A Cross-Section Device To Improve Visualization of Fluorescent Probe Penetration into the Skin by Confocal Laser Scanning Microscopy

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INTRODUCTION

The stratum corneum is an effective barrier, and the low penetration rates of drugs through it is a major problem when attempting to deliver drugs transdermally. One strategy to increase the penetration rate of a drug through the skin is encapsulation of the drug in vesicles. However, contradictory results of the effects of vesicles on drug penetration have been reported (1) and the interactions between liposomes and skin are still not well understood. The effects of vesicles on the ultrastructure of the skin can be visualized by electron microscopy (2–6), and while these methods provide detailed information about the structure of the cells and lipid organization in the skin, they do not provide information on the penetration pathways and penetration depth of a (model) drug.

One technique that can provide information about the localization and the permeation pathway of a model compound in the tissue is confocal laser scanning microscopy (CLSM) (7). In recent studies on vesicular transdermal delivery, a fluorescent probe was incorporated in the liposomal bilayer to track permeation (8–10). Following application of the liposomes, the distribution of the probe in the skin visualized by confocal optical sections made parallel to the plane of the skin surface. 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. While Zellmer *et al.* (8) could only detect the fluorescent probe on the surface of human skin, Cevc *et al.* (9) and Kirjavainen *et al.* (10) could visualize the probe to an apparent depth of 25–30 μm. Simonetti *et al.* (11) have reported the detection of a different fluorescent probe (not encapsulated in vesicles) to an apparent depth of 50 μm in human skin *in vitro*. The major disadvantage of this method is the progressive loss of resolution and signal with depth in the tissue, due to scattering and absorption of both the laser excitation and fluorescence emission light (12,13).

While skin histology has been visualized *in vivo* by reflectance CLSM to a depth of 120–150 μm below the surface, using either white light or laser sources (14–17), reflectance or autofluorescence imaging does not lend itself for detection of permeating compounds Similarly, chemically fixed sections from thick specimens have been used to visualize the penetration of (model) drugs in the deeper layers of the skin, however the processes of fixation and embedding may change the skin lipid organization or can result in redistribution of the label (7,18).

We have developed a device which enables us to rapidly and reproducibly prepare mechanical cross-sections of freshly excised (i.e., unfixed) skin, with the plane of the cut perpendicular to the surface of the skin. The specimen is then oriented so that a CLSM optical section can be obtained parallel to the mechanically-cut surface and several μm below it, thus avoiding artifacts from damaged cells at the surface of the cut. In this way, the distribution of the fluorescent signal at the same site throughout the entire thickness of the skin (stratum corneum, viable epidermis and dermis) can be imaged without loss or distortion, permitting semi-quantitative comparison as well as determination of the penetration pathway and penetration depth of the fluorescent probe in the sample.

MATERIALS AND METHODS

Materials

Dilaurylphosphatidylcholine (DLPC) was a gift from Nattermann Phospholipid (Cologne, Germany). Heptaoxyethylenelaurylether ($C_{12}EO_7$) and N-(5-fluoresceinthiocarbamoyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (FI-DHPE) were purchased from Servo (Delden, The Netherlands) and Molecular Probes (Eugene, OR, USA) respectively. HEPES was from Sigma (Hilversum, The Netherlands) and sodiumchloride was from Merck (Darmstadt, Germany).

Preparation of Liposomes

Liposomes were prepared by the thin film method (19) from dilauryl-phosphatidylcholine (DLPC) and heptaoxyethylenelaurylether ($C_{12}EO_7$) in a molar ratio of 82:18. The phospholipid DLPC has been chosen, because the acyl chains have the same length as $C_{12}EO_7$. A fluorescent probe N-(5-fluoresceinthiocarbamoyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (FI-DHPE, 2.75 mol%) was intercalated in the liposomal bilayer during preparation. The dry lipid film was hydrated with HEPES buffer at pH = 7.4 (20 mM HEPES, 135 mM NaCl) in a sonication bath (Transsonic 460/H from Elma, Singen, Germany) to a final lipid concentration of 50 mg/ml. The size and polydispersity, which ranges from 0.0 (monodisperse) to 1.0 (polydisperse), were determined by

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ABBREVIATIONS: CLSM, confocal laser scanning microscopy; DLPC, dilaurylphosphatidylcholine; FI-DHPE, N-(5-fluoresceinthiocarbamoyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine; $C_{12}EO_7$, heptaoxyethylenelaurylether; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethane-sulphonic acid; SC, stratum corneum; E, epidermis; D, dermis.

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dynamic light scattering (DLS: Malvern 4700C, Malvern Ltd., Malvern, UK). The mean particle size of the liposomes used was 105 nm with a polydispersity index of 0.09.

Application of Liposomes on the Human Skin

Human abdomen skin was obtained after cosmetic surgery. After removal of the subcutaneous fat from the freshly excised skin, 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, in which the acceptor compartment was filled with HEPES buffer and kept at 32°C. Liposomes were applied non-occlusively on the stratum corneum side of the skin (33 μl/cm2). After 1, 3 or 6 hours of application, the skin surface was rinsed 3 times with HEPES-buffer and was blotted dry with a tissue. After the cleaning procedure the skin was immediately examined by CLSM. Optical sections were obtained either parallel to the surface (xy series) or in a plane perpendicular to the surface by using the combined mechanical/optical cross-section technique described below.

The experiments were carried out with at least three skin donors.

Cross-Section Device

Mechanical cross-sections of the skin samples were made by a specially designed cross-section device, shown in Figure 1. In this device the treated skin (A), with the stratum corneum facing downwards, was sandwiched between two silicone sheets (B) and small perspex[R] (polymethylmetha-crylate) holders (C1-2 and D1-2). This assembly was bolted together with light pressure and held in a small vise. The skin and silicone sheets were then cross-sectioned perpendicular to the skin surface with a clean, degreased razorblade (Gillette) that was inserted between the perspex holders C1-2 and D1-2. By unscrewing the vise block in front of the perspex holder D1-2, this block and D1-2 can be pushed a few mm aside (see arrow), thus avoiding contamination of the two cross-sections (clamped in C1-2 and D1-2) during removal of the razor blade.

Confocal Laser Scanning Microscopy (CLSM)

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 was detected using the BioRad blue high sensitivity filterblock, which passes emitted light with a wavelength longer than 515 nm. Confocal images were obtained using a Zeiss Plan Neofluor 25×/0.8 multi immersion objective, on its oil position.

- Parallel-series: A small piece of incubated skin was placed in a sample holder with the stratum corneum facing the lens. By moving the objective lens, thereby shifting the plane of focus in the sample, sequential optical sections of the skin sample were collected (xy-series).
- Cross-sections: However the depth of optical sectioning is ultimately limited by the working distance of the lens used and the absorbance of light in the tissue, see dotted line in Figure 2 ('maximum CLSM depth'). To overcome these limitations and in order to obtain results from the deeper layers of the fresh tissue, optical sectioning was combined with mechanical cross-sectioning perpendicular to the skin surface (Fig. 2). The mechanical cross-section is made using the cross-section device (see above). One of the perspex holders (C1-2 or D1-2) containing a cross-section of the skin was mounted on a sample holder, in such a way that the freshly obtained cutting surface was positioned against the coverglass. The mechanical cross-section of the skin was optically sectioned by CLSM, 10 μm below the cutting surface, to avoid interference by fluorescence from damaged cells (see ---··--- line in Fig. 2).

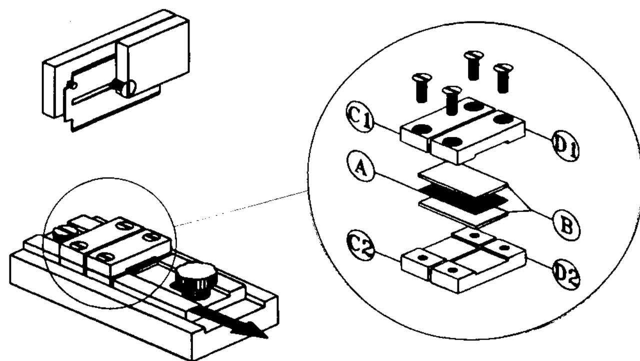

Fig. 1. Diagram depicting the cross-section device. (A) incubated skin with stratum corneum facing downwards. (B) silastic sheeting. (C1-2 and D1-2) perspex[R] holders for the skin and silastic sheeting. This assembly was bolted together and held in a small vise. The skin and silicone sheets were then cross-sectioned with a razor blade inserted between the perspex[R] holders C1-2 and D1-2. By unscrewing the vise block in front of the perspex[R] holder D1-2, this block and D1-2 can be pushed aside (see arrow), thus avoiding contamination of the two cross-sections during removal of the razor blade.

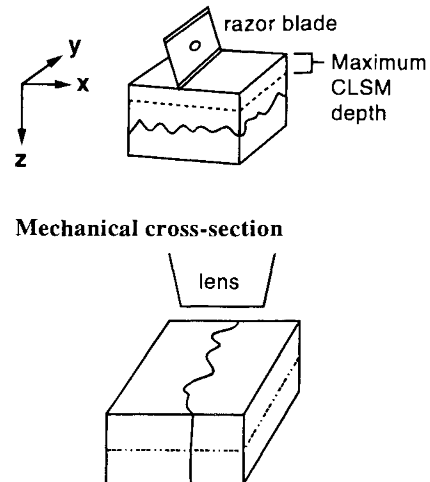

Fig. 2. The orientation of the skin with respect of the CLSM image. Using the conventional method, the CLSM image is parallel to the skin surface (xy-series). The dotted line (-----) represents the maximum CLSM depth. Preparing a mechanical cross-section, a CLSM image is made perpendicular to the skin surface approximately 10 μm below the cutting surface (see ---··--- line).

The CLSM images were corrected for the autofluorescence of the skin with the blacklevel setting. All the pictures were averages of 10 scans.

RESULTS

In figure 3 representative parallel-series of CLSM images of the skin are shown after 6 hours of non-occlusive application with liposomes. The very bright lines (arrows in Fig. 3a) indicate the fluorescence of the label in the wrinkles of the skin. Near to the surface (Fig. 3a and b) the individual corneocytes can be seen, with a hexagonal shape and a diameter of approximately 30 μm. The regions between the corneocytes were strongly fluorescent. Deeper in the skin (fig. 3c and d) the intensity of the fluorescence was extremely decreased. At even a deeper depth only the bright lines of the skin wrinkles could be visualized (data not shown). In this particular parallel series, the label could be detected until an apparent depth of 13–15 μm. Thus the label could only be visualized in the stratum corneum. Using this method of optical sectioning parallel to the skin surface, it was not possible to visualize the label in the viable epidermis and dermis.

To obtain information about the penetration of the label into the deeper layers of the skin cross-sections perpendicular (90°) to the skin were required. This was achieved by first using the cross-section device and subsequently collecting CLSM images parallel to the plane of the mechanical cross-section of the skin. Figure 4 demonstrates representative CLSM images of this technique. After 1 and 3 hours of application (Fig. 4a and b respectively), a bright white band is visible on the left side corresponding to the stratum corneum side. In both Fig. 4a and 4b a few viable epidermis cells are visualized (E). After 6 hours of application (Fig. 4c), the fluorescent label was not only detected in the stratum corneum (SC) with a very high fluorescence intensity, but is also clearly present (although at lower intensity) in the viable epidermis (E) and in the dermis (D) in the same optical section.

Figure 4d shows a high magnification of the stratum corneum and the first cell-layers of the viable epidermis. The stratum corneum can be recognized as a striped structure, in which the intensity of the label in the deeper layers of the stratum corneum is as bright as on the surface. From this observation it was concluded that the intensity of the label is homogeneously distributed in the intercellular regions of the stratum corneum. The arrow in figure 4d shows that the fluorescent label is present around the flattened corneocytes, which is in agreement with the findings obtained with the parallel series. In the viable epidermis 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 (Fig. 4d) (20). The dermis, which is the thickest layer of the skin, shows the structure of the collagen and/or elastic fibres (Fig. 4c).

DISCUSSION

The CLSM images parallel to the surface of the skin provide information about the penetration route of the fluorescent marker in the stratum corneum. These images (fig. 3) and also the high magnification image perpendicular to the skin surface (fig. 4d) reveal that the phospholipid-bound label penetrates the

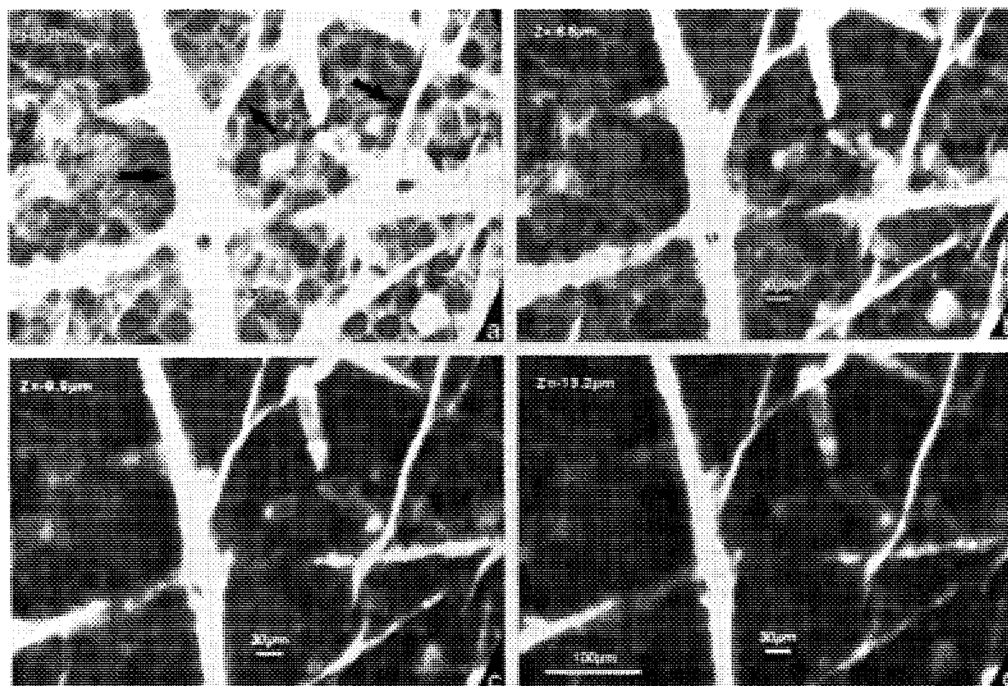

Fig. 3. A CLSM image parallel to the surface of human skin treated with fluorescently labelled DLPC:$C_{12}EO_7$ (82:18) liposomes, non-occlusively for 6 hours. Z indicates the apparent depth below the skin surface. Fig. a is near to the surface and fig. d is ± 13 μm below the skin surface. The skin wrinkles are highly fluorescent (arrows) and the dark hexagonal shapes correspond to the corneocytes. The regions between the corneocytes is strongly fluorescent (a and b). Deeper in the skin (c and d) the intensity of the fluorescence is decreased. Scalebar is indicating 30 μm.

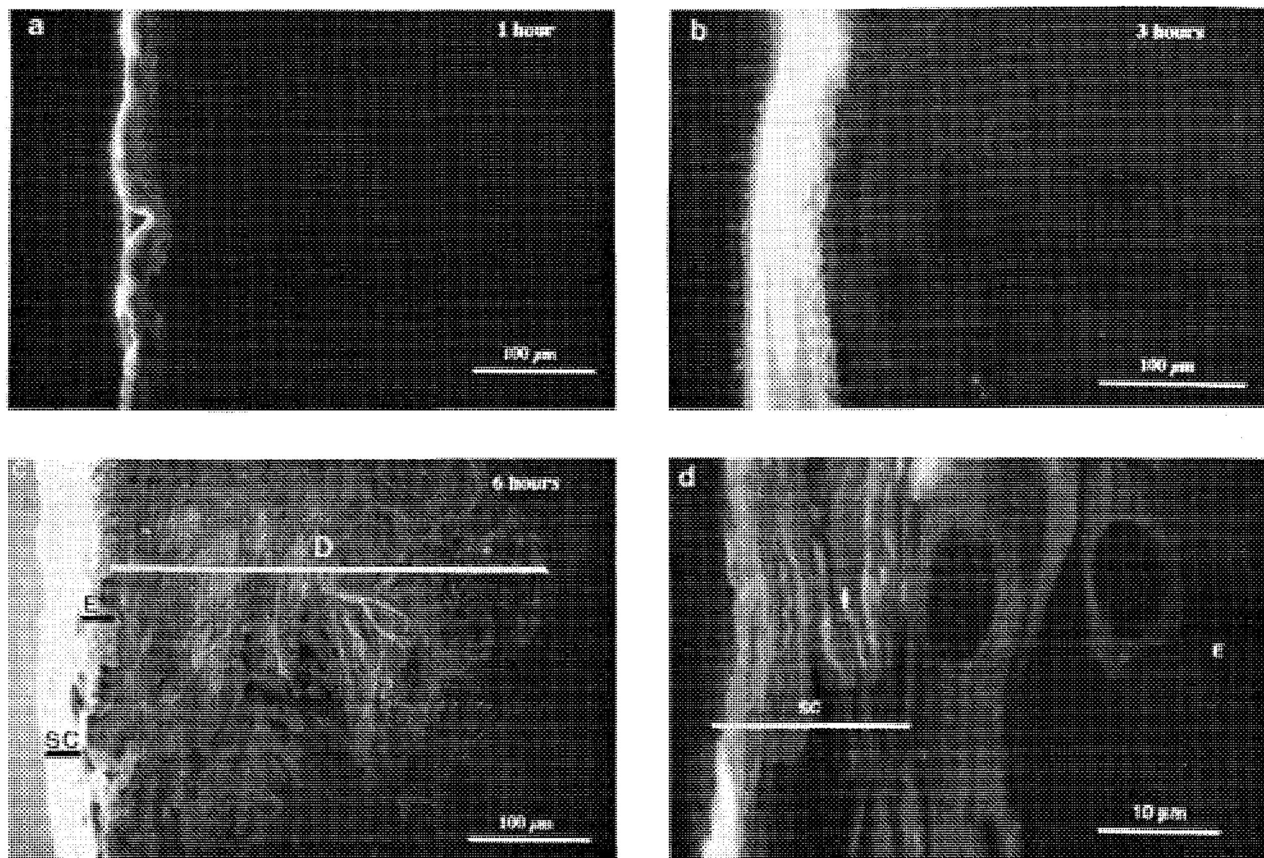

Fig. 4. A CLSM image of a mechanical cross-section of human skin treated with fluorescently labelled DLPC:$C_{12}EO_7$ (82:18) liposomes, non-occlusively for (a) 1, (b) 3 or (c) and (d) 6 hours. The stratum corneum (SC), epidermis (E) and dermis (D) are visualized in the same optical CLSM section. Fig. d. shows a high magnification, which demonstrates that the fluorescent label is present around the corneocytes (see arrow). Also some epidermal cells (E) with dark nuclei are visualized. Scalebar is indicating 100 µm.

stratum corneum primarily via the intercellular route. This route has also been described for the penetration of fluorescein-DHPE and rhodamine-DHPE (originally intercalated in transfersomes™) into murine skin (9) and for Nile Red (dissolved in DMSO) into human skin (11).

The mechanical cross-section perpendicular to the skin surface (Fig. 4d) showed the same dye intensity over the whole stratum corneum, while the parallel series demonstrated an apparent decrease in intensity in the deeper stratum corneum layers, and it was not possible to visualize the viable epidermis and dermis. This was primarily due to scattering and absorption of laser and emission light in the skin. For this reason, the comparison of fluorescence intensities at various depths in the sample is not possible using parallel series. To obtain information about the penetration of the dye in the deeper layers of the skin, another method is required. This study has shown that the fluorescent label can be visualized in deeper layers of the skin by creating mechanical cross-sections perpendicular to the skin surface and subsequently collecting CLSM images several µm below the cutting edge. The method used in this study does not require fixation and embedding of the sample, and therefore redistribution of the diffusing agent or tissue damage is greatly reduced.

Making a mechanical cross-section in unsupported tissue with a sharp razorblade does not result in a flat surface, since the stratum corneum is dense and difficult to cut, while the epidermis and dermis are rather soft. A planar imaging surface is necessary for CLSM examination. Therefore a cross-section device was developed, in which the tissue is held between two silicone sheets in a perspex holder. As shown in figure 4, this method resulted in images in which the stratum corneum, viable epidermis and dermis are visualized in one and the same focal plane, without a decrease in intensity due to intervening layers of tissue. This method thus provides a better estimation of the distribution pattern of the fluorescent probe. In this way the penetration profiles of the label into the skin can be compared after different application periods, as shown by the representative images in Figure 4a, b and c. It is also possible to compare label localization in liposome treated skin as function of liposome composition and application period. Therefore, the cross-section device is a very useful tool, in combination with visualisation techniques (like CLSM), for skin, and probably also for other kinds of tissues.

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