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Interaction of phosphatidylcholine liposomes with the human stratum corneum

Sebastian Zellmer *, Wolfgang Pfeil [1], Jürgen Lasch

Institut für Physiologische Chemie, Martin-Luther-Universität Halle-Wittenberg, D-06097 Halle, Germany

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Abstract

The interaction of dimyristoylphosphatidylcholine liposomes with the human stratum corneum was investigated by confocal laser scanning microscopy and differential scanning calorimetry. Human skin is characterized by a high autofluorescence. By introducing appropriate optical filters the autofluorescence of the skin was depressed and the penetration profile of fluorescence labelled vesicles was investigated. From optical sectioning it was obvious that neither the vesicles nor the fluorophore *N*-(lissamine rhodamine B sulfonyl)diacylphosphatidylethanolamine (Rho-PE) penetrates in detectable amounts into the human skin. Differential scanning calorimetry of human stratum corneum revealed, that the peak positions of the human stratum corneum specific endothermic transitions at 10°C, 35°C, 50°C, 62°C, 73°C and 81°C did not change significantly after 18 h of non-occlusive vesicle application. However, the enthalpy of the transitions at 35°C, 50°C, 62°C and 73°C, estimated through peak heights increased, relative to the protein related peak at 81°C. A novel transition at 10°C was observed. From these data we conclude that DMPC liposomes do not penetrate intact into the human skin. We deduce, however, that the vesicles disintegrate at the surface of stratum corneum after non-occlusive application. The individual lipid molecules then interact with the lipid barrier of the stratum corneum and penetrate into the latter, which results in an increase of the enthalpy, related to the lipid components of the SC.

Keywords: Skin; Liposome; Penetration; Confocal laser scanning microscopy; Calorimetry

1. Introduction

Mezei and Gulasekharam [1] were the first to report that liposomes loaded with triamcinolone acetonide facilitated a 3–5-fold accumulation of the drug within the epidermis and the dermis. The results were ambiguous and the experimental set-up was criticised. However, numerous investigations on the penetration of liposomes through the skin and drug penetration enhancement by liposomes were launched (for a recent summary, see [2]). While Ganesan and coworkers [3] demonstrated in an elegant experiment that neither intact liposomes nor encapsulated material penetrates the human skin at increased rates, Lasch and Wohlrab [4] showed, that egg lecithin/cholesterol liposomes facilitate the penetration of cortisol into the human skin and suggested that the vesicles disintegrate in the deeper strata of the skin. Recent fluorescence microscopy [5] and perturbed angular correlation spectroscopy experiments [6] confirmed that egg lecithin liposomes do not penetrate intact the stratum corneum (SC) and disintegrate at the skin surface. Enclosed hydrophilic markers were released and the encapsulated material was not detected below the SC.

Electron paramagnetic resonance measurements [7] showed that egg lecithin/cholesterol liposomes facilitate the transport of the hydrophilic spin probe ASL into (porcine) skin. However, most of the vesicles disintegrated and only 5% of the encapsulated spin probe was protected from reduction in the stratum corneum. The authors suggested that small amounts of the spin probe are transported encapsulated into the skin, facilitated by liposomal lipids.

Abbreviations: ASL, (*N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)-*N*-dimethyl-*N*-hydroxyethylammonium iodide; CLSM, confocal laser scanning microscopy; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DSC, differential scanning calorimetry; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; FTIR, Fourier transform infrared; Rho-PE, *N*-(lissamine rhodamine B sulfonyl) diacylphosphatidylethanolamine; SC, stratum corneum.

* Corresponding author. Fax: +49 345 5573811.

[1] Present address: Max-Delbrück-Centrum für Molekulare Medizin, Robert-Rössle-Straße 10, D-13125 Berlin, Germany.

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In contrast to these results, Artmann and coworkers [8] claimed that liposomes, prepared from a certain soy-bean phospholipid composition (Nat 106) with a phosphatidylcholine content of 85% transported antibodies into porcine skin and Ghyczy [9] showed in addition that this soy-bean phospholipid composition influenced the skin hydration for 3 h, while other soy-bean phospholipid compositions had no or adverse effects. Small angle X-ray diffraction experiments and electron microscopy confirmed that the skin-vesicle interaction was influenced by soy-bean phospholipid composition [10] and that vesicular structures appeared in the stratum corneum after the application of Nat 106 on skin. The lipid headgroup and the fluidity of the lipid chains seems to have a profound effect on the lipid interaction with the SC, since polar lipid head groups promoted transepidermal water loss [11].

On the basis of physico-chemical considerations, Cevc [12] developed Transfersomes™, phospholipid/detergent mixed vesicles, for enhanced drug transport through the SC into the viable skin. These vesicles seem to penetrate intact into the human skin [13,14]. Recent confocal laser scanning microscopy [15] and immunization experiments (personal communication) support this view.

In this paper we will show by means of confocal laser scanning microscopy that DMPC liposomes are confined to the skin surface and that membrane-bound lipophilic fluorophores are not transported to an appreciable amount through the stratum corneum into the viable skin. Differential scanning calorimetry indicates, however, that the lipid molecules interact with the lipids of the stratum corneum, thereby modifying the thermal transitions of the intact tissue.

2. Materials and methods

2.1. Chemicals

1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) was purchased from Boehringer (Mannheim, Germany). Trypsin (Type II, activity: 1.44 BAEE units/mg solid) and sodium azide were from Sigma (St. Louis, MN). *N*-(Lissamine rhodamine B sulfonyl)diacylphosphatidylethanolamine (Rho-PE) was from Avanti Polar Lipids (Pelham, Alabama). CH_3OH, $CHCl_3$ and NaCl, Na_2HPO_4, NaH_2PO_4, all p.a., were products of Merck (Darmstadt, Germany). The latter were used to prepare 145 mM phosphate buffer, pH 7.3.

2.2. Preparation of liposomes

DMPC was dissolved in chloroform/methanol (1:1) and Rho-PE was added to a final concentration of 0.02% (mol/mol). The organic solvents were removed under a gentle stream of warm nitrogen and the lipids were dried under reduced pressure over night. The dry lipid film was hydrated with 145 mM phosphate buffer (pH 7.3; composed of 1.57 g $NAH_2PO_4 \cdot 1H_2O$, 19.8 g $Na_2HPO_4 \cdot 12 H_2O$, 81 g NaCl in 10 l distilled water) to a final lipid concentration of 50 mg/ml and a transparent vesicle suspension was obtained after tip-probe sonication with a Branson Sonifier W-250 (Danbury, CT) above the chain melting temperature of DMPC (23°C). Unlabelled DMPC vesicles (5%; w/v) for the calorimetric measurements were prepared in the same way by direct hydration of DMPC with 145 mM phosphate buffer (pH 7.3), followed by tip probe sonication.

2.3. Stratum corneum isolation

Fresh human skin from mamma ablatio (breast cancer) was obtained after surgical intervention. The skin was incubated for 24 h in phosphate buffer (145 mM, pH 7.3) with 0.5% (w/v) trypsin. Sodium azide 0.01% (w/v) was added to prevent bacterial growth. After an incubation of 24 h at room temperature the stratum corneum (SC) was separated from the skin and transferred into a separate Petri dish. Excess trypsin and NaN_3 were removed by washing in phosphate buffer (145 mM, pH 7.3). The SC was cut in pieces (approx. 1 × 1 cm) and mounted in a Franz-type diffusion cells in such way that the morphological inner side faced the reservoir. The lid was secured to the reservoir by adhesive tape (Tesafilm, Beiersdorf AG, Hamburg, Germany). The orifice of the lid had a diameter of 7 mm and the volume of the reservoir was 400 μl, which was filled with 145 mM phosphate buffer (pH 7.3). Microscopy confirmed that the SC was intact and free of holes.

2.4. Calorimetric measurements

3 μl DMPC suspension (50 mg/ml) were applied at 20–22°C on the morphological outer side of the SC, mounted into a Franz-type diffusion cell. The suspension covered an area of 2 mm in diameter. 18 h later a SC disk (7 mm in diameter) was cut out along the border of the lid and hydrated for 6 h in 145 mM phosphate buffer (pH 7.3). Subsequently, 15–20 specimen of these SC pieces were transferred into a MicroCal DC-2 differential scanning calorimeter (MicroCal, Northampton, MA) and the sample was equilibrated for 60 min at 2°C. The thermal transitions of these SC pieces were determined in the range of 2–100°C at a scan rate of 30 K/h. At the end of each measurement the dry weight of the SC was determined and all data were expressed per mg dry weight. Six peaks in the stratum corneum and in the stratum corneum + DMPC thermograms were determined. These measurements were repeated with SC samples from 5 different donors and the mean of the peak locations as well as the peak heights as estimates of the enthalpy were determined. Peak heights were measured from a linear baseline between the lowest point in the range of 15–20°C to the lowest point at the

end of the scan. Student's t-test was used to test for significances.

2.5. Confocal laser scanning microscopy

Human skin from mammectomy was cut into small pieces (1 cm^2, 1–2 mm thick) and placed onto a kitchen sieve with the morphological outer side upward. The sieve was placed above a Petri dish, filled with phosphate buffer (145 mM, pH 7.3) to allow contact between the buffer and the lower dermis. 2 μl of DMPC liposomes (5%, w/v), labelled with 0.02% Rho-PE (w/v) were carefully applied on the stratum corneum at 20–22°C. 18 h later the skin sample was mounted, submersed in 145 mM phosphate buffer (pH 7.3), onto a glass slide and the depth of lipid (liposome) penetration into the skin was determined with a confocal laser scanning microscope LSM-410 (Zeiss, Oberkochen, Germany). A Zeiss Plan-Neofluar ×40/1.3 oil immersion objective with a long working distance was used. The 543 nm excitation line of the He/Ne laser (0.5 mW) was used, a 560 nm dicroic beam splitter was inserted into the emission light path and the fluorescence intensity was detected above 590 nm. In order to reduce the intensity of light from tissue autofluorescence an attenuation filter was mounted into the excitation light path, which decreased the light intensity to 1% or 0.3%. The pinhole size was 5% of its maximal aperture. Gain (brightness) and offset (contrast) of the photomultiplier were set to 98% and 26% of its maximal value, respectively. These values were varied by 2%, depending on the autofluorescence intensity of the tissue but were kept constant throughout one measurement session for data comparison. The measurements were repeated with skin samples from 5 different donors. It was always assured that the inspected skin area was parallel to the cover glass, by taking images in the xy-mode. Therefore all cross section images are perpendicular to the stratum corneum surface.

3. Results

In order to determine the interaction of liposomes with the intact human stratum corneum (SC) we applied DMPC liposomes non-occlusively on isolated SC, mounted into Franz-type diffusion cells (same model as in [16]). The purity of DMPC was verified by calorimetry, since the pretransition T_p at 14°C and the lipid chain-melting transition at 23°C (cf. Fig. 1) were clearly detected. The endothermic profile of the treated and the untreated SC was determined 18 h after the application of the vesicles. In general, 6 endothermic transitions were observed at approx. 10°C, 35°C, 50°C, 62°C, 73°C and 81°C (Fig. 1 and Table 1). One novel transition at 10°C (Fig. 2) was detected. The location of the peaks varied by a few degree in each experiment, due to the biological variability of the samples. In all lipid treated samples the endothermic transition of DMPC at 23°C was resolved. However, we could not detect any significant temperature shift of the 6 main endothermic transitions after the liposomal treatment (Table 1). The endothermic profile of the SC, treated with liposome suspension, changed markedly, compared to control. In general, the profile of the DMPC-treated SC flattened after the addition of the vesicles (Fig. 1 and Fig. 2, Nos. 1 and 2).

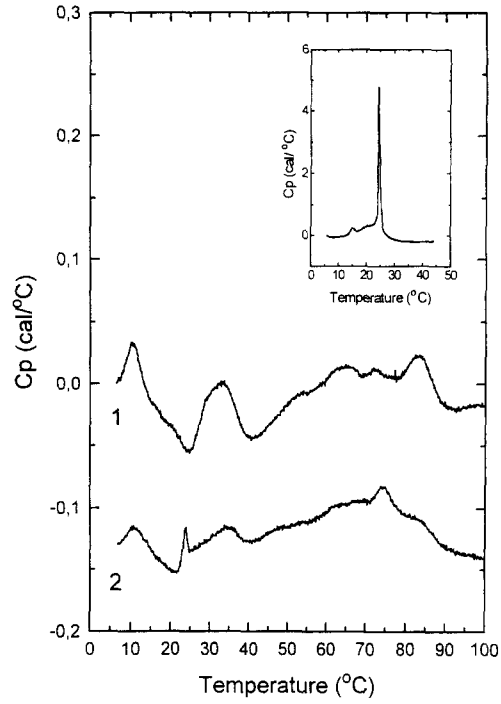

Fig. 1. Endothermic profile of trypsin-isolated human stratum corneum. Curve 1 represents the untreated stratum corneum with 6 transitions at approximately 10°C, 35°C, 50°C, 62°C 73°C and 81°C. Upon non-occlusive application of 3 μl DMPC liposome suspension (50 mg/ml) on the stratum corneum (mounted into Franz-type diffusion cells), the height of the endothermic transition of lipid-related peaks changed, relative to the peak height at 81°C (curve 2). All thermograms were corrected for stratum corneum dry weight per DSC cell volume. (Inset) Thermogram of the DMPC liposome suspension applied on the stratum corneum. The pretransition T_p at 14°C and main transition T_m at 23°C are well resolved, indicating the high purity of the lipid.

Table 1
Thermal transitions temperatures of pure human stratum corneum and stratum corneum after 18 h of non-occlusive application of DMPC vesicles

Thermal transition temperature [a] (°C) of	
Stratum corneum	stratum corneum + DMPC liposomes
10.4 ± 0.5	10.7 ± 0.6
34.3 ± 1	35.5 ± 1.6
51.6 ± 2	48.7 ± 1.4
62.4 ± 1.6	63 ± 1.7
72.1 ± 1.2	74.5 ± 2.2
81.2 ± 0.7	82.7 ± 2

[a] Values are expressed as the mean ± S.D. of 2–5 separate measurements.

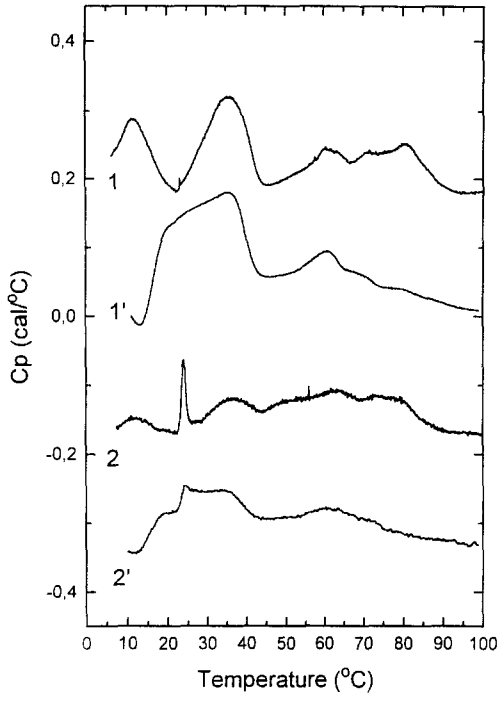

Fig. 2. Endothermic profile of trypsin-isolated human stratum corneum before (1) and after (2) 18 h non-occlusive application of 3 μl DMPC liposome suspension (50 mg/ml). Reheating of the stratum corneum resulted in a markedly different endothermic profile, due to irreversible disturbances of the stratum corneum structure and its components (curves 1′ and 2′).

Table 2
Changes in the SC characteristic peak heights after 18 h of non-occlusive application of DMPC vesicles, relative to the keratin related peak height at 81°C

Transition temperature	increase/decrease of the peak height
10°C	↓
35°C	↑/±
50°C	↑
62°C	↑
73°C	↑

The transition at 81°C has been related to proteins [17] and the other transitions to intercellular lipids. Since the protein transition should be independent of DMPC-stratum corneum interactions we decided to compare the heights of the 5 lower transitions to this protein-related transition. In all experiments the height of the SC transition at 10°C decreased and the height of the transition at 50°C, 62°C and 73°C increased, relative to the transition at 81°C (Table 2). The transition at 35°C decreased in one experiment and did not change in another experiment, relative to the transition of the SC proteins. The non-occlusive application of DSPC liposomes (T_m = 55°C), which are in the gel state at ambient temperature, resulted also in a disappearance of the SC transition at 10°C (data not shown).

Fig. 3. Optical tangential section (xy-direction) through human skin in the plane of the stratum corneum by means of confocal laser scanning microscopy. Corneocytes are clearly visualized due to the high auto-fluorescence of the tissue (Ex. 543 nm, Em. > 590 nm).

An irreversible alteration of the SC thermogram occurred during the calorimetric measurement, since a reheating of the same sample resulted in a different endothermic profile (Fig. 2, Nos. 1′ and 2′). The transition at 81°C decreased, indicating its protein-related origin. The transition at 35°C broadened. These reheated SC did not reorganize within one week (data not shown). In order to evaluate the influence of the liposomal suspension on the SC we analyzed always the endothermic profile of the first heating scan.

Confocal laser scanning microscopy was used to determine whether fluorescent labelled DMPC vesicles penetrate into the human skin. In contrast to conventional microscopy CLSM can provide optical cross sections of tissue in situ and allows high resolution in the z-direction. Fig. 3 is a xy (tangential) section through the human stratum corneum. Even without any fluorophore on the SC the corneocyte boundaries can be clearly resolved at an excitation wavelength of 543 nm and an emission wavelength above 590 nm, due to tissue autofluorescence. Autofluorescence was also detected at an excitation wavelength of 488 nm and 514 nm (data not shown). The individual corneocytes had the shape of an hexagon with a diameter of approx. 30 μm, which corresponds to literature data [18]. The high autofluorescence of the tissue, which allowed the visualization of the individual corneocytes, hampered the determination of liposome distribution in the skin. In order to depress noise from autofluorescence and to resolve all fluorescence labelled lipid vesicles we developed a stringent experimental protocol: a clear autofluorescence image of the corneocytes was prepared by setting gain and offset of the photomultiplier to the appropriate values. Then an 0.3% attenuation filter was inserted into the excitation light path, which suppressed the autofluorescence. The penetration profile of Rho-PE labelled DMPC liposomes was then determined in the xz-measurement mode (cross-section) of the microscope. Fig. 4 consists of 4 optical cross-sections, obtained in the xz-measurement mode, through human skin. The skin surface exhibited a high autofluorescence and, as a consequence, light reflection at the cover glass occurred (Fig. 4A). Decreasing the excitation intensity to 1% or 0.3% eliminated the signal from autofluorescence (Fig. 4B).

Application of fluorescent labelled liposomes without reduced excitation intensity resulted in a extremely bright signal (Fig. 4C). This signal consisted of skin autofluorescence and fluorescence of Rho-PE labelled vesicles. It was not possible to distinguish between autofluorescence of human skin and fluorescence of the labelled vesicles. The insertion of an attenuation filter reduced the autofluorescence of the skin (Fig. 4B) and allowed for a clear resolution of the fluorescent labelled vesicles on top of the skin (Fig. 4D). No fluorescence could be detected in the deeper skin layers.

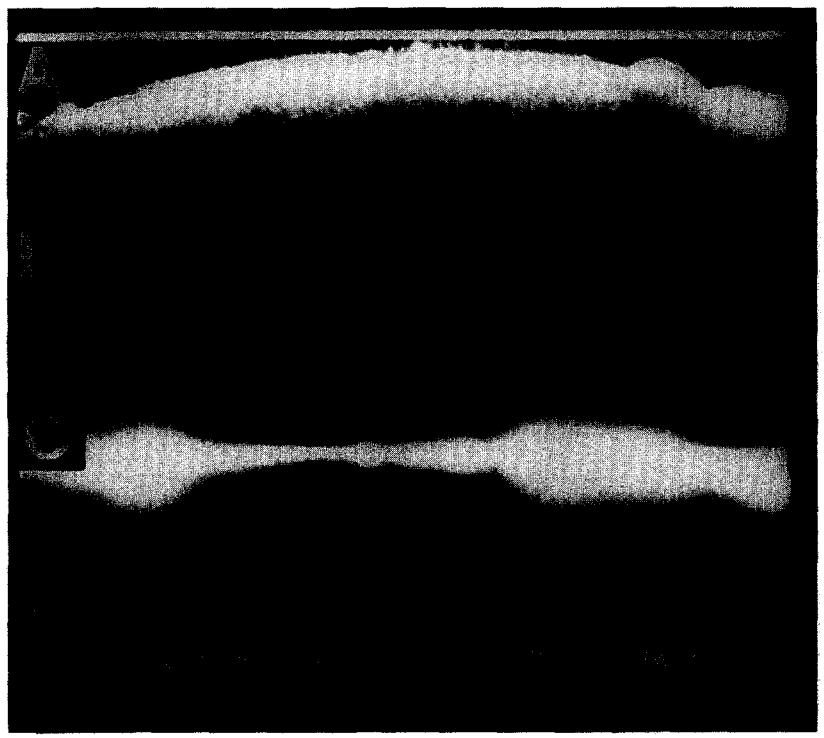

Fig. 4. Optical cross-section in xz-direction through human skin. The skin exhibited a high auto-fluorescence (A), which can be suppressed by inserting a 0.3% attenuation filter into the excitation light path (B), thereby reducing the light intensity of the excitation wavelength. Without filters the auto-fluorescence and the fluorescence of Rho-PE labelled liposomes, applied on the stratum corneum give a very bright signal (C). After insertion of the filter, only the fluorescent labelled vesicles are detected on the skin (D). This indicates that neither intact DMPC liposomes nor Rho-PE penetrates in appreciable amounts into the human skin.

4. Discussion

It is generally accepted that liposomes do not penetrate intact into the skin [2–4,6]. An exception are some experimental findings of Artman and coworkers [8] and of Mezei [19]. Recently developed ultradeformable vesicles, Transfersomes™ [12,13], however, might be able to penetrate the skin. Experimental findings support this view [15]. The current available data, however, allow the conclusion that standard liposomes do not penetrate intact into the skin. An enhancement of lipophilic drug penetration, however, is generally accepted. Either due to high concentration of the lipophilic drug at the liposome/skin interface [3], lipid induced alteration of the SC with facilitated liposome penetration or by direct vesicle/stratum corneum lipid fusion, which was reported for niosomes [20]. We have used confocal laser scanning microscopy to confirm earlier data [5,6] that standard liposomes do not penetrate intact the stratum corneum. After 18 h non-occlusive application of Rho-PE labelled DMPC liposomes, the skin was mounted on a glass slide and confocal laser scanning images were taken. An unexpected high autofluorescence occurred, which was eliminated by introducing an attenuation filter into the excitation light path (Fig. 4). From these data we conclude that care should be taken by analyzing CLSM images and the penetration profile of fluorescence labelled particles in tissues. In order to obtain good images one should avoid tissues with high autofluorescence and the use of labels with high fluorescence intensity is advisable.

The optical *xz*-sections showed that the lipophilic fluorophore remains confined at the skin surface (Fig. 4D). Therefore, we conclude that the non-occlusively applied DMPC liposomes dry on the surface of the skin, which results in a deposition of a lipid film on the stratum corneum. Neither intact DMPC vesicles nor Rho-PE, which was incorporated in the vesicle membrane, penetrated in substantial amounts into the skin. This is in agreement with recent perturbed angular correlation spectroscopy measurements [6].

Differential scanning calorimetry, however, provided evidence for interaction of DMPC with SC. The purity of DMPC was high, since the lipid pretransition, which is very sensitive towards impurities [21], was clearly resolved at 14°C (Fig. 1). Six thermal SC transitions at 10°C, 35°C, 50°C, 62°C, 73°C and 82°C were detected (Table 1). In all liposome treated samples the transition of DMPC was resolved (Fig. 1). The measurement, however, did not allow to distinguish between DMPC on the surface of the SC and DMPC in the SC.

The transitions at 40°C, 75°C and 85°C were reported earlier by Van Duzee [22]. The author reported that the transitions of different skin samples may vary by as much as 7°C, which can explain the differences between his and our results. Individual differences were also reported by Bouwstra and coworkers [23]. The transitions at 35°C and 62°C are most likely due to SC lipids [17], since the transition is reversible upon reheating (Fig. 2, Nos. 1 and 1′).

The transition at 35°C (Fig. 1 and Fig. 2) may correspond to the transition at 40°C, determined by X-ray scattering analysis of human SC [23]. The authors suggested that it may be related to a change in the packing of the lipids from an orthorhombic structure to a hexagonal packing. In a recent study, however, Gay and coworkers [24] concluded from DSC and FTIR measurements that the transition at 35°C is related to a solid-to-fluid phase change for a discrete subset of stratum corneum lipids. A similar transition temperature was found in porcine stratum corneum at 25°C and also related to a solid-to-fluid transition [25] and in mouse SC between 31°C and 43°C, probably related to a solid phase transition [26].

The transition at 73°C originates from a lipid and a protein component, since it is only partly reversible [17,22]. The transition at 10°C has never been reported before. The reason for this might be that most stratum corneum DSC scans covered a different temperature range and used porcine or mice SC. An exception is the recent study of Tanojo and coworkers [28]. The transition at 10°C seems not to be related to melting of water, since this transition occurs at approx. −2°C [22]. Since this transition decreases after the application of vesicles (Fig. 1 and Fig. 2, Table 2) we conclude that it does originate partially from lipid. The transition is not reversible (Fig. 2) and therefore a protein component might be involved also. It is highly unlikely that this transition is an artefact, since the samples were equilibrated for 60 min at 2°C. However, preparation of the SC and liposome treatment occurred at room temperature. Therefore, we can not exclude that the transfer of the SC samples from ambient temperature to 2°C induced a lipid transition which then is detected upon the subsequent measurement. Only recently Tanojo and coworkers [28] determined the endothermic profile of human stratum corneum in the range of −130°C to 120°C and did not find a transition at 10°C. However, they stored the SC after trypsin isolation under dehydrated conditions, while in our experiments the SC was used immediately after preparation and was never fully dehydrated. This difference in preparation might explain the different results.

The transition at 81°C originates from SC proteins, mainly keratins. When taking the peak height of individual transitions relative to the transition at 81°C as a measure of lipid transition enthalpy, the application of DMPC vesicles on human stratum corneum results in an increase of enthalpy at 50°C, 62°C and 73°C (Table 2). This indicates that DMPC penetrates into the intact stratum corneum. One has to keep in mind that the keratin-related transition depends on the hydration of the SC sample [27,28]. In this study, however, the SC was never fully dehydrated. During the non-occlusive treatment the physiological inner side was always hydrated and before the calorimetric measurement each sample was hydrated for 6 h in 145 mM

phosphate buffer (pH 7.3). Therefore, we believe that the keratins were fully hydrated and peak height of the latter can be used for standardisation.

The finding that DMPC penetrates into the SC is supported by the following data. Lipids with polar headgroups enhance the transepidermal water loss [11] and vesicular structures were visualized in the stratum corneum after the application of liposomes from soy-bean lipids with a high phosphatidylcholine content [10]. Lasch and Wohlrab [4] found facilitated penetration of cortisol into the SC, when applied with soy-bean lipid/cholesterol liposomes.

The penetration of DMPC into the SC, however, did not result in a significant shift of the SC transition temperatures (Table 1). The reason for this might be the heterogeneous composition of the components, responsible for the endothermic transition. Since every transition is related to a complex mixture of several lipids or lipid structures, the incorporation of a minor amount of a single lipid component (e.g., DMPC) does not necessarily shift the transition temperature significantly. In addition, the high concentration of free sterols in the SC (25–50%) results in generally broad endothermic transitions.

From the CLSM measurements we conclude, that DMPC liposomes do not penetrate intact the SC. No fluorophore could be detected in deeper layers of the skin. Individual DMPC molecules, however, can penetrate into the stratum corneum and disturb the well organized structure of the intercellular lipids. This results in a smoothening of the SC endothermic profile and in a change of the lipid-related transition enthalpy. However, no shift of the endothermic transitions occurred, most likely due to the complex composition of the SC lipids.

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