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In vivo comparison of various liposome formulations for cosmetic application☆

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Abstract

The interaction of liposome formulations, prepared with phospholipids of different origins (egg and soya), with skin were compared in terms of their effects on skin water content, skin barrier function, and skin elasticity. Short-term effect of four different liposome formulations and two references during 3.5 h was investigated non-occlusively on the volar side of the forearm of 10 volunteers, ranging in age from 24 to 32 years. Liposomes composed of different phospholipids showed differing effects on skin humidity. The maximal effect was achieved within 30 min and constant values were reached after 1.5 h for all formulations, however values remained significantly higher than without treatment ($p < 0.05$) during the whole application time. The best results were obtained with liposome formulations prepared from egg phospholipids, which exhibited a 1.5-fold increase in skin water content ($p < 0.05$), whereas liposome formulations prepared from soya phospholipids showed no advantage compared to the references. Skin barrier function showed greatest influence within 30 min after application and remained constant after 1.5 h for all formulations. Within the liposome formulations, egg phospholipids showed the highest transepidermal water loss values during the first 30 min, representing the strongest interactions with the skin barrier function, whereas for the other liposome formulations lower transepidermal water loss values were measured. Skin elasticity and tiring effect of the skin was not influenced by any of the formulations, due to the young skin tested. Long-term effect of two different liposome formulations mixed with base cream DAC in two different concentrations during 14 days was investigated non-occlusively on the volar side of the forearm of 10 volunteers, ranging in age from 20 to 25 years. Skin water content was measured daily and the results showed that skin humidity was increased significantly ($p < 0.05$) for the formulation containing 20% egg phospholipids during 6 days.

Liposome formulations prepared with egg phospholipids showed significantly higher ($p < 0.01$) hydration effects during 3.5 h of application on human skin compared with liposome formulations prepared with soya phospholipids.

Keywords: Liposomes; Skin barrier; Transepidermal water loss; Skin hydration; Skin aging; In vivo comparison

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1. Introduction

Bangham discovered liposomes in 1963 and the use of lipid vesicles as systemic and topical drug delivery systems has attracted increasing attention (Bangham, 1963; Sharate and Katz, 1996). The application in skin treatment is based on the similarity of the bilayer structure of lipid vesicles to that of natural membranes. This includes the ability of lipid vesicles, depending on lipid composition, to alter cell membrane fluidity and to fuse with cells. Thus, delivering active drugs to the target site (Gregoriadis, 1994). Typically, conventional dosage forms, such as solutions, creams, and ointments, deliver drugs in a concentration-dependent manner across the stratum corneum. Multilamellar liposomes can deliver drugs within 30 min to the stratum corneum, epidermis, and dermis in significantly higher concentrations than conventional preparations.

However, it is generally accepted that classic liposomes are of minor value as transdermal drug delivery systems due to the fact that they do not penetrate deeply into the skin (Braun-Falco et al., 1992; Touitou et al., 2000a). Only specially designed lipid vesicles were shown to penetrate into deeper layers of the skin. The group of Cevc (Cevc et al., 1998) reported about ultradeformable material carriers, the Transfersomes™, optimized to overcome the skin transport barrier spontaneously due to the natural transepidermal water activity gradient. The drug is delivered to the deeper epidermal layers through dehydration of the lipid vesicles within the stratum corneum. Therefore, liposome uptake is driven by the hydration gradient that exists across the epidermis, stratum corneum, and ambient atmosphere (Cevc and Blume, 1992).

Furthermore, the presence of ethanol in systems of lipid vesicles termed ethosomes was reported to influence the stratum corneum penetration and permeation of drugs (Touitou et al., 2000a,b).

Liposomes based on a natural marine lipid extract containing a highly polyunsaturated fatty acid ratio were recently introduced as Marinosomes® for the prevention and treatment of skin diseases (Moussaoui et al., 2002).

The intercellular lipids of the stratum corneum play a key role in establishing the permeability barrier of the skin (Elias, 1981; Landmann, 1988; Cevc, 1996). Liposomes, which are vesicles having a phospholipids 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). Further studies were aimed to induce a local rather than a systemic effect (Schmid and Korting, 1994).

In our previous works, the penetration behavior of heparin across the skin from various liposome formulations was investigated in vitro (Betz et al., 2001a). Furthermore, the distribution of lipids in the skin was studied by confocal laser scanning microscopy using a fluorescence labelled phospholipid marker (Betz et al., 2001b).

As mentioned before, the stratum corneum is the main barrier of the skin and prevents dehydration. Water plays an important role in respect to the normal function of the epidermal barrier, which is also reflected in the differing water contents of the stratum corneum (10–15%) and viable epidermis (about 60%). The water content in the stratum corneum is mostly bound to the head group of ceramides and to proteins and is an essential prerequisite for the barrier function. Thus, a decrease in water content below the minimum level is associated with skin malfunction (Gregoriadis, 1994). The moisture content of the skin is of particular interest in cosmetic applications. Cosmetic care is therefore concerned to equilibrate the moisture balance of the skin.

In the work of Schmid and Korting (1993), stratum corneum lipid liposomes have been used in the treatment of atopic dry skin in order to restore the barrier function and to provide a drug delivery system at the same time. The composition and properties of liposomes play an important role in their interaction with and possible penetration into the epidermis. In addition, liposomes provide valuable raw material for the regeneration of skin by replenishing lipid molecules and moisture. Lipids are well hydrated and, even in the absence of active ingredients, humidify the skin. Often this is enough to improve skin elasticity and barrier function, which are the main causes of aging of the skin.

The main objective of the present study was to investigate the influence of liposomes prepared with phospholipids from different origins (egg and soya) on skin,

in vivo. This interaction of phospholipids with skin was evaluated by non-invasive biophysical techniques using the Corneometer, Tewameter, and Cutometer, measuring the skin water content, skin barrier function, and skin elasticity, respectively.

Furthermore, the results should lead to an improved understanding of liposome penetration and their effects on skin. Thus, the results will be useful in designing specific liposome formulations for cosmetical and dermatological application with the aim to equilibrate the moisture balance of the skin. Finally the in vivo results of the present study will be compared with the in vitro results of our previous work.

2. Materials and methods

2.1. Materials

Two different types of phospholipids of different origins were used to prepare the liposome formulations. Lipoid E 80 and Lipoid S 80 were a gift from Lipoid AG, Cham, Switzerland. Lipoid E 80 is an egg lipid extract with 82% (w/w) phosphatidylcholine and Lipoid S 80 is a soybean lipid extract with 76% (w/w) phosphatidylcholine, see Table 1 for complete composition. As a reference formulation DAC (Deutscher Arzneimittel Codex) base cream (Thoma, 1989) was prepared, the composition is given in Table 2. Glycerol monostearate was obtained from Sandoz, Switzerland, Cetyl alcohol from Hänseler AG, Swizerland, Miglyol 812N from Hüls AG, Switzerland, Vaselinum album from Hänseler AG, Switzerland, Polyoxyethylenglycerylmonostearate from Goldschmidt AG, Germany, and Propylene glycol was purchased from Hänseler AG, Switzerland.

Table 1
Composition of Lipoid E 80 and S 80

	Lipoid S 80 (%)	Lipoid E 80 (%)
Phospholipids		
Phosphatidylcholine	76.7	81.8
Phosphatidylethanolamine	5.9	7.7
Lysophosphatidylcholine	5.0	2.0
Lysophosphatidylethanolamine	–	<0.5
Sphingomyelin	–	2.0
Triglyceride	–	2.5
Cholesterine	–	0.7
Free fatty acids	–	<0.05

Table 2
Composition of DAC base cream (Thoma, 1989)

Excipient	(g)
Glycerol monostearate	4.0
Cetylalcohol	6.0
Miglyol 812N	7.5
Vaselinum album	25.5
Polyoxyethylenglycerylmonostearate	7.0
Propylene glycol	10.0
Water double distilled	to 100.0

All other chemicals and reagents purchased from commercial sources were of analytical grade.

2.2. Methods

2.2.1. Preparation of formulations

2.2.1.1. Preparation of base cream DAC. Base cream DAC is an oil in water (O/W) emulsion and was prepared by melting the lipophilic phase consisting of glycerolmonostearate, cetylalcohol, Miglyol 812, and vaselinum album and separately melting the hydrophilic compounds polyoxyethylenglycerolmonostearate, propylenglycol, and water in a water bath at 60 °C. Subsequently, the water phase was gently poured into the lipophilic phase under constant stirring until the cream reached room temperature; evaporated water was complemented.

2.2.1.2. Preparation and characterization of liposomes for the in vivo study, single application. Liposome formulations were prepared by a modified ethanol injection method described by Batzri and Korn, 1973. This is a well established method that was shown to produce phospholipid vesicles using electron microscopy (Kremer et al., 1977). The ethanol solution containing the lipids was poured rapidly into the well-stirred aqueous solution. The liposome formulations contained 25% (v/v) ethanol 96% (Betz et al., 2001a,b). As lipids, Lipoid E 80 and Lipoid S 80 were used to prepare liposome formulations of 1 and 2% lipid concentration, respectively. The liposome formulations were homogenized by Polytron (Kinematica AG, Switzerland) at 15 000 rpm for 15 min. The composition of the prepared formulations are shown

Table 3
Formulations used for the in vivo study, single and multiple application

Formulation source of phospholipid/ final lipid concentration single/ multiple application (mg/ml)	In vivo	
	Study single application	Multiple application
Lipoid E 80/10/100	PLE 1%	BLF 10%
Lipoid E 80/20/200	PLE 2%	BLF 20%
Lipoid S 80/10	PLS 1%	
Lipoid S 80/20	PLS 2%	
Reference		
Negative control	0.9% NaCl	
Positive control	Base cream DAC	

in Table 3. The *z*-average mean and polydispersity of the liposomes were determined routinely by photon correlation spectroscopy using a Malver Zetasizer 1000 HS (Malvern instruments Ltd., Malvern, UK) after dilution with double distilled water and 25% (v/v) ethanol 96%, respectively. Size determination was performed at 25 °C and the viscosity value of pure water was used in the calculations. The measurements were carried out in triplicate. The following parameters were used to characterize the size of liposomes:

z-average mean: The intensity weighted mean derived directly from PCS data by the cumulant method. It is inversely proportional to the slope of the logarithm of the normalized correlation function.
Polydispersity: The measure of the width of the particle size distribution obtained from the cumulants analysis. Typically 0.02–0.05 for latex standards and 0.20 or more for broad size distributions.
Cumulants analysis: A method of calculating the *z*-average mean size and polydispersity of a sample from the correlation function. The correlation function is fitted to a power series of the delay time τ: $a + b\tau + c\tau^2$, where b is related to the *z*-average mean size and c to the polydispersity.

Furthermore, images were taken after freeze fraction in liquid nitrogen and sputtering with gold using a Philips freeze fracture transmission electron microscope FF-TEM, ESEM XL-30 (Philips, Netherlands). The phase transition temperature was determined by differential scanning calorimetry using a Differential Scanning Calorimeter (Pyris 1, Perkin-Elmer, US Instruments Division, Norwalk).

2.2.1.3. Preparation and characterization of liposomes for the in vivo study, multiple application. The liposome formulation for the in vivo study following multiple application was prepared and characterized as described in Section 2.2.1.2 using Lipoid E 80 with the final phospholipid concentration of 400 mg/ml. Subsequently, the liposome formulation was mixed with base cream DAC (see Section 2.2.1) in the weight ratio 1:1 to receive a final phospholipid concentration of 200 mg/ml (BLF 20%) and 1:3 to receive a final phospholipid concentration of 100 mg/mL (BLF 10%) (see Table 3).

2.2.2. In vivo studies

The in vivo studies were carried out in an examination room under constant environmental conditions of 45% relative humidity and 25 °C.

2.2.2.1. In vivo study, single application. Four liposome formulations and two references were applied non-occlusively to the volar side of the forearm of 10 male and female volunteers, ranging in age from 24 to 32 years (see Table 4). The two references, physiologic sodium chloride solution and base cream DAC were used as negative and positive control, respectively. A single dose corresponding to 100.0 mg of each formulation was applied to the defined test area delimited by a 6 cm × 10 cm window cut from a plaster sheet and

Table 4
Protocol of the in vivo study, single application during 210 min

Number of volunteers	10 (7 female and 3 male volunteers);
Age of volunteers	24–32 years old
Application	Single application on the volar side of the forearm
Formulations	PLE 1%, PLE 2%, PLS 1%, PLS 2%,
References	NaCl 0.9% and base cream DAC
Application dose	100 mg formulation/10 cm^2 skin (1 mg PL/cm^2 skin for PL 1%; 2 mg PL/cm^2 for PL 2%)
Evaluation	0–210 min, according to prefixed time points
Parameters	Skin water content (Corneometer CM 820), TEWL (Tewameter TM 210), elasticity (Cutometer SEM 474)
Skin characterization	Skin-pH (Skin-pH-meter PH 900), sebum content (Sebumeter SM 810).

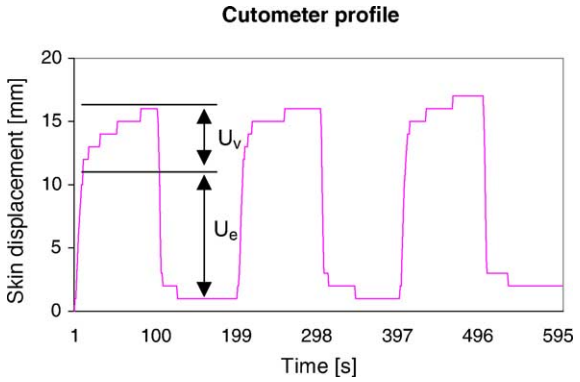


Fig. 1. Description of elastic and viscoelastic component of the skin by Cutometer.

applied to the cleansed volar side of the forearm of 10 dermatologically normal subjects. On each subject the test area was further partitioned into 6 test fields using a specially perforated self-adhesive fabric (Mefix®) in order to separate the application of each formulation and reference.

The skin type of each volunteer was characterized by the pH-value (Skin-pH-meter PH 900, Courage Khazaka, Germany) and the sebum content (Sebumeter SM 810, Courage Khazaka, Germany).

Skin water content (Corneometer CM 820, Courage Khazaka, Germany), skin barrier function (Tewameter TM 210, Courage Khazaka, Germany), and skin elasticity (Cutometer SEM 474, Courage Khazaka, Germany) were measured and monitored for all test fields before application and at prefixed time points after application (15, 30, 45, 60, 90, 120, 150, 180, and 210 min). Five individual measurements were carried out for each formulation at any time point using the Corneometer and the average value was used to calculate the results. The rate of transdermal water loss (TEWL) was determined using the Tewameter with a measuring time of 1 min for each formulation at any time point. The value with the lowest standard deviation was taken for the comparison of the formulations. Skin elasticity was measured with the Cutometer, where the skin is drawn into the aperture of the probe at a constant negative pressure of 500 mbar. The resulting curve of each measurement (see Fig. 1) represents the elastic and viscoelastic qualities of the skin. The first phase (U_e), represents the elastic component and the second phase (U_v) the plastic component of the skin.

Table 5
Protocol of the in vivo study, multiple application during 14 days

Number of volunteers	10 (3 men and 7 female)
Age of volunteers	20–25 years old
Application	Multiple application, twice a day, on the volar side of the forearm during 14 days.
Application dose	Roughly 100 mg formulation/10 cm^2 skin (10 mg PL/cm^2 skin for BLF 10%; 20 mg PL/cm^2 for BLF 20%)
Formulations	BLF 10% and BLF 20%
Evaluation	Every morning, except on weekends
Parameter	Skin water content (Corneometer CM 820)

From these curves, the parameters, such as skin elasticity (portion between the maximum amplitude and the ability of the skin to recover from deformation), plasticity (U_v/U_e), and tiring effect (highest point of the last curve compared to the maximum amplitude of the first curve) were calculated.

Volunteers were requested not to use moisturizers, body lotions, soaps or occlusive cosmetic preparations on the tested skin areas 12 h prior to the in vivo study.

2.2.2.2. In vivo study, multiple application. An in vivo study after multiple application was performed during 14 days using two formulations containing 10 and 20% egg phospholipids (BLF 10% and BLF 20%), for preparation and composition see Section 2.2.1.3. Each formulation was applied non-occlusively to the volar side of the forearm of five volunteers, in total 10 male and female volunteers, ranging in age from 20 to 25 years (see Table 5). The volunteers were requested to apply the cream twice daily over 14 days after showering and to maintain a record about the time schedule. Every morning at the same time, except on weekends, the skin water content was measured using the Corneometer CM 820 (Courage Khazaka, Germany). Five individual measurements were carried out for each formulation at any time point and the average value was used to calculate the results.

2.2.3. Analysis of variance

Statistical analysis of variance was performed using the SPSS program for Windows. ANOVA test was applied and *p*-values less or equal to 0.05 were considered as statistically significant.

3. Results and discussion

3.1. Characterization of liposome formulations

The particle size and the polydispersity of size distribution of the liposome formulations measured by photon correlation spectroscopy is shown in Table 6. Dilution of the sample with different media prior to measurement greatly influenced the result. The larger size of liposomes measured in the liposome solution containing ethanol compared to double distilled water is consistent with previous results of our research group. In the work of Kirjavainen et al. (1999), the same phenomenon was observed and the increased liposome size in the presence of ethanol was suggested to be due to a decrease in interfacial tension or the induction of interdigitation. Dilution with double distilled water yielded up to two-fold smaller particles than dilution with the solvent used in the formulation. It was concluded that the measurements in 25% (v/v) ethanol reflect the real particle size of the liposome formulations.

The formulation prepared with PLE 1% showed a 2- to 3-fold larger *z*-average mean and a higher polydispersity index than the other formulations using both dilution media. Liposomes prepared with PLE 2% and PLS 2% showed particle sizes in the same range, whereas liposomes prepared from PLS 1% are up to 100 nm smaller, irrespectively of the dilution media. This observation can be due to the different lipid composition (see Table 1) where Lipoid E 80 contains triglycerides and cholesterole. Generally for liposomes prepared from soya phospholipids, the size of the liposomes increased with increasing phospholipid concentration. This is in agreement with the results reported in the work of Memoli (Memoli et al., 1995), where the soya liposomes were on an average smaller than egg liposomes.

At ethanol <25%, the polydispersity of size distribution increased (results not shown). The composition and preparation process of the formulations used in the present study was optimized with respect to homogeneity and stability of the liposomes.

The liposome dimension and the morphologies measured by freeze fracture transmission electron microscopy are given in Fig. 2a. Liposomes prepared from egg phospholipid showed a multilamellar structure with a broad size distribution. The liposomes depicted in Fig. 2a had a diameter in the range of 1–3 μm. However, it is not a size distribution and the probability to fracture larger vesicles than smaller ones is higher (Braun-Falco et al., 1992). The micrographs of soya liposomes (see Fig. 2b) were completely different, exhibiting a homogeneous structure, which was smaller in size. The different consistency was already visible to the naked eye, where the PLS 1% and PLS 2% formulation exhibited a clear semi-solid structure and the PLE 1% and PLE 2% a clear liquid solution.

The phase transition temperature of the Lipoid S® 80 containing liposomes was determined to be −25 °C for the extrapolated peak onset temperature (T_e) and –14 °C for the peak maximum temperature (T_P) and that of Lipoid E® 80 containing liposomes was –36 and −14 °C, respectively. This difference of 11 °C in the extrapolated peak onset temperature is suggested to be due to the different composition of the phospholipids. Egg phospholipids contain free fatty acids in comparison with soya phospholipids. All liposome formulations were in the liquid crystalline state at the

Table 6
Particle size and polydispersity of size distribution of liposomes

	Formulation			
	PLE 1%	PLE 2%	PLS 1%	PLS 2%
Dilution with double distilled water				
z-average mean (nm)	510.3	228.2	169.7	261.9
±S.D.	16.6	2.5	3.5	3.7
Polydispersity index	0.27	0.20	0.23	0.30
Dilution with 25% (v/v) ethanol in water				
z-average mean (nm)	921.3	445.3	343.8	471.1
±S.D.	19.3	5.9	12.3	3.8
Polydispersity index	0.33	0.15	0.11	0.21

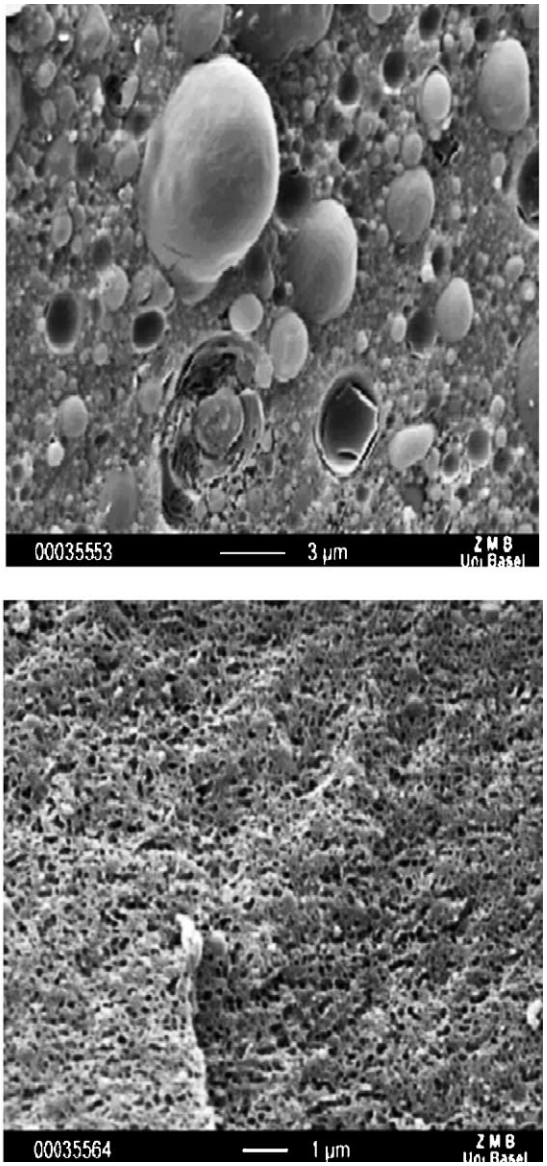


Fig. 2. Characterization of liposomes by freeze fracture technique, magnification 10 000: (a) liposomes prepared from egg phospholipid (PLE 1%), (b) liposomes prepared from soya phospholipid (PLS 2%).

temperature of the in vivo experiments, which is related to the fact that the chief constituent of Lipoid S® and E® 80, phosphatidylcholine, contains mainly linoleic acid. The low phase transition temperature is of advantage for liposomes as drug carrier systems due to the fact that actives stored in liposomes with high phase transition temperature are generally set free slowlier than those in liposomes with lower phase transition temperature (Lautenschläger, 1990). Furthermore, the melting point of the lipids contained in the liposome also affects the depth of penetration (Sharate and Katz, 1996).

The state of the bilayer is responsible for the interaction with the stratum corneum. Van den Bergh et al. (1999) showed by freeze-substitution electron microscopy that liquid crystalline liposomes induce interactions with intercellular lipids in deeper stratum corneum layers, whereas gel state liposomes aggregate, fuse and adhere on the stratum corneum surface. 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.

3.2. In vivo study, single application

3.2.1. Skin characterization in vivo

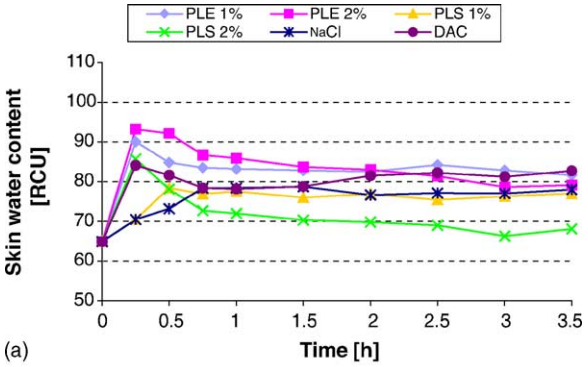
The skin of the 10 volunteers was characterized by the skin pH-value and the sebum content on the volar side of the forearm and is given in Table 7. The pH-value of all volunteers ranges between 4.8 and 5.8. The average pH-value of skin ranges from approximately 5 (men) to 5.5 (women). This value is dependent on the tested skin area and increases with age. This is also true for the sebum content, which reaches its maximum between the age of 20 and 40 years. Nine volunteers showed sebum values below 6, which corresponded to dry skin and one volunteer (8) had a sebum content of 8, which corresponded to normal skin.

3.2.2. Short-term effects of liposomes on human skin in vivo

The results of the skin water content measurements after single application of six formulations averaged over all 10 volunteers are represented in Fig. 3a. The maximal increase of skin water content was achieved within 30 min after application and constant values were reached after 1.5 h for all formulations. The skin water content remained significantly higher for the formulations PLE 1%, PLE 2%, and PLS 1% than without treatment ($p < 0.05$) (see Fig. 3b). The greatest increase in skin water content was obtained with PLE 2% and 1% exhibiting a 1.5 and 1.4-fold increase ($p < 0.05$), respectively. Liposomes prepared from PLE were found

Table 7
Skin characterization by pH-value and sebum content

	Volunteer									
	1	2	3	4	5	6	7	8	9	10
pH-value	5.0	5.1	5.4	5.7	5.2	5.5	5.8	4.8	5.8	4.8
Sebum ($\mu g/cm^2$)	1	1	4	1	1	1	1	8	1	1



(b)

Time [h]	PLE 1% [RCU]	PLE 2% [RCU]	PLS 1% [RCU]	PLS 2% [RCU]
0	64,8	65,0	64,9	64,9
0,25	90,1 ★★	93,0 ★★	70,5	85,7 ★★
0,5	84,8 ★★	92,1 ★★	78,4 ★★	78,1 ★
0,75	83,5 ★★	86,7 ★★	76,4 ★★	72,7
1.0	83,2 ★★	85,9 ★★	77,5 ★★	72,0
1,5	82,8 ★★	83,7 ★★	76,5 ★★	70,3
2,0	82,5 ★★	79,0 ★	76,9 ★★	69,8
2,5	84,2 ★★	81,4 ★★	75,4 ★★	69,0
3,0	82,8 ★★	78,6 ★	79,3 ★★	66,3
3,5	81,6 ★★	77,1 ★	77,0 ★★	68,1

★ p< 0,05 ★★ p< 0,01

Fig. 3. (a) Skin water content measurement after single application of six formulations averaged over all 10 volunteers. (b) Statistical evaluation of the results of skin water content compared with untreated skin $t = 0$.

to be multilamellar vesicles (MLV). This structure is suggested to have a positive influence on the increase of skin water content. Whereas the PLS formulations showed no significant advantage compared to the references. A comparison between the female and the male volunteers yielded to interesting results. Men had higher skin water content than women. This can be seen in the skin water content measured before any application of the formulations ($t = 0$) at the volar side of the forearm, where men had an average skin water content of 71 RCU (relative corneometer units) and women of 62 RCU. The same trend was observed at the maximal skin water content values measured for the different formulations. Thus, men showed a maximum skin water content of 100 RCU after 30 min application of PLE 2%, whereas women exhibited a maximum skin water content of 92 RCU after 15 min application for the same formulation.

The results of the transepidermal water loss measurements of all female volunteers compared with all male volunteers showed no remarkable difference between the sexes. However, when comparing two individual volunteers, the female volunteer 4 and the male volunteer 8 (see Table 7), a significant difference in the measured TEWL values was observed (see Fig. 4).

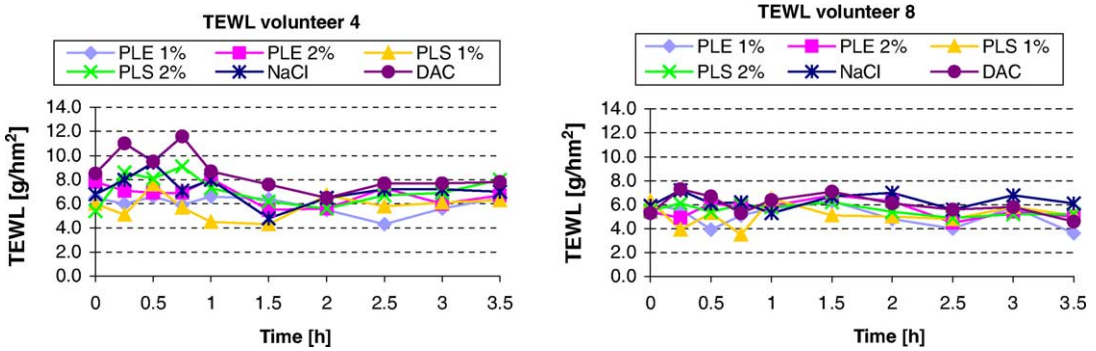


Fig. 4. Skin barrier function measurement (TEWL) after single application of six formulations to volunteer 4 and 8.

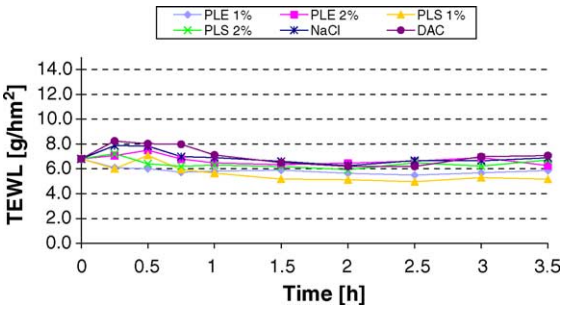


Fig. 5. Skin barrier function measurement (TEWL) after single application of six formulations averaged over all 10 volunteers. Statistical analysis showed no significant difference between treated and untreated skin due to the strong interindividual differences of the TEWL values measured.

TEWL reflects the ability of skin to restrict diffusion of water and the measured values were roughly in the range of in vivo measurements cited in the literature (Barel and Clarys, 1995). The stronger variation of TEWL values seen for volunteer 4, represented the increase of interactions between the formulations and the skin barrier, especially for DAC base cream and PLS 2%. Volunteer 4 has dry skin and a lower initial skin water content value of 60 RCU compared with volunteer 8 having normal skin with the initial value of 80 RCU. Dry skin seems to be more sensitive for the interaction with the tested formulations, especially with DAC base cream and PLS 2%. These variations in TEWL values were not seen for volunteer 8, where the formulations applied to the skin showed no interaction with the skin barrier. The skin barrier function, measured in TEWL values (g/hm^2) averaged over all 10 volunteers, showed greatest influence within 1.0 h after application and remained constant after 1.5 h. Maximal values were achieved with DAC and 0.9% NaCl solution, due to occlusive effects and remaining water on the skin surface, respectively. Within the liposome formulations PLE 2% showed highest TEWL values during the first 30 min, representing the strongest interactions with the skin barrier function, whereas for the other liposome formulations, lower TEWL values were measured (see Fig. 5). A possible explanation are structural changes induced in the intercellular lipids by egg phospholipids. It was already reported that the incorporation of enhancers or fluid lipids into the intercellular domains can dramatically interfere with the barrier function of the stratum corneum, thus lowering its phase transition temperature and increasing fluidity, facilitating diffusion and transport through the skin (Coderch et al., 1999).

Skin elasticity, plastic component and skin tiring effect was not influenced by any of the formulations.

3.3. In vivo study, multiple application

3.3.1. Long-term effects of liposomes on human skin in vivo

The skin water content averaged over all volunteers for both liposome creams is shown in Fig. 6. The long-term application of the formulations showed the maximal increase of skin water content after 3 days. The maximum value achieved within the first week roughly remained stable during the second week of the study. A dose dependent effect of the liposomes could not be detected within the investigated concentration range of 100 mg PLE/ml and 200 mg PLE/ml. In comparison with the results achieved in the in vivo study, single application (see Section 3.2), the maximum skin water content observed in the long-term study was not as high as in the first study. This can be explained with the different measuring time points chosen for the two studies. In the in vivo study, multiple application, the first measuring time point was 24 h after the first application of the liposomal cream, compared with the in vivo study, single application, where the first values were measured 15 min after the first application of the formulations.

In comparing male and female volunteers, a difference in their skin water content behavior throughout the study was observed. For the lower concentrated liposomal cream (BLF 10%) male skin showed stronger fluctuations in their skin water content value than female skin and also reached higher water content

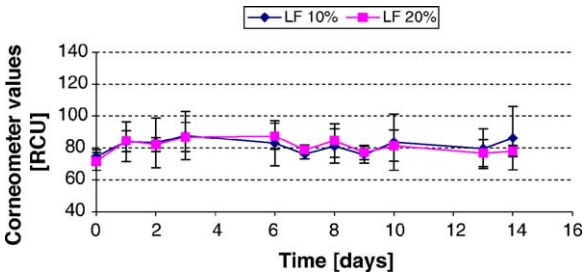


Fig. 6. Skin water content values averaged over all 10 volunteers after a two weekly application of BLF 10% and BLF 20%.

levels during the 2-week study period, whereas female skin showed more consistent skin water content values, which are slightly lower. For the higher concentrated liposomal cream (BLF 20%) the fluctuations are more balanced for both, men and women volunteers, and therefore more constant skin water content values were observed.

Based on the statistic results, it can be concluded, that the formulations are not significantly differing. However, liposomal cream with higher lipid concentration (BLF 20%) showed more reliable results and is therefore in preference to the liposomal cream with lower lipid concentration (LF 10%). BLF 20% increased the water content significantly ($p < 0.05$) during the first week (6 days) compared with the water content at $t = 0$, whereas for BLF 10% none of the results were significant due to the great interindividual fluctuations.

The results of the long-term in vivo study, multiple application confirmed the results of the short-term in vivo study, single application. The results showed that skin humidity was increased significantly by the application of liposomes over a period of 6 days compared without treatment. These observations correlate well with the findings of Röding and Ghyczy (1991), where an in vivo study demonstrated that the long-term application of liposomes caused an increased hydration of the skin. Liposomes with a high proportion of phosphatidylcholine increase skin humidity after a single application, as well as after a long-term application over a 28-day period.

3.4. In vivo versus in vitro comparison

Previous in vitro results of our research group showed that penetration of fluorescence labelled phospholipids into deep dermis layers was achieved within a relatively short period of time regardless of the composition of the liposomes. The interaction of phospholipid with the skin was studied by confocal laser scanning microscopy (CLSM). The results provided clear evidence that targeting of individual phospholipid molecules to hair follicles has occurred. The fluorescent label reached a penetration depth of approximately 1000 μm, corresponding to the dermis, within 30 min (Betz et al., 2001a,b). The quickness of penetration found was of the same order but somewhat greater than the one reported by other groups, i.e. 1–3 h (Meuwissen et al., 1998), or 3–6 h (Kuijk-Meuwissen van et al., 1998).

Since only the fluorescence labelled phospholipid is visualized by CLSM, this technique provides reliable information about the penetration of phospholipids into the skin but not about the question whether liposomes can penetrate as intact vesicles.

The hair follicle likely plays a role as a pathway for this penetration. The inner root sheath surrounding the hair shaft presents an opportunity for molecules to diffuse along the hair follicle. The distribution of phosphlipids within the lipid matrix of the stratum corneum depends on liposome composition. The liquid crystalline state of the unsaturated phosphatidylcholine bilayer membrane in the presence of ethanol seems to favor this distribution.

The results of the present study showed that all liposome formulations tested in the in vivo study, single application caused a significant increase in skin water content with a maximum after 30 min compared without treatment. The results indicated the influence of liposome formulations on skin humidity and skin barrier function. Thus, the liposomes are suggested to have penetrated the stratum corneum and the epidermis at least fragmentarily. Furthermore a significant difference was detected between the two phospholipid types (PLE and PLS). Liposomes prepared from egg phospholipids showed the best results in increasing skin water content. These results are in agreement with the in vitro study using fluorescent labelled phospholipids.

4. Conclusion

The liposome formulations prepared from PLE showed significantly higher ($p < 0.01$) hydration effects on human skin compared with liposome formulations prepared from PLS. With decreasing PL content in the liposome formulation, a decrease in the hydration effect and in the influence on skin barrier function was observed. It follows that the liposomes must have penetrated the stratum corneum and the epidermis at least fragmentarily. Consistently, all four liposome formulations in the in vivo study after single application caused a significant increase in skin water content. This increase reached a maximum after 30 min. In this context, the formulation of the liposomes seems to be of importance, since a significant difference between the two

phospholipid types (PLE and PLS) could be demonstrated. Liposomes prepared from egg phospholipids showed better results overall, especially they induced a highly significant increase in skin water content. This is of great interest for the dermatologist, since the hydration of the skin is considered to be a marker of its state of health, in the same way that skin dryness is a sign of malfunction. Therefore liposomes and liposome formulations have been implied for skin moisturization, due to the potential occlusive effect of the phospholipid film deposited on the skin surface. In order to increase the skin water content significantly, egg phospholipids are suggested to be used for the preparation of the topical formulation.

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