomes, a single chain surfactant, $C_{12}EO_7$, was incorporated to achieve more deformable bilayers [18,20]. Two DLPC:$C_{12}EO_7$ suspensions with different molar ratios were used.

The penetration profiles of the label applied in the various DLPC suspensions were very similar (Fig. 2). Application of the label in DLPC:$C_{12}EO_7$ (60:40) liposomes resulted in a slightly higher fluorescence intensity in the skin compared to application of the label in DLPC:$C_{12}EO_7$ (82:18) (all application periods) and the conventional DLPC:CHEMS liposomes (6 and 16 h). From this, it was concluded that intercalation of a surfactant in the liposomal bilayer, which increased the flexibility of this bilayer (Van Kuijk-Meuwissen et al., unpublished data), does not result in a dramatic increase of penetration of the fluorescent label into human skin.

This is in contrast with the CLSM results, reported by Schätzlein and Cevc [18]. They concluded that the label originally intercalated in transfersomes™ penetrated much further into murine skin than the label applied in conventional liposomes. The differences in findings between the former experiments and the present study may be due to (i) different composition of the vesicles, (ii) differences in the skin tissue (murine stratum corneum consists only of 5–6 cell layers, while human stratum corneum contains 20–30 cell layers), or (iii) difference between in vitro and in vivo. However, we also carried out in vivo experiments with rats (Van Kuijk-Meuwissen et al., unpublished data). In these studies, a very similar label distribution was observed as in the present investigation.

4.3. Comparison between micelles and liquid-state vesicles

The effect on label penetration of pure $C_{12}EO_7$, forming micelles in the buffer solution, was also studied in order to assess if the $C_{12}EO_7$ micelles can increase the penetration of the label in the skin to the same extent as the DLPC:$C_{12}EO_7$ suspensions, or that the presence of vesicles is responsible for the increase in penetration of the label.

Skin treated with micelles revealed a much weaker fluorescence intensity than skin incubated with DLPC liposomes. These findings are in agreement with those published before [18], in which also a lower label penetration into the skin after application of micelles has been reported, despite the smaller size of micelles.

The present findings are also in agreement with earlier studies where the estradiol flux through stratum corneum was investigated, in vitro [21]. In these studies, it was found that estradiol-loaded vesicles resulted in a higher estradiol flux compared to estradiol-loaded micelles.

It seems that the vesicular bilayer form is more favourable than a micellar form for increasing the penetration depth of the fluorescent label in the skin.

4.4. Occlusive vs. non-occlusive application

The fluorescent dye distribution after occlusive application was compared to non-occlusive application. In these experiments, we chose for the DLPC:$C_{12}EO_7$ (60:40) suspension, since this suspension revealed the highest fluorescence intensity in the deeper layers of the skin. From Figs. 3 and 4, it is clear that large differences in fluorescence intensity are observed in favour of the non-occlusive method. These results are in agreement with the theory that the natural transepidermal water activity gradient acts as a strong driving force to get substances across the skin barrier [17,20]. During occlusive application, the water gradient is expected to be absent.

4.5. Skin wrinkles

Fig. 5a shows fluorescence in between the hexagons and Fig. 1c also reveals fluorescence in the intercellular spaces. From this, it can be concluded that the fluorescent label, applied in the liposomal bilayer, penetrates the stratum corneum mainly via the intercellular route. Cevc et al. [17] also described this route. In the same study also, a second type of passage through the skin was described, namely wide clefts between clusters of 3–10 corneocytes with a width of ≥ 0.1 μm [17]. In Fig. 5a of this paper, similar wide lines are depicted (see * in Fig. 5a). In our experiments, these regions are filled with fluorescent material, due to pooling of the liposome suspension. We strongly have the impression that these lines correspond to wrinkles in the skin. This means that the fluorescent label detected in these regions is still on the surface of the skin. The fact that these lines remained dark after application of the same vesicle suspension on the dermal side of the skin (see Fig. 5b) confirms this suggestion.

4.6. Intact liposome penetration?

CLSM does not provide reliable information about the question whether or not liposomes can penetrate as intact vesicles, because only the fluorescent marker is visualized and not the entire liposome. There are at least three mechanisms by which the label can penetrate the skin: (i) the label penetrates associated with the intact liposomal bilayer (intact liposome), (ii) the label penetrates solitary (liposomal phospholipids penetrate molecularly dispersed) or (iii) the label penetrates associated with liposomal bilayer fragments (after disruption of the liposomes).

One way to distinguish intact liposomes from disrupted ones is using two fluorescent labels suitable for resonance energy transfer (RET), and to intercalate both labels in the liposomal bilayers. In our experiments for this purpose, fluorescein–DHPE (donor) and Texas Red–DHPE (acceptor) were used. That both labels penetrated via the same intercellular route was anticipated, since they both are lipophilic. In an ideal RET experiment, all the emission light from fluorescein would be absorbed by Texas Red; thus, only the fluorescence from Texas Red would be detected. In the case that also disrupted liposomes are present, not only fluorescence from Texas Red, but also from the fluorescein label will be observed. However, we were not able to incorporate both labels in such concentrations, that only the fluorescence of Texas Red was detected by dual CLSM imaging of the liposomes, and without disturbing the physical properties of the vesicles. Therefore, with the set-up of these CLSM experiments, no final answer can be given about the state in which liposomes are able to penetrate the human skin.

5. Conclusions

From the present results, it can be concluded that both the vesicular form and the thermodynamic state of liposomes play an important role in (*trans*)dermal penetration of model compounds. A lipophilic substance penetrates deeper into the skin when it is applied in liquid-state liposomes than when it is applied in gel-state vesicles. A high deformability of the liposomal bilayer resulted in a slightly better penetration of the lipophilic probe compared to conventional liposomes. In addition, non-occlusive application resulted in deeper penetration of the label into the skin than occlusive application. Furthermore, it should be stated that CLSM does not provide reliable

information about the question whether or not liposomes can penetrate as intact vesicles.

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