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Targeting of liposome-associated calcipotriol to the skin: Effect of liposomal membrane fluidity and skin barrier integrity

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ABSTRACT

Many dermal diseases like psoriasis are characterized by major changes in skin barrier function, which challenge the reproducible delivery of drugs into specific layers of diseased skin. The purpose of this study was to elucidate how liposomal bilayer fluidity and barrier integrity affected the delivery of liposome-associated calcipotriol to the skin. Calcipotriol-containing gel state and liquid state dipalmitoylphosphatidyl-choline:dilauroylphosphatidylcholine liposomes were prepared by extrusion. Using Langmuir monolayers, calcipotriol was shown to affect the packing of the lipid membrane. The penetration of radioactively labeled lipid and calcipotriol into pig skin was examined using the Franz diffusion cell model, and tape stripping was applied to impose an impaired barrier. Distorting the skin barrier resulted in an enhanced penetration of lipid from both gel and liquid state liposomes. In addition, increased penetration of lipid from liquid state liposomes was observed compared to gel state liposomes into barrier-impaired skin. For barrier-impaired skin, an elevated calcipotriol-to-lipid ratio was found in the receptor fluid for both liposome compositions indicating that calcipotriol is released from the vesicles. This suggests that the liposome-mediated delivery of calcipotriol to the epidermis of diseased skin is affected by the fluidity of the liposomal membrane.

1. Introduction

Several skin diseases are characterized by the dysfunction of keratinocytes in the lower epidermis, making the epidermis a promising target for topically applied drugs. However, dermal diseases such as psoriasis are often associated with major, patient-specific changes in the skin barrier function, which challenge the prediction of penetration and accumulation of drugs used for treatment of skin diseases *in vivo*. An example is the reduced barrier function in psoriatic plaques due to alterations in ceramide composition as well as increased keratinocyte proliferation and incomplete differentiation, causing a highly irregular stratum corneum (SC) (Choi and Maibach, 2005). An impaired skin barrier function often results in an increased transepidermal water loss (TEWL) and an enhanced percutanous permeation (Levin and Maibach, 2005). Several studies have shown that increased penetration occurs in diseased or damaged skin (Gattu and Maibach, 2010). A commonly used *ex vivo* approach to study further the effect of a distorted barrier on drug disposition in the skin is to remove the SC by tape stripping, which significantly increases the drug permeation through the skin (Simonsen and Fullerton, 2007).

Liposomes can be used to increase the delivery of drugs into the skin upon dermal administration, but to date the underlying mechanism(s) is not fully understood (Honeywell-Nguyen and Bouwstra, 2005; Korting and Schäfer-Korting, 2010). The drug delivery properties of liposomes are largely affected by the physico-chemical characteristics of the lipid bilayer, which are determined by factors such as the lipid composition, the particle size and the drug loading. In addition, the specific lipid composition is highly decisive for the drug release and for how the vesicles interact with the skin barrier (Schafer-Korting et al., 2007). It is important to consider the thermotropic phase behavior of the lipid bilayer when the liposomes are applied topically at the skin temperature of 32 °C. Liposomes with a phase transition temperature (T_m) above the temperature of the skin will be in a rigid gel state at 32 °C. In contrast, liposomes with a T_m below 32 °C are in a fluid liquid state at the skin temperature. Several *ex vivo* and *in vivo* studies have shown that liquid state liposomes have increased penetration into the skin, as compared to

Abbreviations: BSA, bovine serum albumin; DLPC, 1,2-dilauroyl-sn-glycero-3-phosphatidylcholine; DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine; DSC, differential scanning calorimetry; HEPES, 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid; PDI, polydispersity index; RF, receptor fluid; SC, stratum corneum; SUV, small unilamellar vesicles; TEWL, transepidermal water loss.

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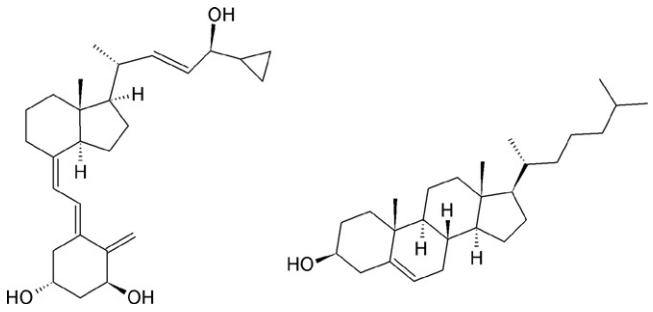

Fig. 1. Molecular structure of calcipotriol (left) and cholesterol (right).

gel state liposomes (Honeywell-Nguyen and Bouwstra, 2005; Ogiso et al., 1996; Perez-Cullell et al., 2000). The fluidity of SC lipids is significantly decreased by treatment with liposomes composed of solid saturated phospholipids due to intercellular lipid deposition, whereas treatment with unsaturated phospholipids dramatically raises the fluidity of SC lipids due to perturbation of the lipid organization (Ogiso et al., 1996).

Even though many studies have confirmed the drug penetration-enhancing effects of liposomes into the skin, the responsible mechanism(s) is still widely debated. One explanation for this is the highly diverse lipid compositions applied for the studies, which complicates the comparison. Often, several different lipids and surfactants are used for the preparation of liposomes for dermal application. The present study is based on a reductionistic approach using a simplified binary liposome composition consisting of well-tolerated dipalmitoylphosphatidylcholine (DPPC) and dilauroylphosphatidylcholine (DLPC). By adjusting the molar ratio of these two components systematically, it is possible to investigate the effect of membrane fluidity. The aim of this study was to examine how the disruption of the skin barrier integrity influenced the interaction with liposomes *ex vivo*, and to elucidate how this interaction was affected by the fluidity of the liposomal membrane. In addition, the ability of the liposomes to deliver drug into barrier-impaired skin was examined by including the synthetic D-vitamin analogue calcipotriol (Fig. 1), which is a secosteroid commonly used for the treatment of psoriasis, into the lipid bilayer structure. The target site of action of calcipotriol is the D-vitamin receptor expressed by keratinocytes present in the lower epidermis, where it inhibits proliferation and normalizes differentiation of keratinocytes during psoriasis (Bury et al., 2001; Cirunay et al., 2001).

2. Materials and methods

2.1. Materials

1,2-Dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC) (≥99% purity) and 1,2-dilauroyl-sn-glycero-3-phosphatidylcholine (DLPC) (≥99% purity) were provided by Lipoid GmbH (Ludwigshafen, Germany) and Sigma–Aldrich (Brøndby, Denmark). Calcipotriol monohydrate (≥94% purity, Mw = 430.6 g/mol) and ^{3}H-calcipotriol (13 MBq/mL, 1.9 MBq/μg in 2-propanol) were obtained from LEO Pharma A/S (Ballerup, Denmark). Phosphatidylcholine, L-a-dipalmitoyl [dipalmitoyl-1-14C] (^{14}C-DPPC) with a specific activity of 4.11 GBq/mmol (0.93 MBq/mL) in ethanol/toluene (50:50, w/w) (≥99% purity) was acquired from American Radiolabeled Chemicals Inc. (Missouri, USA). Soluene-350 and Hionic-fluor were provided by Perkin Elmer (Massachusetts, USA). Bovine serum albumin (BSA) was from Merck KKGaA (Darmstadt, Germany). All other general chemicals and reagents were obtained commercially at analytical grade.

2.2. Preparation of liposomes

The liposomal formulations were prepared according to the technique described by Bangham et al. (1965). For liposomes not loaded with drug, DPPC and DLPC were dissolved in chloroform and mixed at different molar ratios (100:0, 90:10, 80:20, 50:50 and 40:60) in a round-bottomed flask. The organic solvent was evaporated, and the lipid film was stripped with ethanol, which was removed under vacuum over night. The film was hydrated at 55 °C with 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) buffer (10 mM HEPES, 0.5 mM KCl, 1 mM NaN_3, pH 7.4) for 1 h to a final lipid concentration of 2 mM followed by 1 h of annealing at room temperature. To obtain small unilamellar vesicles (SUVs), the large multilamellar vesicles were extruded 10 times through two stacked polycarbonate membrane filters with 100 nm pore sizes (Whatman, Herlev, Denmark) and stored at 4 °C. Drug-containing liposomes were prepared in a similar way, except that calcipotriol was added to the chloroform phase before evaporation of the organic solvent to a final calcipotriol concentration of 50 μg/mL (5.7 mol%) or 200 μg/mL (19.5 mol%), and the lipid-calcipotriol film was hydrated in 13 mM Tris buffer at pH 8.5, since calcipotriol is less stable at the lower pH provided by the HEPES buffer. For examination of skin penetration, the liposomes were labeled with trace concentrations of ^{14}C-DPPC (0.1 MBq/mL) and ^{3}H-calcipotriol (0.5 MBq/mL), which were added to the lipid-containing chloroform phase before evaporation of the organic solvent. The amount of ^{14}C-DPPC was 1.2% of the total amount of lipid and the amount of ^{3}H-calcipotriol was 0.5% of the total amount of calcipotriol in the formulations used for the skin penetration studies.

2.3. Characterization of liposomes

The lipid concentrations of the liposomal dispersions were assessed by the Phospholipids B enzymatic kit (MTI-Diagnostics Gmbh, Idstein, Germany). The concentration of calcipotriol in the formulations was verified by high pressure liquid chromatography (HPLC) using a Sunfire C18, 3.5 μm, 150 mm × 4.6 mm column with acetonitrile:water (60:40, v/v) as the mobile phase. The detection was done at 264 nm. The incorporation of calcipotriol was also analyzed by light microscopy: calcipotriol is practically insoluble in water at concentrations above 0.1 μg/mL, and consequently, it forms visible crystals unless it is incorporated into the lipid bilayer. No crystals were found in any of the formulations during the period of the experiments, which indicates the incorporation of calcipotriol into the lipid bilayer of the liposomes. For the radioactive labeled formulations for the skin penetration studies, the formulations were characterized from recovery of the radioactive label. The average particle size distribution and polydispersity index (PDI) were analyzed by dynamic light scattering using the photon correlation spectroscopy technique. The surface charge of the particles was estimated by analysis of the zeta-potential (Laser-Doppler Electrophoresis). The measurements were performed in triplicate at 25 °C using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) equipped with a 633 nm laser and 173° detection optics. For viscosity and refractive index, the values of pure water were used. Malvern DTS v.5.10 software was used for data acquisition and analysis. The particle size distribution was reflected in the PDI, which ranges from 0 for a monodisperse to 1.0 for an entirely heterodisperse dispersion.

2.4. Phase transition temperature

The gel-to-liquid crystalline T_m of the undiluted vesicles in suspension was determined by differential scanning calorimetry (DSC). For liposomes without drug in HEPES buffer, thermograms were obtained with a VP-DSC calorimeter (Microcal, Milton Keynes,

UK) at a scanning rate of 1 °C/min in the temperature range from 20 °C to 80 °C. For calcipotriol-loaded liposomes in Tris buffer, thermograms were obtained with a Nano DSC (TA Instruments, New Castle, DE, USA) at a scanning rate of 0.5 °C/min from 20 °C to 60 °C. VPViewer 2000 and Origin® 7 scientific plotting software were used for data analysis, which was performed on the first of three scans of each sample ($n = 3$).

2.5. Langmuir monolayers

Surfactant monolayers were formed at room temperature by spreading a total amount of 36 nmol lipid mixture in chloroform onto an aqueous subphase consisting of 13 mM Tris buffer (pH = 8.5) in a KSV Minitrough 1 (KSV Instruments Ltd., Helsinki, Finland) with a surface area of 243 cm^2 using a Hamilton microsyringe. The compression of the monolayer was initiated 10 min after spreading the lipids to allow the organic solvent to evaporate. The monolayer was compressed with a barrier speed of 10 mm/min, and the surface pressure was measured using a Wilhelmy platinum plate (KSV Instruments Ltd.). Each sample was compressed once in three independent experiments ($n = 3$). KSV software (KSV Instruments Ltd.) was used for data analysis.

2.6. Skin preparation

Porcine skin was used for the skin penetration studies. Ears were obtained from the local slaughterhouse (Roskilde Slagteriskole, Roskilde, Denmark) and frozen at −20 °C upon arrival. Whole ears were thawed at 4 °C over night, and full thickness skin was removed from the back of the ear. The subcutaneous tissue was removed, and the excised skin was cut into appropriate pieces and re-frozen at −20 °C. The skin was thawed for 2 h at room temperature before use.

2.7. Penetration studies

The penetration of liposomes was investigated using Franz diffusion cells (six cells per formulation) with nominal surface areas of 3.14 cm^2 and a receiver compartment with a capacity of approximately 10 mL. The skin was mounted with the SC side of the skin exposed to ambient conditions while the dermal side was pre-hydrated for 30 min in an isotonic NaCl solution followed by equilibration in receptor fluid (RF) (phosphate-buffered saline, pH 7.4, containing 4% (w/w) BSA). BSA was added to the receptor fluid to increase the solubility of calcipotriol and lipid to obtain sink conditions during the experiment. More than ten times the applied amount of drug was soluble in the receptor fluid. The solubility of the lipids in the receptor fluid was not examined, but spectroturbidimetry and dialysis experiments have been used in the literature to determine the DLPC solubility in water as 4 ± 1 ppm and the DPPC solubility as essentially zero (Pinazo et al., 2002). Therefore the solubility of DLPC/DPPC is expected to be very low in the receptor fluid. After 1 h, the RF was renewed, and 15 μL of the undiluted liposomal formulations (2 mM lipid, 50 μg/mL calcipotriol) was applied onto the SC The applied lipid dose of the gel state formulation was thus 21.7 μg (6.9 μg/cm^2 skin), whereas the applied lipid dose of the liquid state liposomes was 20.0 μg (6.4 μg/cm^2 skin). For both formulations the applied dose of calcipotriol was 0.75 μg (0.24 μg/cm^2 skin). To impose an impaired barrier to the skin, the SC was removed by adhesive D-Squam® tape (CuDerm, TX, US) applied with a pressure of 225 g/cm^2 for 5 s. This procedure was repeated 25 times, which results in the removal of most of the SC, as confirmed by light microscopy (results not shown). The RF was agitated with a magnetic stirring bar and kept at 35 ± 1 °C by placement in a water bath resulting in a temperature of 32 °C at the skin surface. The cells were incubated at non-occlusive conditions in the dark for 24 h, after which the penetration of the formulations was examined.

2.8. Skin layer separation

After incubation, the receptor fluid was collected and the excess formulation was removed by wiping the skin with a cotton pad followed by tape stripping twice with Transpore™ tape (VWR International, Herlev, DK). The Transpore™ tape and D-Squam® tape have been shown to remove equal amounts of the SC (Bashir et al., 2001), but the Transpore™ tape was preferred for this specific purpose since the physical dimensions of this tape is larger than the D-Squam® tape, which is pre-cut in disc shapes covering only the diffusion area. The Transpore™ tape therefore also removes the small amounts of formulation present outside the diffusion area, which might otherwise cause errors due to lateral diffusion of the formulation in the skin when the non-applied skin is collected. The skin was mounted on cork discs using small pins by stretching the skin to overcome problems of furrow in the subsequent tape stripping procedure. For intact skin, the SC was removed by tape stripping: Adhesive D-Squam® tape was applied with a pressure of 225 g/cm^2 for 5 s and stripped off rapidly 10 times, which is sufficient for removal of the SC after 24 h of hydration. For both intact and barrier-impaired skin, the viable epidermis was isolated by heat separation; the skin was incubated at high humidity for 5 min at 60 °C. All skin samples were dissolved using the strong organic base formulated with toluene named Soluene-350 (PerkinElmer, MA, USA), and the excess formulation present on the cotton pad, the tape and the surface of the empty Franz diffusion cells was extracted using heptane:ethanol (70:30, w/w). The amounts of ^{3}H-calcipotriol and ^{14}C-DPPC were quantified by incubating the samples with 10 mL Hionic–Fluor scintillation liquid (PerkinElmer, MA, USA) for 24 h in the dark followed by analysis using a Tri-CARB 2100TR Liquid Scintillation Analyzer (Packard Instrument Company Inc., USA). The mass balance of each cell was calculated based on the applied amount of formulation, and more than 77% of the theoretically applied amount was recovered for each formulation. The percentages of ^{3}H-calcipotriol and ^{14}C-DPPC in the different skin layers were calculated based on the recovered amount of the compound in each cell.

2.9. Statistics

The statistical significance of the result for the formulation data, the Langmuir data and the comparison of the penetration from two different formulations were determined using the unpaired t-test comparing two variations assuming equal variation ($p < 0.05$ was considered significant). For the comparison of the penetration of ^{3}H-calcipotriol and ^{14}C-DPPC applied onto the same cell, the statistical significance of the result was determined using the paired t-test comparing two variations ($p < 0.05$ was considered significant).

3. Results and discussion

3.1. Characterization of liposomes

Liposomes were prepared from binary mixtures of DPPC and DLPC at different molar ratios, and the thermotropic phase behavior of the liposomes was characterized by DSC (Fig. 2). Membrane bilayers undergo a transition from a gel phase to a liquid crystalline phase at the main T_m. Only a single phase transition appeared in the investigated temperature range for all lipid mixtures, which indicates a homogenous distribution of the lipids in the membrane. The T_m of the gel-to-liquid phase transition was decreased with increasing content of DLPC due to the shorter length of the acyl

Table 1
Particle size, PDI and zeta-potential of gel state and liquid state DPPC:DLPC liposomes with 0, 5.7 and 19.5 mol% (0, 50 and 200 μg/mL) calcipotriol in 13 mM Tris buffer. Results denote mean ± SD ($n = 3$). Results significantly different from liposomes with the same composition without calcipotriol are indicated: $^{*}p < 0.05$ and $^{**}p < 0.01$.

DPPC:DLPC molar ratio	Calcipotriol mol%	Size (nm)	PDI	Zeta-potential (mV)
90:10	0.0	87.2 ± 4.0	0.100 ± 0.016	−9.64 ± 1.77
90:10	5.7	88.1 ± 3.3	0.131 ± 0.026	−8.12 ± 3.97
90:10	19.5	82.1[a]	0.105[a]	−4.83[a]
40:60	0.0	88.3 ± 1.6	0.141 ± 0.013[b]	−5.99 ± 1.00[b]
40:60	5.7	79.6 ± 3.0[**b]	0.111 ± 0.026[*]	−4.06 ± 0.70[*]
40:60	19.5	77.0[a]	0.124[a]	−6.60[a]

[a] $n = 1$.
[b] Significant difference between gel state and liquid state liposomes with the same calcipotriol content (either 0.0 or 5.7 mol%): $p < 0.05$.

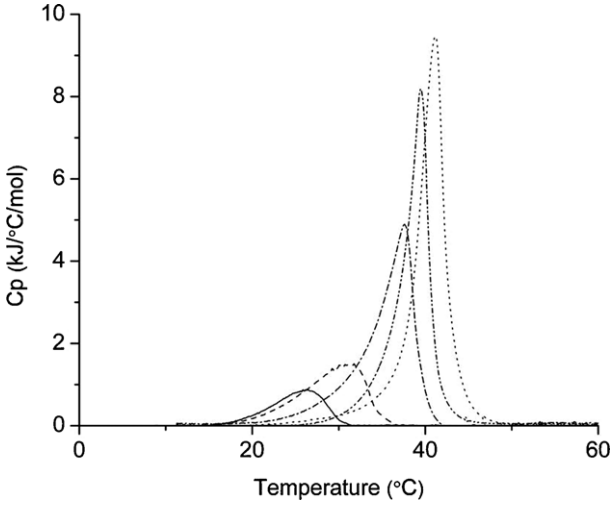

Fig. 2. Representative thermograms of liposomes composed of different molar ratios of DPPC:DLPC in HEPES buffer. Solid: 40:60, dash: 50:50, dash-dot: 80:20, dash-dot-dot: 90:10 and dot: 100:0. The curves have been normalized to the molar content.

chains of DLPC with reduced potential for hydrophobic interactions (Fig. 2). The sharpness of the peak for pure DPPC liposomes suggests a highly cooperative gel-to-fluid phase transition in which the majority of the lipid membrane molecules undergo a simultaneous phase transition. In addition, the addition of DLPC broadened the phase transition due to the less cooperative phase transition for binary DPPC:DLPC mixtures. Two lipid compositions were selected for further skin penetration studies; a composition in the gel state at the skin temperature of 32 °C (DPPC:DLPC, 90:10) and a composition in the liquid-crystalline state composition at skin temperature (DPPC:DLPC, 40:60). These two formulations were prepared and further characterized with respect to particle size, PDI, zeta potential and thermodynamic properties (Tables 1 and 2). The thermodynamic parameters included the T_m, the width of the phase transition peak at half height ($T_{1/2}$), the phase transition enthalpy (ΔH) and the phase transition entropy (ΔS). The non-loaded liposomal preparations had an average particle diameter of approximately 88 nm and there was no significant difference between the 90:10 composition and the 40:60 composition. In addition, the liposomes were monodisperse with PDI values below 0.15 (Table 1), and the PDI for the DPPC:DLPC 40:60 liposomes (0.141 ± 0.013) was significantly higher than the PDI for the DPPC:DLPC 90:10 liposomes (0.100 ± 0.016). The zeta potential was approximately −6 to −10 mV and did not vary significantly between the two formulations (Table 1). The T_m was 26.8 ± 0.2 and 39.4 ± 0.1 °C for DPPC:DLPC 40:60 and 90:10 liposomes, respectively (Table 2). The $T_{1/2}$ was 3.3 ± 0.2 and 6.2 ± 0.4 for DPPC:DLPC 40:60 and 90:10 liposomes, respectively, reflecting a broader and less cooperative phase transition for the 40:60 composition.

Calcipotriol was included in the bilayer of the liposomes at a concentration of 5.7 mol% (50 μg/mL), which corresponds to the therapeutic calcipotriol concentration used in products for treatment of psoriasis (e.g. Daivonex®, LEO Pharma A/S, Ballerup, Denmark). This did not affect the particle size, PDI and zeta-potential of the 90:10 composition significantly, whereas all three parameters were decreased significantly for the DPPC:DLPC 40:60 liposomes (Table 1). An additional molar ratio of calcipotriol (19.5 mol%) was included in the study, and there was a tendency of decreased particle size at 19.5 mol% calcipotriol for both lipid compositions. The overall appearance of the thermograms for the calcipotriol-loaded liposomes (results not shown) was very similar to the appearance of the thermograms for the non-loaded DPPC:DLPC 40:60 and 90:10 compositions presented in Fig. 2. However, there was a significant decrease in the T_m (38.9 ± 0.2 °C) and $T_{1/2}$ (2.9 ± 0.3) for the DPPC:DLPC (90:10) liposomes whereas the T_m and $T_{1/2}$ for the DPPC:DLPC (40:60) liposomes remained largely unchanged upon addition of 5.7 mol% calcipotriol (Table 2). The decrease in $T_{1/2}$ upon incorporation of calcipotriol into the DPPC:DLPC (90:10) bilayer suggests a more cooperative phase transition in the presence of calcipotriol, whereas there is a tendency of less cooperativeness for the 40:60 composition. Interestingly, the ΔH was increased for the DPPC:DLPC (90:10) liposomes from 31.7 ± 0.6 to 33.4 ± 1.0 kJ/mol and decreased for DPPC:DLPC (40:60) liposomes from 8.4 ± 0.9 to 7.0 ± 0.3 kJ/mol by the inclusion of calcipotriol (Table 2), and the data for liposomes with 19.5 mol% calcipotriol confirmed this trend. This suggests overall that calcipotriol is indeed intercalated in the lipid membrane. The data also indicates that calcipotriol affects the packing of the molecules in the lipid membrane at the investigated calcipotriol concen-

Table 2
Thermodynamic properties of gel state and liquid state DPPC:DLPC liposomes with 0, 5.7 and 19.5 mol% (0, 50 and 200 μg/mL) calcipotriol in 13 mM Tris buffer. Results denote mean ± SD ($n = 3$). Results significantly different from liposomes with the same composition without calcipotriol are indicated: $^{*}p < 0.05$ and $^{**}p < 0.01$.

DPPC:DLPC molar ratio	Calcipotriol mol%	T_m (°C)	$T_{1/2}$ (°C)	ΔH (kJ/mol)	ΔS (kJ/mol/°C)
90:10	0.0	39.4 ± 0.1	3.3 ± 0.2	31.7 ± 0.6	0.804 ± 0.017
90:10	5.7	38.9 ± 0.2[**]	2.9 ± 0.3[*]	33.4 ± 1.0[*]	0.860 ± 0.024[*]
90:10	19.5	38.9[a]	2.5[a]	38.2[a]	0.982
40:60	0.0	26.8 ± 0.2[b]	6.2 ± 0.4[b]	8.4 ± 0.9[b]	0.312 ± 0.033[b]
40:60	5.7	26.4 ± 0.4[b]	6.3 ± 0.1[b]	7.0 ± 0.3[*b]	0.264 ± 0.007[*b]
40:60	19.5	27.5[a]	9.4[a]	6.3[a]	0.229

[a] $n = 1$.
[b] Significant difference between gel state and liquid state liposomes with the same calcipotriol content (either 0.0 or 5.7 mol%): $p < 0.001$.

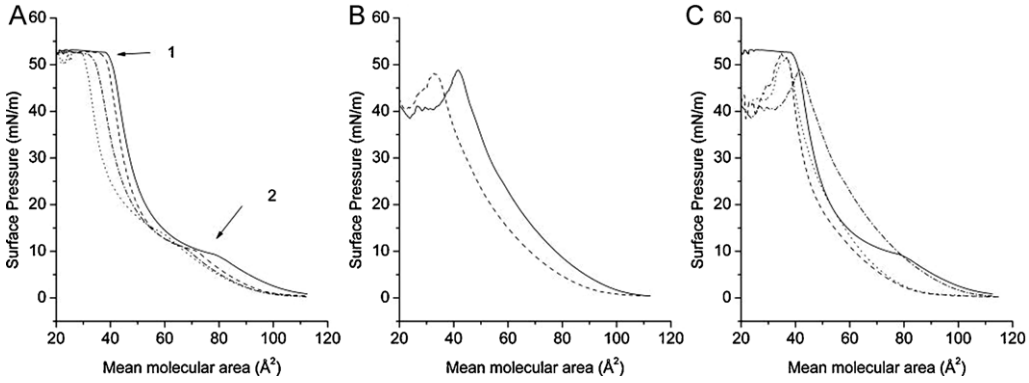

Fig. 3. Pressure/area isotherms of Langmuir monolayers of DPPC:DLPC containing calcipotriol and cholesterol, respectively, on Tris buffer subphases. The total lipid concentrations of the different monolayers are identical for all experiments. The curves represent averages of three experiments. (A) DPPC:DLPC (90:10) with 0 (solid), 5.7 (dash), 10.8 (dash-dot) and 19.5 (dot) mol% calcipotriol. (B) DPPC:DLPC (40:60) with 0 (solid) and 19.5 mol% calcipotriol (dash). (C) DPPC:DLPC (90:10) with 0 (solid) and 20.6 mol% cholesterol (dash), and DPPC:DLPC (40:60) with 0 (dash-dot) and 20.6 mol% cholesterol (dot). Arrows: 1: collapse and 2: phase transition.

trations. The increased ΔH of the main phase transition for the DPPC:DLPC (90:10) liposomes upon addition of calcipotriol can be explained by a significant increase in the phase transition entropy ($\Delta S = \Delta H/T_m$) from 0.804 ± 0.017 kJ/mol/°C without calcipotriol to 0.860 ± 0.024 kJ/mol/°C in the presence of 5.7 mol% calcipotriol (Table 2), suggesting that the lipid molecules in the membrane become less ordered in the presence of calcipotriol when the membrane bilayer goes from gel to liquid state, leading to increased positional disorder and increased lateral diffusion, resulting in a larger degree of motional freedom and thereby a higher entropy. For the DPPC:DLPC (40:60) composition with a higher degree of chain asymmetry there is a significant decrease in the main phase transition entropy from 0.312 ± 0.033 kJ/mol/°C without calcipotriol to 0.264 ± 0.007 kJ/mol/°C in the presence of 5.7 mol% calcipotriol, suggesting decreased changes in positional disorder and lateral diffusion in the presence of calcipotriol upon phase transition from gel state to liquid state, resulting in a lower degree of motional freedom and thereby a lower change in entropy of the main transition, suggesting a more structured liquid phase and/or a less structured gel phase.

3.2. The incorporation of calcipotriol affects the lipid membrane packing

The effect of the calcipotriol loading on the membrane characteristics of the liposomes with the two lipid compositions was investigated further using the Langmuir technique since monolayers at the air–water interface are ideal model membrane systems for measuring membrane properties and lipid interactions according to the monolayer–bilayer correspondence theory (Feng, 1999). Langmuir monolayers of DPPC:DLPC at a molar ratio of 90:10 and 40:60, respectively, were compressed in the presence of different concentrations of calcipotriol. The results are shown in Fig. 3, and in Table 3 the values for the surface pressure (Π) and the mean molecular area (A) at the monolayer collapse and at the phase transition from the liquid-expanded to the liquid-condensed phase are listed. The phase transition and the collapse point were estimated from the compression modulus (C_S^{-1}) versus Π dependency, where C_S^{-1} is defined according to Eq. (1):

$$C_S^{-1} = -A\left(\frac{d\pi}{dA}\right) \quad (1)$$

A characteristic minimum for the C_S^{-1} versus Π dependency for the monolayer reflects the phase transition from the liquid-expanded to the liquid-condensed states of the monolayer, whereas the surface pressure at $C_S^{-1} = 0$ reflects the monolayer collapse. Monolayers of DPPC:DLPC (90:10) collapsed at a surface pressure of 52.7 mN/m and a mean molecular area of 38.1 Å^2 (Fig. 3A, arrow 1). The surface pressure at the monolayer collapse was not affected by the presence of calcipotriol for the DPPC:DLPC (90:10) composition, whereas the mean molecular area at the collapse was significantly decreased in the presence of calcipotriol to 29.2 Å^2 at 19.5 mol% calcipotriol (Fig. 3A and Table 3). Similarly, the mean molecular area at the phase transition between the liquid-expanded phase and the liquid-condensed phase was significantly decreased in a calcipotriol content-dependent manner from 78.1 Å^2 for DPPC:DLPC

Table 3
Surface pressures (Π) and mean molecular areas (A) at the phase transition and the collapse point for DPPC:DLPC monolayers with calcipotriol or cholesterol. Results denote mean ± SD ($n = 3$). Results significantly different from 0 mol% calcipotriol/cholesterol are indicated: $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

DPPC:DLPC molar ratio	Calcipotriol mol%	Collapse point		Phase transition	
		Π (mN/m)	A (Å^2)	Π (mN/m)	A (Å^2)
90:10	0.0	52.7 ± 0.01	38.1 ± 0.2	9.4 ± 0.3	78.1 ± 1.7
90:10	5.7[a]	52.7	32.2	10.3	70.7
90:10	10.8	$52.4 \pm 0.2^{**}$	$32.6 \pm 1.3^{**}$	$11.4 \pm 0.3^{***}$	$64.9 \pm 0.6^{***}$
90:10	19.5	$52.5 \pm 0.03^{***}$	$29.2 \pm 0.7^{***}$	$14.2 \pm 1.0^{***}$	$58.1 \pm 0.4^{***}$
40:60	0.0	48.9 ± 0.4[d]	41.5 ± 0.3[d]	[b]	[b]
40:60	19.5	48.4 ± 0.2[d]	$32.4 \pm 1.1^{**}$[e]	[b]	[b]
90:10	20.6[c]	$53.7 \pm 1.3^{***}$	$35.6 \pm 1.5^{*}$	[b]	[b]
40:60	20.6[c]	$51.1 \pm 0.2^{***}$	$36.8 \pm 0.4^{***}$	[b]	[b]

[a] $n = 2$.
[b] No detectable phase transition.
[c] Calcipotriol was replaced with cholesterol.
[d] Significant difference between gel state and liquid state liposomes with the same calcipotriol content (either 0.0 or 19.5 mol%): $p < 0.001$.
[e] Significant difference between gel state and liquid state liposomes with 19.5 mol% calcipotriol: $p < 0.01$.

(90:10) to 58.1 Å^2 for monolayers containing 19.5 mol% calcipotriol (Fig. 3A, arrow 2). In addition, the surface pressure at the phase transition was increased from 9.4 mN/m at 0 mol% calcipotriol to 14.2 mN/m at a calcipotriol content of 19.5 mol%. This suggests that calcipotriol has an area-condensing effect on the lipid monolayers at this specific concentration, which results in an improved packing of the lipids. This can explain the increased ΔH of the main phase transition in the presence calcipotriol observed by DSC. At the other calcipotriol molar ratios tested (5.7 and 10.8 mol%) the effects were similar but less pronounced, and the 19.5 mol% was therefore selected for further studies.

For the DPPC:DLPC (40:60) composition, no phase transition was observed (Fig. 3B). The surface pressure at the collapse point was slightly decreased for DPPC:DLPC (40:60, 48.8 mN/m) and the mean molecular area was increased to 41.5 Å^2 compared to DPPC:DLPC (90:10) due to the presence of a larger amount of DLPC with a shorter chain length. However, a significant decrease in the mean molecular area to 32.4 Å^2 at the monolayer collapse was observed upon addition of 19.5 mol% calcipotriol due to condensation and improved packing of the membrane in the presence of calcipotriol (Fig. 3B and Table 3). The improved packing of the lipids together with the decreased enthalpy of the main transition suggests an increased order of the lipid bilayer in the liquid state.

For comparison, the structurally similar and well-studied sterol cholesterol was included in the monolayer, which has evolved to decrease the permeability of membrane bilayers by filling in the flickering spaces among the acyl chains of the phospholipids (Mouritsen and Jorgensen, 1994; Ohvo-Rekila et al., 2002). Cholesterol and calcipotriol had to some extent comparable area-condensing effects on the monolayer at the tested concentrations. The major difference, though, was that while cholesterol completely eliminated the cooperative liquid expanded-liquid condensed phase transition for the 90:10 molar ratio at 20.6 mol%, the phase transition was still detectable for calcipotriol at a similar concentration (Fig. 3C and Table 3). In addition, the mean molecular area at the collapse point was increased for the cholesterol-containing monolayers to 35.5 Å^2 for the 90:10 composition and 36.8 Å^2 for the 40:60 composition compared to the calcipotriol-containing monolayers (29.2 and 32.4 Å^2, respectively) showing that the calcipotriol molecule has a stronger area-condensing effect than cholesterol (Demel et al., 1967; Gong et al., 2002).

This may be explained by the differential interaction with the membrane phospholipids between cholesterol and calcipotriol at the specific molar ratio (Fig. 1): whereas the two hydroxyl groups of calcipotriol interact with the polar head groups of the membrane phospholipids and the aqueous subphase, the bulky secosteroid group and the hydrocarbon chain are embedded in the membrane and interact with the hydrophobic phospholipid acyl chains. The larger bulkiness of the planar sterol group of cholesterol, as compared to the secosteroid group of calcipotriol, could explain the larger mean molecular area at the collapse point and suggests that calcipotriol has a higher membrane-condensing effect than cholesterol at the tested molar ratio. However, the hydroxyl group and the cyclopropyl group present on the hydrocarbon chain of calcipotriol might disturb the packing of the membrane compared to the less bulky hydrocarbon chain of cholesterol, explaining the detection of a phase transition upon inclusion of calcipotriol, compared to the complete elimination of the phase transition with cholesterol. However, further studies with additional molar ratios of calcipotriol and different membrane compositions are needed to conclude on the effects of calcipotriol on membranes in general. Such studies are though hampered by the fact that pure calcipotriol does not form a Langmuir monolayer, which complicates the calculation of excess molecular areas and free energies.

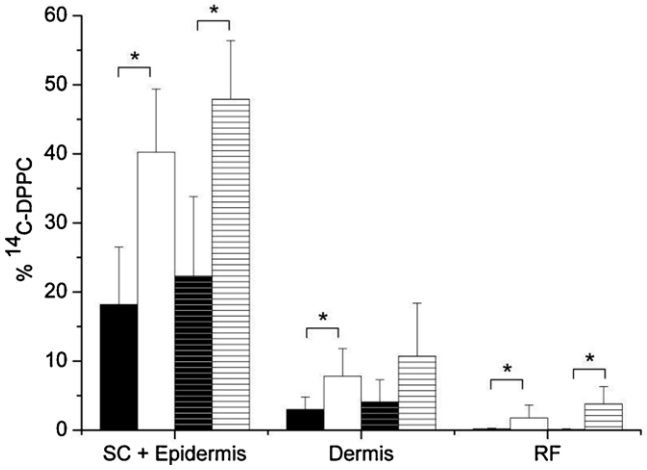

Fig. 4. Skin penetration of ^{14}C-DPPC-labeled gel state and liquid state liposomes into the SC + viable epidermis (left), the dermis (middle) and the RF (right) in percent of the total amount of ^{14}C-DPPC recovered after the experiment. The bars show the penetration of gel state liposomes into intact (grey) and barrier-impaired skin (white), and the penetration of liquid state liposomes into intact (grey-striped) and barrier-impaired skin (white-striped). The remaining ^{14}C-DPPC fraction was recovered from the excess formulation in the acceptor chamber and from the non-applied skin (results not shown). Bars denote mean values ± SD ($n = 6$). Significant differences between penetration into intact and barrier-impaired skin are indicated: $p < 0.05$*.

3.3. Increased lipid penetration into barrier-impaired skin

The skin penetration of lipid from gel and liquid state liposomes was examined (Fig. 4). The penetration of lipid into intact skin was significantly decreased compared to skin where most of SC has been removed by tape stripping, suggesting that the SC is an important barrier for penetration of both gel and liquid state liposomes. For intact skin, the lipid penetration from liquid state liposomes was not significantly elevated in the different skin layers, as compared to the lipid penetration from gel state liposomes, but a trend towards increased accumulation was observed (Fig. 4). The same was the case for the penetration of the gel and liquid state liposomes into barrier-impaired skin, where most of the SC was removed by tape stripping before the formulation was applied. This trend was reflected by a significant increase ($p < 0.05$) in the total amount of lipid penetrated into barrier-impaired skin and RF from liquid state liposomes (62.5 ± 8.2%) compared to gel state liposomes (49.8 ± 9.9%, Table 4).

These data support previous studies reported in the literature, which generally suggest that the penetration into barrier-impaired is increased compared to intact skin (Gattu and Maibach, 2010; Simonsen and Fullerton, 2007). The results also substantiate the usefulness of tape stripping to impose skin barrier damage. In the current study, lipid accumulation was most pronounced in the epidermis, both for liquid and gel state liposomes. In addition, a trend towards penetration of liquid state liposomes compared to penetration of gel state liposomes into intact skin was observed (Table 4), which has been reported previously in the literature (Perez-Cullell et al., 2000). The fluidity of SC lipids has been shown to be sig-

Table 4
Total recovery of ^{14}C-labeled DPPC from gel state and liquid state DPPC:DLPC liposomes in the skin + RF (SC, viable epidermis, dermis and RF). Results denote mean ± SD ($n = 6$). Significant difference between gel and liquid state liposomes in barrier-impaired skin is indicated: *$p < 0.05$.

DPPC:DLPC molar ratio	Intact skin (%)	Barrier-impaired skin (%)
90:10	21.4 ± 5.1	49.8 ± 9.9
40:60	26.5 ± 10.6	62.5 ± 8.2*

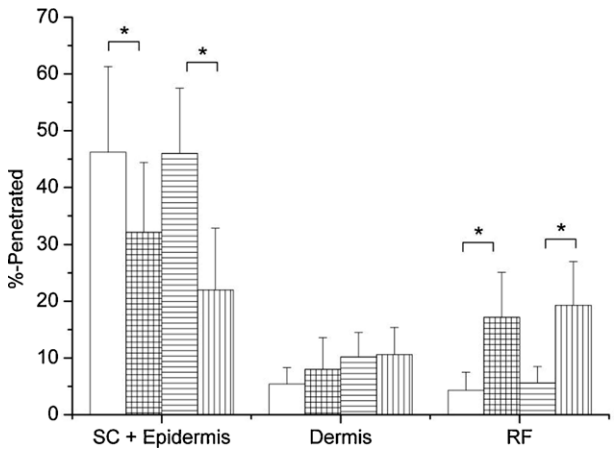

Fig. 5. Comparison of the skin penetration of ^{14}C-DPPC and ^{3}H-calcipotriol from gel and liquid state liposomes into the SC + viable epidermis (left), the dermis (middle) and the RF (right) of barrier-impaired skin in percent of the total amount of ^{14}C-DPPC and ^{3}H-calcipotriol, respectively, recovered after the experiment. The bars show the penetration of lipid (white bars) and calcipotriol (squared bars) from gel state liposomes, and the penetration of lipid (vertical lined bars) and calcipotriol (horizontal lined bars) from liquid state liposomes. The amount of lipid/calcipotriol in the different skin layers is represented as the percentages of total recovered amount. The figure only show the amount penetrated into the skin. The remaining ^{14}C-DPPC and ^{3}H-calcipotriol fractions were recovered in the excess formulation and in the non-applied skin (results not shown). Bars denote mean values ± SD ($n = 6$). Calcipotriol penetrations significantly different from the lipid penetrations are indicated: $^{*}p < 0.05$.

nificantly decreased by treatment with liposomes composed of solid saturated phospholipids due to intercellular lipid deposition, whereas treatment with unsaturated phospholipids dramatically raise the fluidity of SC lipids due to perturbation of the lipid organization (Ogiso et al., 1996) explaining the effect on intact skin. However we observed a significantly increased penetration of liquid state liposomes into barrier-impaired skin where most of the barrier had been removed by tape stripping (Table 4), suggesting that mechanisms different than a change in the fluidity of SC lipids are involved. In addition, it is often claimed that the SC is the main barrier for liposomal permeation, but after removal of most of the SC still, less than 5% of the lipid has permeated to the RF during the 24-h incubation period, suggesting that the other barriers in the skin might reduce the permeation.

3.4. Increased penetration of calcipotriol into the deeper layers of barrier-impaired skin

The penetration of calcipotriol and lipid into barrier-impaired skin was compared between gel and liquid state liposomes (Fig. 5). The initial ratio between ^{3}H-calcipotriol and ^{14}C-DPPC of the formulations applied into the skin was set to 1. For both gel and liquid state liposomes, the calcipotriol-to-lipid ratio was decreased in the upper layers of the skin (SC + viable epidermis) compared to the ratio for the formulations applied on the skin (Table 5). In contrast, a significantly increased ($p < 0.05$) calcipotriol-to-lipid ratio was detected in the RF for both formulations after 24 h. The highest amount of calcipotriol (32.1 ± 12.3%) was found in the epidermis upon application of calcipotriol-containing gel state liposomes, but no significant difference was found compared to the amount of calcipotriol measured in the epidermis (22.0 ± 10.8%), when calcipotriol was formulated in liquid state liposomes ($p = 0.16$).

Table 5
Ratio between ^{3}H-calcipotriol and ^{14}C-DPPC in barrier-impaired skin and RF. The initial ratio between ^{3}H-calcipotriol and ^{14}C-DPPC of the formulations applied onto the skin was set to 1. The ratios are calculated from the mean values presented in Fig. 4.

DPPC:DLPC molar ratio	SC + viable epidermis	Dermis	Receptor fluid
90:10	0.70	1.47	3.96
40:60	0.48	1.04	3.44

The increased calcipotriol-to-lipid ratio in the dermis and in the receptor fluid as compared to the applied formulation suggests that calcipotriol is released from the liposomes in the lower layers of the skin. Consequently it is most likely that the liposomes disintegrate and that penetration occurs as single molecules. The higher calcipotriol permeation can be explained by the log P value of calcipotriol (4.9) compared to DPPC (13.5), which favors penetration/permeation of the less lipophilic calcipotriol into the more hydrophilic dermis and receptor fluid (Bouwstra et al., 2003). Similar results have been reported for cholesterol-containing liposomes (Coderch et al., 2000), where the log P value for cholesterol is 7.17.

No significant difference in the delivery of calcipotriol from gel state liposomes was observed compared to the delivery of calcipotriol from liquid state liposomes, but there was a trend towards increased targeting of calcipotriol to the epidermis from the gel state liposomes. This might be due to a fluidity-decreasing effect of solid saturated lipids on the lipid components present in the epidermis resulting in an increased retention of calcipotriol in the epidermis. Liquid state lipids, on the other hand, increases the fluidity of lipids present in the skin and might thereby enhance the diffusion of calcipotriol through the skin. Another factor, which could influence deposition in the skin, is the intrinsic bilayer-fluidizing effect of calcipotriol, which might increase the penetration as shown for cholesterol-containing liposomes (Coderch et al., 2000; Vrhovnik et al., 1998). Finally, it has been proposed that liquid-state liposomes penetrate the SC through channels and are able to penetrate deeper into the skin compared to gel-state liposomes, which has been observed as bilayers mixed with SC lipids (Honeywell-Nguyen and Bouwstra, 2005). Hence, it has been suggested that liquid-state liposomes may act as drug carriers since they have been observed intact in the deeper SC layer, whereas gel-state liposomes may function as penetration enhancers due to the effect on the upper SC.

Many treatment strategies for skin diseases often aim at delivering drugs to target sites in the viable epidermis. One example is calcipotriol therapy since the target site of action is the D-vitamin receptor expressed by keratinocytes present in the lower epidermis where calcipotriol inhibits proliferation and normalizes differentiation of keratinocytes during psoriasis (Bury et al., 2001; Cirunay et al., 2001). Consequently, gel state liposomes seem to be favorable for the targeting of calcipotriol to the epidermis.

4. Conclusion

From this study it can be concluded that enhanced penetration of lipid from both gel and liquid state liposomes occurs into barrier-impaired skin compared to intact skin, since significantly increased amounts of lipid were found in the skin after application of both gel and liquid state liposomes to the skin surface where most of the SC had been removed. In addition, significantly increased penetration of lipid from liquid state liposomes was observed into barrier-impaired skin compared to gel state liposomes. This suggests that liposomal delivery to diseased skin is affected by the fluidity of the liposomal membrane and/or the lipophilicity of the skin barrier. Calcipotriol intercalated into the membrane bilayer of liposomes, and calcipotriol formulated in liposomes seems to be released in the skin from the vesicles, since a significantly increased calcipotriol-to-lipid ratio was found in the receptor fluid. The highest amount of calcipotriol in the viable epidermis of distorted skin was found when calcipotriol was formulated in gel state liposomes,

as compared to liquid state liposomes. The evaluated amount of calcipotriol in the epidermis was not significant, but data suggests that it is possible to optimize the physiochemical properties of the liposomes to target liposomal delivery to specific layers of the skin.

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