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# Interaction of liposome formulations with human skin in vitro

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**Abstract**

The interaction of liposome formulations consisting of Phospholipon® 80 and sphingomyelin with human skin was investigated. These formulations were shown previously to have a composition-dependent effect on the penetration of Heparin into the skin. Fluorescence labelled phosphatidylethanolamine (PE-NBD) was incorporated in the liposomes and the depth in which the fluorescent phospholipid label enters into epidermal membrane and full thickness skin was studied by confocal laser scanning microscopy (CLSM). Confocal sections parallel to the surface of the skin were recorded in heat separated epidermis. An even distribution of phospholipid in the lipid matrix of the stratum corneum surrounding the corneocytes was observed with Phospholipon 80 but not when sphingomyelin was included in the formulation. The addition of Heparin which formed a coating around the liposomes, caused a strong localization of fluorescence within the epidermis. For full thickness skin, mechanical cross sections of skin were made and optical sections were recorded parallel to the plane of cut. Phospholipid penetrated and was distributed fairly homogeneously in the lower dermis layers within 30 min of application regardless of liposome composition and the presence of Heparin. This rather quick penetration process seemed to follow distinct pathways along the epidermis and the upper dermis, notably the hair follicle route. Thus, a strong and in some respects composition-dependent interaction of phospholipids with skin is evident. These observations, however, are limited to the level of phospholipid molecules, rather than of entire liposomes interacting with skin. 

*Keywords:* Heparin sodium salt; Liposome; Confocal laser scanning microscopy; Skin penetration; ζ-potential

## 1. Introduction

The stratum corneum, representing the chief transport barrier of the skin, has been structurally described by the brick-and-mortar-model (Elias, 1987) comprising two compartments, the bricks which correspond to the protein-rich corneocytes and the mortar which corresponds to the lipid-rich intercellular substance. The intercellular lipids play a key role in establishing the permeability barrier of the stratum corneum (Elias, 1981; Landmann, 1988; Cevc, 1996). The structural organization of stratum corneum lipids has been studied by freeze-fracture electron mi-

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croscopy (Wertz et al., 1986) and X-ray diffraction (Bouwstra et al., 1996). Liposomes, which are vesicles having a phospholipid bilayer membrane identical to that of natural cell membranes have been suggested as a promising vehicle for many drugs enhancing their penetration into the skin. The first work using liposomes as a drug delivery system for topical administration was performed by Mezei and Gulasekharam, 1980. Subsequent studies were aimed at inducing a local rather than a systemic effect (Schmid and Korting, 1994). Studies in which Heparin was encapsulated in liposomes in order to enhance its permeation across the skin were carried out using radiolabelled Heparin (Artmann et al., 1990; Bonina and Montenegro, 1994).

In our previous work, we investigated the penetration behaviour of Heparin across the skin from various liposome formulations using a biological assay (Betz et al., 2001). The results showed that native Heparin penetrated into epidermal membrane from liposomes prepared with Phospholipon® 80 while no penetration was found with a purely aqueous formulation. The mechanism of stratum corneum–liposome interaction is not entirely clear yet, although many investigations have been performed so far using differential scanning calorimetry (Blume et al., 1993; Zellmer et al. 1995), freeze-fracture electron microscopy (Abraham and Downing, 1990; van den Bergh et al., 1999), fluoromicrography (Lasch et al., 1991) and confocal laser scanning microscopy (Zellmer et al., 1995; Kirjavainen et al., 1996; Cevc et al., 1998; Meuwissen et al., 1998; Kirjavainen et al., 1999). Furthermore, the presence of ethanol in systems of phospholipid vesicles termed ethosomes was reported to influence the stratum corneum permeation of drugs (Touitou et al., 2000a,b). The use of confocal laser scanning microscopy (CLSM) has the advantage that optical sectioning can be performed with unfixed specimens, making lengthy preparation procedures unnecessary. Lipid penetration into skin studied by CLSM was reported to depend greatly on liposome composition (Zellmer et al., 1995; Kirjavainen et al., 1996), thermodynamic state of the bilayer (Meuwissen et al., 1998) and presence of ethanol in the formulation (Kirjavainen et al., 1999), while Schätzlein and Cevc (1998), reported about ultradeformable material carriers, the Transfersomes™ optimized to overcome the skin transport barrier spontaneously due to the natural transepidermal water activity gradient. Studies performed by fluoromicrography (Lasch et al., 1991) and CLSM are not conclusive as to whether intact liposomes are able to penetrate into the skin.

The main objective of this study was to investigate the interaction of liposomal formulations with different phospholipid compositions (Phospholipon 80, Sphingomyelin, and a mixture of both at a 2:1 mass ratio) with skin. These formulations were previously used in skin transport experiments with Heparin and were found to have a composition-dependent effect on the penetration of Heparin into human skin (Betz et al., 2001). The distribution of lipids of the liposome formulations in the skin was studied by CLSM using a fluorescence labelled phospholipid marker that was incorporated in the liposomes and the dependence of the liposome-skin interaction on the presence of Heparin was evaluated.

## 2. Materials and methods

### 2.1. Materials

Heparin (UH) with a molecular weight between 6000 and 30 000 was obtained as sodium salt in its native (unfractionated) form from commercial sources. Its anti-factor Xa activity was 172.9 IU per mg as determined by the USP 24 assay against the WHO 4th international standard. Phospholipon® 80 (PL80) was a kind gift from Nattermann Phospholipid GmbH, Cologne, Germany. This is a soy bean lipid extract with 76% (w/w) content of phosphatidylcholine. Sphingomyelin (SM) was a kind gift from Lipoid AG, Ludwigshafen, Germany. It is obtained from egg yolk and has a purity of $>98\%$. Phosphatidylethanolamine,dipalmitoyl,*N*-(4-nitrobenz-2-oxa-1,3-diazolyl) (PE-NBD) was used as fluorescent label of the liposomes and was purchased from Sigma (Buchs, Switzerland). In PE-NBD, the fluorescent molecule NBD is covalently

linked to the headgroup of the phospholipid by a chemically and enzymatically stable bond. All other chemicals and reagents purchased from commercial sources were of analytical grade.

### 2.2. Skin

Excised human cadaver skin from the abdominal region of female donors (mean age of the donors $74 \pm 2$, $n = 4$) was obtained from Pathologisches Institut, University of Basel, Switzerland. Biopsies were taken within 24 h post mortem and stored at $-70$ °C. Before use, the skin was thawed for 1 h at room temperature and the subcutaneous fat was removed with a scalpel. The epidermal membrane was separated from the dermis by immersing the skin for 60 s in water at 60 °C (Kligmann and Christophers, 1963). The epidermal membrane was then peeled off in the beginning by forceps and then with the finger tips. In the experiments with the liposomes, both, the heat separated epidermal membrane and full thickness skin were used.

### 2.3. Preparation and characterization of liposomes

Three liposome formulations (Table 1) were prepared by a modification of the ethanol injection method described by Batzri and Korn (1973). For preparation of liposomes containing PE-NBD, thin-film hydration preceded ethanol injection. PE-NBD was dissolved in chloroform at a concentration of 2 mg/ml and phospholipid (Phospholipon® 80, Sphingomyelin or a mixture thereof) was added to a concentration of 375 mg/ml. The solution was thoroughly mixed and 800 μl of it were dried by rotary evaporation. The molar ratio of PE-NBD to phospholipid was 0.005 (0.5 mole%). The lipid film was hydrated in 2.5 ml ethanol 96% and water (containing Heparin when desirable) was poured rapidly into the ethanol. The two solutions were mixed at room temperature except for the SM formulation where mixing took place at approximately 45 °C in order to be above the phase transition temperature of the SM liposomes (37 °C). The final liposome formulation contained 25% (v/v) ethanol 96% and (where applicable) a Heparin concentration of 2000 IU/ml. For formulations without PE-NBD, the lipids were directly dissolved in 2.5 ml ethanol 96% and the same procedure was applied as above. For $\zeta$ potential measurements, formulations with Heparin concentrations of 50 and 200 IU/ml were also prepared. All liposome formulations were finally homogenized by Polytron (Kinematica AG, Switzerland) at 13 000 rpm for 12 min.

Table 1
Composition of liposome formulations (LF)

| | LF1 | LF2 | LF3 |
|---|---|---|---|
| Phospholipid | PL80 | PL80/SM | SM |
| Final lipid concentration (mg/ml) | 30 | 20/10 | 30 |
| Heparin (UH) (IU/ml) | 2000 | 2000 | 2000 |

The $z$-average mean and the polydispersity of size of the liposomes were determined by photon correlation spectroscopy using a Malvern Autosizer 2c with Autocorrelator 8, Type 7032 CN (Malvern Instruments Ltd, Malvern, UK). The samples were prepared by diluting the formulations 100-fold with double distilled water, or an aqueous medium containing 25% (v/v) ethanol or this medium containing in addition the same concentration of Heparin as the sample. The measurement was performed at 25 °C. The viscosity value of pure water was used in the calculation. The particle charge was assessed by $\zeta$ potential measurements, which were based on the electrophoretic mobility determined by Laser Doppler anemometry using a Zetasizer 3 (Malvern Instruments Ltd, Malvern, UK). The samples were prepared by diluting the formulations 100-fold with an aqueous medium containing 25% (v/v) ethanol and (where applicable) the same concentration of Heparin as the sample. The determination was performed at 25 °C. All formulations were measured in triplicate.

### 2.4. Skin sectioning device

A device for reproducibly preparing mechanical cross sections of excised skin perpendicularly to the skin surface without previous fixation was

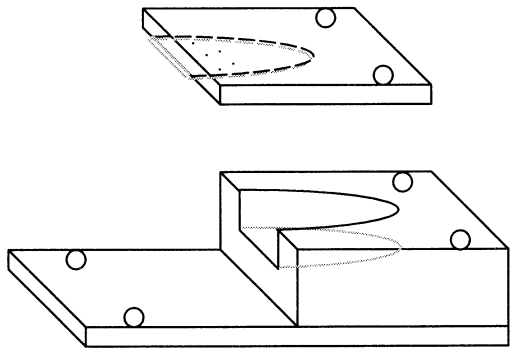

Fig. 1. Schematic drawing of the skin sectioning device. The base plate is shown with one rectangular PVC block bearing a semi circular indentation on its upper surface and one plexiglas plate used to clamp the skin on the block. Screw holes are shown.

designed based on a modification of the work by Meuwissen et al. (1998) (Fig. 1). Two rectangular blocks of PVC with a side length of 2 cm were fixed with screws on a base plate at a distance of approximately one half of a millimeter from each other. The upper surface of each block had a semi circular indentation (depth $\approx 2$ mm) and the two indentations were placed facing each other. A circular piece of fresh, full thickness skin was sandwiched between two teflon membranes (PTFE Membrane Filter, Gelman Sciences, Michigan, USA) and transferred flat into the indentation formed by the two blocks with the stratum corneum facing upwards. The skin was clamped onto the PVC blocks with two plexiglas plates which were fixed with screws one on each block leaving an approximately one half of a millimeter wide slit between them. The plexiglas plates had holes which were used to pass pins through them that perforated the skin specimen and prevented it from sliding during sectioning. The skin and the teflon membranes were cut along the slit perpendicularly to the skin surface with a razor blade. After sectioning, the two blocks, each containing one half of the original skin specimen, were separated exposing the planes of cut. These were inspected by confocal laser scanning microscopy in the blocks.

### 2.5. Confocal laser scanning microscopy (CLSM)

#### 2.5.1. Stack of confocal images parallel to the skin surface

Heat separated epidermal membrane was exposed to liposomes in modified Franz-type diffusion cells described in detail previously (Betz et al., 2001). The liposome formulations were applied occlusively and under light protection on the stratum corneum side of the epidermal membrane for 158 h, this time corresponding to the duration of the permeation/penetration experiments carried out before (Betz et al., 2001). The diffusion cells were kept in a water bath maintained at 37 °C, which resulted in a temperature on the skin surface of 33 °C. After application, the liposomes were rinsed away thoroughly with Sörensen buffer pH 6.4 (Wissenschaftliche Tabellen Geigy, 1979) and the epidermal membrane was transferred on a glass slide. The membrane was embedded in Mowiol solution (Hoechst, Frankfurt, Germany) containing glycerol and an antioxidant to prevent photo bleaching, covered with cover glass and kept for 24 h in the dark at room temperature for polymerization. This is a standard procedure employed in the interdepartmental electron microscopy unit of the Biocenter of the University of Basel. After embedding, the samples were kept in the dark at 4 °C until inspection by CLSM. A Noran Odyssey XL confocal microscope mounted on a Zeiss Axiophot 135 TV equipped with an Ar–Kr laser and a Plan Neofluar 40 × objective lens in fluorescence mode with illumination at 488 nm and emission at 525 nm was used.

#### 2.5.2. Optical cross sections perpendicular to the skin surface

Full thickness skin was exposed to liposome formulations as described in the previous section. Following exposure times of 30 min and 144 h, the liposomes were rinsed away with buffer and the skin was cross sectioned using the device described above. The resulting two cross section planes were used for CLSM imaging without further preparation and with the skin remaining mounted in the blocks of the sectioning device. For the skin treated for 30 min, optical sections were made parallel to the plane of mechanical cut

at a depth of 75–125 μm below the surface. An optical slice was recorded every 2 μm and these were combined to a 3D stereo image. For the skin treated for 144 h, images were taken 100 μm below the mechanical section plane. A Leica TCS NT SP confocal microscope, mounted on a Leica DM RXE and equipped with Ar, Kr, He–Ne lasers was used and images were recorded by $5\times/10\times$ HC Flustar APO CORR objective lens in fluorescence mode with illumination at 488 nm and emission at 525–550 nm without cover glass.

The CLSM imaging of cross sectioned skin mounted in the blocks of the sectioning device made it possible to record the distribution of the fluorescent label throughout the entire thickness of the skin. CLSM optical sections were obtained below the plane of the mechanical section thus avoiding artifacts from damaged cells or tissue at the surface of the cut.

#### *2.5.3. Autofluorescence of skin and stability of fluorescence*

Isolated epidermal membrane and full thickness skin were incubated with Sörensen buffer pH 6.4 with and without 25% (v/v) ethanol and with Heparin solution with a concentration of 2000 IU/ml under the same conditions as described above but in the absence of PE-NBD and inspected by CLSM. Also, epidermal membrane was bleached with a 5% (w/v) sodium boro-hydride solution in Sörensen buffer for 10 min prior to incubation with fluorescence labelled liposomes and compared with untreated membrane by CLSM. The fluorescence of PE-NBD in skin tissue was compared with that of a fluorescence labelled liposome solution in the presence and absence of 2000 IU/ml Heparin using the same microscope.

## 3. Results

### *3.1. Particle size of liposomes*

The particle size and the polydispersity of size distribution of the liposome formulations measured by photon correlation spectroscopy is shown in Table 2. Dilution of the sample with different media prior to measurement greatly influenced the result. Thus, dilution with distilled water yielded up to two-fold smaller particles than dilution with the solvent that was used in the formulation and contained ethanol or ethanol with 2000 IU/ml UH. The liposomes of formulations containing Heparin were markedly larger in size than those of formulations without Heparin. The formulation with pure SM liposomes (LF3) showed a larger particle size and a higher polydis-

Table 2
Particle size and polydispersity of size distribution of liposomes

| Dilution with | | Particle size $z$-average mean (nm) $\pm$ S.D. | Polydispersity index |
|---|---|---|---|
| | | *Formulations with heparin* | |
| Double distilled water | LF1 | $315.9 \pm 16.1$ | 0.21 |
| | LF2 | $251.8 \pm 4.6$ | 0.21 |
| | LF3 | $422.5 \pm 39.9$ | 0.44 |
| 25% (v/v) ethanol in water | LF1 | $440.9 \pm 12.0$ | 0.21 |
| Containing 2000 IU/ml UH | LF2 | $572.8 \pm 125.3$ | 0.19 |
| | LF3 | $743.2 \pm 104.0$ | 0.50 |
| | | *Formulations without heparin* | |
| Double distilled water | LF1 | $225.0 \pm 8.3$ | 0.17 |
| | LF2 | $200.9 \pm 8.2$ | 0.22 |
| | LF3 | $327.4 \pm 20.4$ | 0.80 |
| 25% (v/v) ethanol in water | LF1 | $375.6 \pm 7.9$ | 0.19 |
| | LF2 | $382.3 \pm 23.2$ | 0.25 |
| | LF3 | $693.6 \pm 173.1$ | 0.72 |

Table 3
ζ potential of liposome formulations

| | ζ potential [mV], mean, $n=3$ | | |
|---|---|---|---|
| | LF1 | LF2 | LF3 |
| Without Heparin | −11.80 | −8.82 | −3.41 |
| Heparin 50 IU/ml | −20.56 | −20.80 | −12.48 |
| Heparin 200 IU/ml | −20.77 | −17.87 | 1.23 |
| Heparin 200 IU/ml + 0.09% NaCl | −8.52 | | |
| Heparin 2000 IU/ml | −11.03 | −7.88 | −8.17 |

persity than the other formulations. The fluorescent label incorporated in the phospholipids for the CLSM studies at a concentration of 0.5 mole% did not affect the particle size of the different liposome formulations (result not shown).

### 3.2. ζ potential

The ζ potential of the liposomes of the formulations prepared without addition of Heparin was negative (Table 3). The presence of PL80 augmented the negative charge of the liposomes because of the phosphatidic acid that is contained in PL80 at a concentration of 8 mole%. Phosphatidylcholine, the major constituent of PL80, and other lipids contained in small amounts in PL80 are neutral in terms of charge. Pure SM liposomes had a small ζ potential because of the electroneutrality of SM. In the presence of 50 IU Heparin/ml, the ζ potential of all three liposome formulations was negative but its magnitude increased considerably compared with the formulations without Heparin. Upon increase of the Heparin concentration to 200 IU/ml the values of ζ potential leveled off and at 2000 IU/ml they declined. The addition of NaCl to the formulation with 200 IU Heparin/ml also depressed the ζ potential of the liposomes. The slightly positive value obtained for LF3 with 200 IU/ml seems to be an outlier that does not fit into the general pattern. Addition of Heparin in the medium used for diluting the samples prior to the measurement at a concentration corresponding to that of the original formulation was essential for obtaining stable ζ potential values.

### 3.3. Confocal laser scanning microscopy images

#### 3.3.1. Autofluorescence of skin and stability of fluorescence

The control experiments showed only a low level of autofluorescence of the skin specimens used in the present study contrary to findings of other groups (Zellmer et al., 1995). This was corrected by the photomultiplier setting. After the correction, skin exhibited no fluorescence of its own when excited either with the 488 nm line or with the 525 nm line of the laser. The fluorescence properties of PE-NBD were not influenced by the presence of Heparin, the used phospholipids, or both as a liposomal Heparin formulation.

#### 3.3.2. Stack of confocal images parallel to the skin surface

Two CLSM experiments with every liposome formulation each with and without Heparin were carried out. Representative images are shown in Fig. 2. Each image depicts an optical slice parallel to the surface of the skin with an area of 250 × 250 μm. Different penetration patterns of the fluorescent label from the four formulations into the epidermis were observed. For liposomes prepared with PL80 without Heparin, fluorescence was fairly uniformly distributed in the skin around non-fluorescent spots in depths of around 5 μm. From this it is evident that fluorescent phospholipid is distributed in the lipid domain of the stratum corneum outlining the corneocytes that do not show affinity for the label. Individual corneocytes with a roughly hexagonal shape and a diameter of 25–35 μm can be seen. The bright lines on the outermost surface indicate accumulation of the fluorescent label in the wrinkles of the skin. Deeper in the skin the intensity of the fluorescence label decreased and all but disappeared at depths $>10$ μm.

When SM was included in the formulation, no homogeneous distribution of fluorescence in the lipid matrix and, therefore, no corneocytes could be seen. The label appeared rather unevenly distributed across the plane of the skin. In the pres-

ence of Heparin, loci of high fluorescence intensity into depths $>10$ μm within the stratum corneum were observed for all phospholipid compositions indicating a strong localization and possibly aggregation of the label in contact with skin.

### 3.3.3. Optical cross sections perpendicular to the skin surface

Two CLSM experiments with every liposome formulation each with and without Heparin were carried out with exposure times of skin to liposomes of 30 min and 144 h. Representative 3D stereo images of the 30 min exposure are shown in Fig. 3. These images were recorded 12 h after the experiment. When images were recorded immediately after the experiment the results were identical (not shown). It is demonstrated that within 30 min the fluorescent phospholipid penetrated into deep layers of the dermis where the fluorescence was homogeneously distributed and particularly strong. In upper dermis layers and in the epidermis, fluorescent phospholipid was apparent only along distinct thread-like channel pathways. Hair shafts were strongly fluorescence labeled along their entire length reaching into the dermis layer which showed the homogeneous and intense fluorescence. These observations were the same for all formulations and independent of the pres-

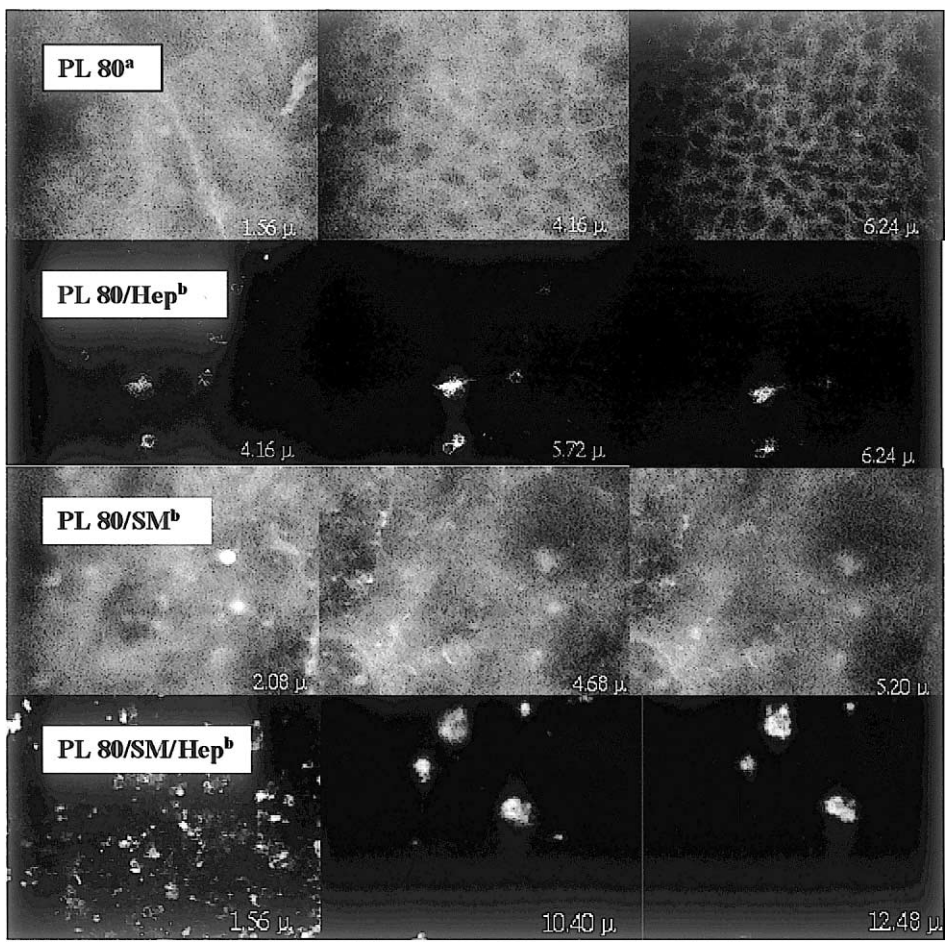


Fig. 2. Stack of confocal images parallel to the skin surface for PL80 and PL80/SM formulations each without and with Heparin. The size of an individual image is $250 \times 250$ μm. The depth of the image is indicated on each frame. [a]Penetration into epidermal membrane mainly by the lipid matrix as penetration pathway. [b]Aggregation of liposomes on the surface of skin.

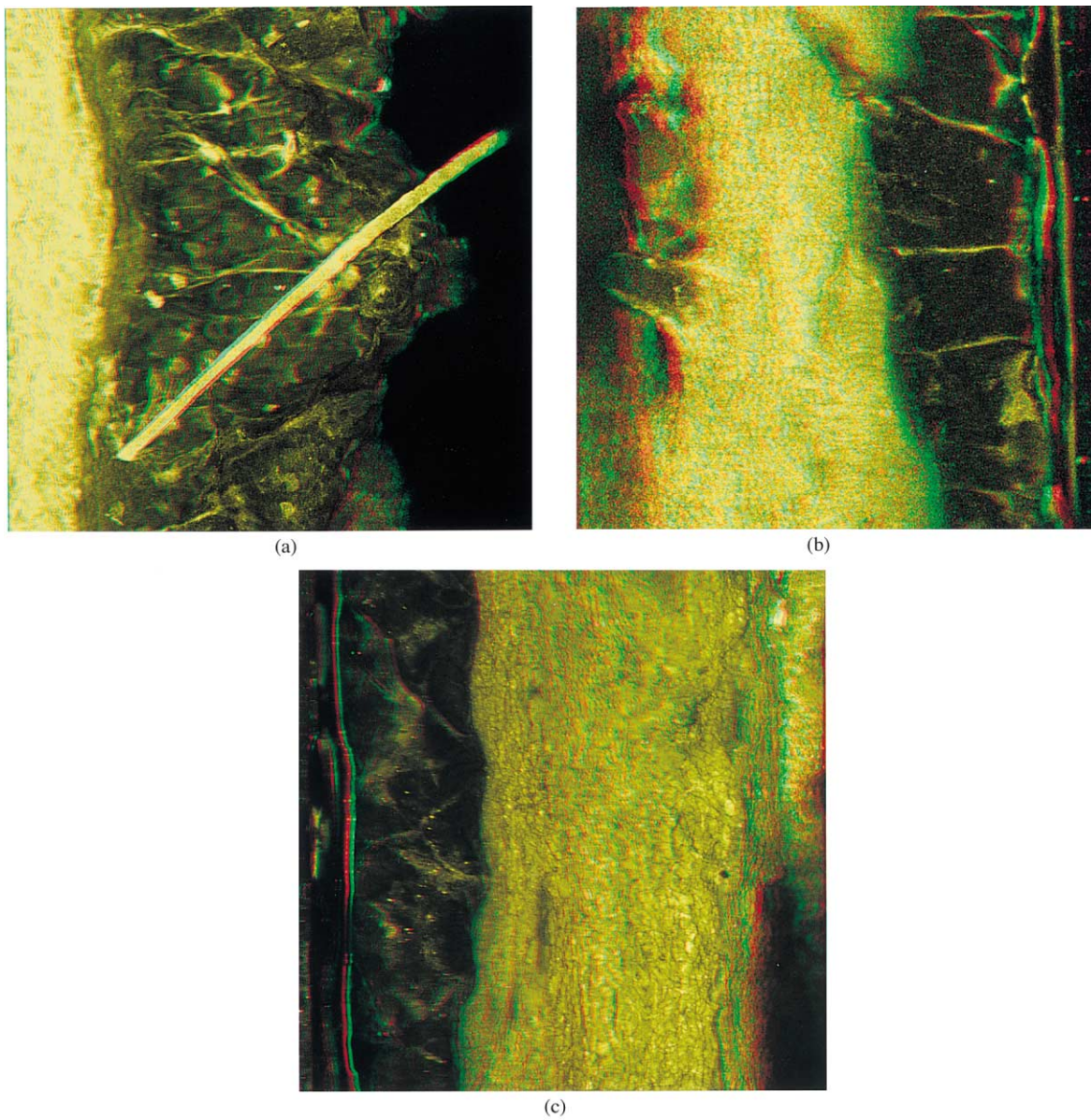


Fig. 3. Optical cross sections perpendicular to the skin surface. (a): PL80/SM formulation with Heparin. (b): PL80 formulation with Heparin. (c): PL80 formulation without Heparin. Scale: (a): $1000 \times 1000$ μm; (b) and (c): $2000 \times 2000$ μm. The stratum corneum surface of the skin faces to the left hand side of the image. Three-dimensional stereo anaglyph generated by superimposed images shifted by $-5°$ and $+5°$ which are represented by red and green color and produce the yellow color when combined.

ence of Heparin. Fluorescent label appeared uniformly in an outermost superficial layer of the skin only for the PL80 formulation.

For the 144 h exposure of skin to the liposomes, images were recorded at maximum 48 h after the experiment. This lapse of time until imaging was not expected to influence label distribution since no such influence was observed for the much shorter exposure time of 30 min when imaging was carried out immediately and 12 h after the exposure.

The penetration depth of the fluorescent phospholipid into skin after 144 h (representative images in Fig. 4) exhibited no difference to that after 30 min. The deeper layers of the dermis were homogeneously and intensely fluorescent. Above this layer, a very faint or no fluorescence at all was observed. In contrast to the 30 min exposure, the thread-like fluorescent pathways were not evident here. For the PL 80 formulation only, fluorescence was detected to penetrate homogeneously in considerable depth into the epidermis which is in agreement with the findings from the images taken parallel to the skin surface.

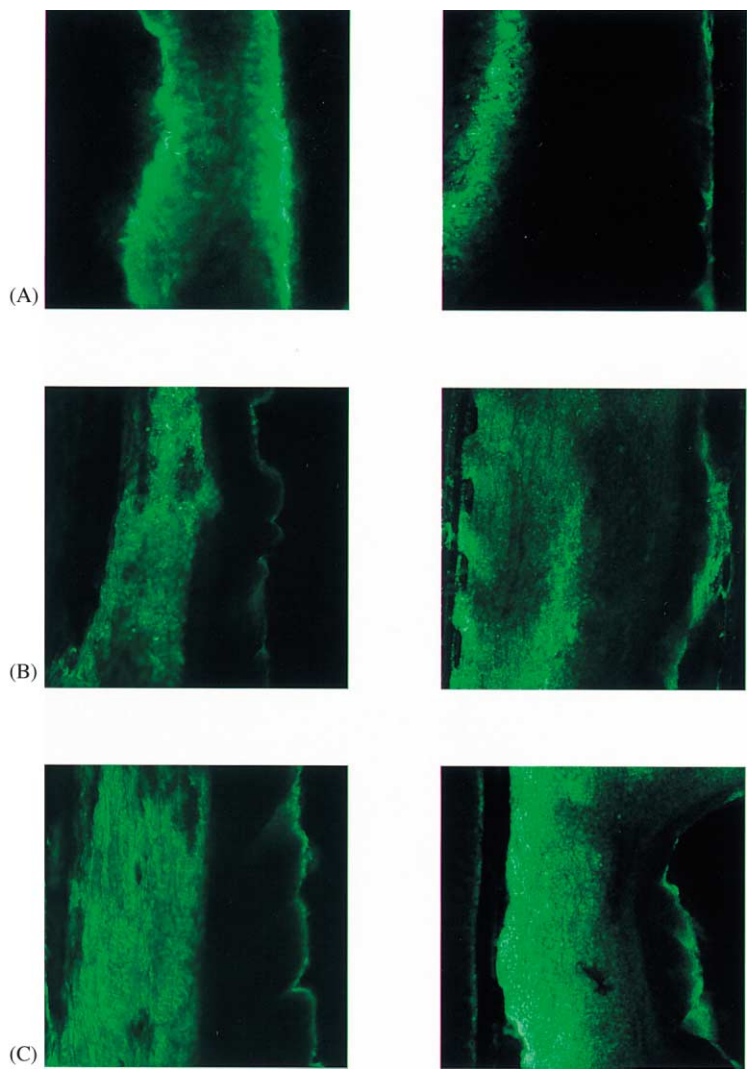


Fig. 4. Optical cross sections perpendicular to the skin surface. A: PL80 formulation. B: PL80/SM formulation. C: SM formulation. The left hand side images correspond to formulations without Heparin and the right hand side images to formulations with Heparin. Scale: A: 1000 × 1000 μm. B and C left hand side: 1000 × 1000 μm. B and C right hand side: 2000 × 2000 μm. The stratum corneum surface of the skin faces to the right hand side of the image. Recorded in the green channel.

## 4. Discussion

### 4.1. Liposome characterization

The larger liposome size found for all formulations after dilution with 25% (v/v) ethanol in water compared with dilution with double distilled water is consistent with the finding of Kirjavainen et al. (1999), who suggested that the increased liposome size in the presence of ethanol might be due to a decrease in interfacial tension or the induction of interdigitation. The size of liposomes in the formulations, therefore, corresponds to that measured after dilution with 25% (v/v) ethanol in water.

The increased liposome size in the presence of Heparin suggests an adsorption of Heparin molecules onto the surface of the liposomes.

The $\zeta$ potential measurements confirm this hypothesis. The increase of magnitude of the $\zeta$ potential in absolute terms elicited by the addition of 50 IU/ml Heparin to the liposome dispersion is consistent with a covering of the liposome surface with Heparin molecules that bear a negative charge because of their free sulfate groups. Upon further increase of the Heparin concentration in the liposome dispersion, the negative $\zeta$ potential of the liposomes remained constant, at first, and then diminished. This is probably due to a combination of two phenomena. Firstly, surface adsorption is generally a saturable process implying that the amount of Heparin adhering to the liposome surface will not increase steadily with concentration, reaching instead a plateau. Secondly, $\zeta$ potential depends on the ionic strength of the solution. Thus, increasing Heparin concentration results in an increase of the ionic strength which suppresses $\zeta$ potential. This probably causes the $\zeta$ potential to diminish, in absolute terms, at the Heparin concentration of 2000 IU/ml. The 0.09% NaCl solution, which contained roughly the same concentration of electrical charges as the 2000 IU/ml Heparin solution, caused the same drop of $\zeta$ potential when included in the 200 IU Heparin/ml liposome formulation, supporting this interpretation. Hence, these results provide indirect but clear evidence that the surface of the liposomes is covered by Heparin molecules. This also explains the fact that samples diluted prior to $\zeta$ potential measurement with a medium containing no Heparin gave continuously changing readings.

A high surface charge of liposomes enhanced their physical stability. Liposomes of the LF3 formulation had a much higher polydispersity index, particularly in the absence of Heparin, than the other formulations. The high polydispersity indicates aggregation and possibly fusion of the SM liposomes which is attributed to the virtual lack of a net surface charge of these liposomes. The physical state of the phospholipid bilayer at the temperature of the experiment, however, also plays a role for the stability of the liposome dispersion (see below).

### 4.2. Gel-state versus liquid crystalline-state liposomes

The PL80 phospholipid bilayer is at the temperature of the experiment in the liquid crystalline state which is related to the fact that its chief constituent, phosphatidylcholine, contains mainly linoleic acid, a doubly unsaturated fatty acid (see Betz et al., 2001). The SM bilayer, on the other hand, is at the temperature of the experiment in the gel state because of the content of palmitic acid, a saturated fatty acid, in SM. This consideration may be responsible for the different fashion in which the liposome formulations interacted with the stratum corneum. van den Bergh et al. (1998), showed by freeze-substitution electron microscopy that gel state liposomes aggregate, fuse and adhere on the stratum corneum surface, thereby depositing stacks of lamellar sheets and forming lipid bilayer networks. On the other hand, liquid crystalline liposomes did not aggregate or fuse on the surface of the stratum corneum but were reported to induce interactions with intercellular lipids in deeper stratum corneum layers. The greater flexibility of the bilayer and freedom of movement of the individual phospholipid molecules in the liquid crystalline state compared with the gel state may be related to this difference of interaction of the liposomes with the stratum corneum. Similarly, Zellmer et al. (1995), reported that phosphatidylcholine liposomes remained homogeneously dispersed within

reconstructed human epidermis while phosphatidylserine and human stratum corneum lipid liposomes aggregated on the surface and within the epidermal cell layers. Finally, in the work of Kuijk-Meuwissen van et al. (1998), label applied non-occlusively in liquid crystalline state vesicles on rat skin in vivo penetrated deeper into the skin than when applied in gel state vesicles. The presence of 20–50% ethanol in liposomal formulations was reported to lower the phase transition temperature and increase the fluidity of the liposomal membrane thereby promoting interaction with the stratum corneum. Thus, ethanol containing phospholipid vesicle preparations termed ethosomes were shown to improve the permeation properties of drugs through animal skin (Touitou et al., 2000a Touitou et al., 2000b).

The findings of the CLSM images recorded parallel to the skin surface in the present work are consistent with these literature results. It is considered that distribution of fluorescence labelled phospholipid in tissue reflects the distribution of liposome-forming lipids. An homogeneous distribution in the lipid domains of the stratum corneum was observed only for the liquid crystalline state PL80 liposomes whose behavior may be enhanced by the presence of ethanol. SM liposomes showed an uneven distribution on the skin which could possibly be related to their aggregating on the skin surface. The strong localization of lipids observed in the presence of Heparin may be due to the fact that liposomes are covered with Heparin, not allowing individual phospholipid molecules to interact with the stratum corneum.

### *4.3. How deep do liposomes penetrate into the skin*

Since only the fluorescence labelled phospholipid is visualised by CLSM, this technique provides reliable information about the penetration of phospholipids in the skin but not about the question whether liposomes can penetrate as intact vesicles. A penetration and accumulation of lipids deep into the dermis took place within 30 min and the penetration profile was essentially the same after 144 h. This was independent of the composition of the liposome formulation and of the presence of Heparin. The quickness of penetration found here was of the same order but somewhat greater than the one reported by other workers, i.e. 1–3 h (Meuwissen et al., 1998), or 3–6 h (Kuijk-Meuwissen van et al., 1998).

The penetration of lipids through the epidermis and the upper dermis appears to follow distinct thread-like pathways. Similar images were reported before (van den Bergh et al., 1999) and one might hypothesize that in the upper skin layers these pathways correspond to the lipid matrix domain near the edges of the corneocytes in the stratum corneum (Cevc, 1996) and the intercellular domains of the viable epidermis. Furthermore, the intense fluorescence staining of the hair shaft in its entire length suggests that lipids may follow the route of the hair follicle to penetrate into the deep dermis layers. The inner root sheath surrounding the hair shaft presents an opportunity for molecules to diffuse along the hair follicle. This compartment is filled with sebum which does not form a barrier to diffusion comparable to the intercellular domain present in the SC. The hair follicle has a diameter of approximately 40 μm (Dawber, 1985). The entrance of the hair follicle may serve as a trap for particles (Schaefer and Redelmeier, 1996). The rather quick penetration of phospholipids into the dermis is consistent with the follicular pathway. The intensely fluorescent lower layer of the dermis can roughly correspond to the reticular dermis, which is made of densely packed thick fibrous bundles of collagen.

It is generally accepted that standard liposomes do not penetrate intact into the skin. The distance between the intercellular lipid bilayers is very small compared with the size of standard liposomes, the average size of one Landmann unit being $\pm 12.8$ nm (Madison et al., 1987). It has been argued, however, that transfersomes™ might be able to penetrate into and permeate across skin. Over all, the extent of interaction between lipid vesicles and skin seems to be highly dependent on the lipid composition of liposomes. Thus, the fluorescent label of DMPC vesicles applied non-occlusively on human cadaver skin remained on top of the skin (Zellmer et al., 1995), while with DLPC and $C_{12}EO_7$ vesicles fluorescent label

was detected mainly in the stratum corneum after 1 and 3 h of application and in the dermis after 6 h (Meuwissen et al., 1998) and the addition of DOPE and octaoxyethylene laurate ester to liposome compositions enhanced the penetration of the fluorescence label into the stratum corneum (Kirjavainen et al., 1996, 1999; van den Bergh et al., 1999).

## 5. Conclusion

Penetration of phospholipids into deep dermis layers is achieved within a relatively short period of time regardless of the composition of the liposomes used in this study. The hair follicle likely plays a role as a pathway for this penetration. The distribution of phospholipids within the lipid matrix of the stratum corneum depends on liposome composition. The liquid crystalline state of the unsaturated phosphatidylcholine bilayer membrane of Phospholipon 80 in the presence of ethanol seems to favor this distribution. Heparin forms a coating around liposomes and influences the interaction of phospholipids with the lipid matrix of the stratum corneum but not their penetration into the deeper dermis. However, the penetration of phospholipids into the dermis is not accompanied by Heparin penetration that stays in the upper epidermis layers (Betz et al., 2001), indicating that a dissociation of the Heparin from the liposomes takes place.

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