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Lipid Nanostructures: Self-Assembly and Effect on Skin Properties

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Abstract: This work evaluates the relation between the composition and the self-assembly of some lipid aggregates with their effects on the skin. To this end, liposomes, bicelles and micelles formed by dipalmitoylphosphatidylcholine (DPPC), dimyristoylphosphatidylcholine (DMPC) and dihexanoylphosphatidylcholine (DHPC) were characterized by electron microscopy and dynamic light scattering techniques, and applied on the skin. The results revealed that nanostructures with similar assembly but different composition caused different effects on the skin parameters. In general, samples containing DMPC affected the barrier function to a greater extent than systems containing DPPC. Additionally, our results showed that samples with the same lipid composition but different assembly exerted different effects on the skin. Liposomes decreased or did not modify the transepidermal water loss (TEWL), while bicelles and micelles increased this parameter. Hydration of the skin diminished especially after the application of micellar and bicellar samples. *In vitro* experiments showed structures like vesicles inside cutaneous SC (stratum corneum) incubated with DPPC/DHPC bicelles. These structures were not detected in SC samples incubated with DMPC/DHPC bicelles probably due to the different thermotropic behavior of DMPC and DPPC at physiological temperatures. Results reported in this work should be considered in terms of design of more efficient and specific skin delivery systems.

Keywords: Freeze-substitution transmission electron microscopy; TEWL; skin hydration; biophysical properties of skin; stratum corneum permeability; stratum corneum microstructure

Introduction

Phospholipid systems comprising mixtures of phosphatidylcholines with long and short hydrocarbon chains show high morphological diversity as a function of hydration, temperature and composition.[1] Spherical micelles, discoidal micelles also called bicelles, two-dimensional networks of branched flattened cylindrical micelles, perforated lamellar sheets and vesicles have been detected in these systems.[2] The unique combination of biocompatibility and spontaneous magnetic alignment exhibited by some of these structures justifies their growing importance as membrane models for the characterization of macromolecules by nuclear magnetic resonance (NMR) studies.[3] The discs formed by the combination of the dimyristoylphosphatidylcholine (DMPC) constituting the flat bilayer plus the dihexanoylphosphatidylcholine (DHPC) stabilizing the rim of the structures have

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been the most applied systems for these kinds of studies.[1,3] However, the formation of bicelles with other phospholipids as well as the inclusion of cholesterol, ceramides, cardiolipin and polyethylene glycol (PEG) lipids on the systems was also reported.[4−6] Thus, bicelles are replacing lipid based liposomes and surfactant based micellar systems as models to study membrane proteins. These two nanostructures have often been used for skin treatment[7,8] although their application is debated due to the large size of liposomes (with diameters ranging from 25 nm to several micrometers[9]) that makes difficult their penetration in the skin (interlamellar spaces between 3 and 10 nm[10]), and due to the presence of surfactant in micelles, which usually promotes skin irritation. The skin penetration is a key factor that determines the efficacy of skin delivery systems.[11,12] In this field the bicellar systems seem to offer advantages over liposomes because of their dimensions (diameters in the range of 10−50 nm and thickness about 4−6 nm[1]), small enough for passing through the SC lipid lamellae; and over micelles due to their bilayered structure and exclusively lipidic composition. In addition, bicelles exhibit the possibility of inclusion of different drugs, peptides and polymers, as was demonstrated by Boija et al.[13] The inclusion of ceramides in bicellar systems was also recently reported.[14]

In previous works we studied the structure, composition and lipid behavior of the SC[15−17] as well as the effect of different nanostructures and molecules, such as liposomes, surfactants, organic solvents etc., on the skin, particularly on the SC.[18−20] We demonstrated the disturbing effect of surfactants on SC microstructure and on skin conditions and, inversely, the beneficial effect of structures containing lipids similar to those present in SC. Additionally, the effects of bicellar systems formed by DMPC/DHPC and DPPC/DHPC bicelles on skin have been recently investigated in our group.[21,22] We have demonstrated that DMPC/DHPC bicelles increase the transepidermal water loss (TEWL) and decrease skin hydration without promoting local signs of irritation and without affecting either elastic parameters or SC lipid microstructure.[21] The DPPC/DHPC systems seem to penetrate inside the skin SC and grow forming vesicles.[22] In this work, we seek to evaluate the relevance of lipid self-assembly and composition on effects of different nanoaggregates in skin properties. TEWL measurements and changes in skin hydration reported on the barrier integrity

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and were useful to determine the different effects of each system. Furthermore, erythema and melanin measurements were used as indications of skin tolerance to the different systems tested.

Experimental Section

Chemicals. Phospholipid systems were formed using 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC) purchased from Avanti Polar Lipids (Alabaster, Al).

Preparation and Characterization of the Lipid Systems. Seven systems were formed, including discoidal bicelles, micelles and liposomes with different composition. It is expected that discoidal bicelles with different characteristics will induce different effects on the skin. Thus, discoidal bicelles DMPC/DHPC $q = 2$ and discoidal bicelles DPPC/DHPC $q = 3.5$ were expressly chosen by the different T_m of the long-chain phospholipids and by the different amount of the hydrophilic lipid (DHPC) in both systems.

The discoidal DMPC/DHPC and DPPC/DHPC bicelles (BicDMPC/DHPC and BicDPPC/DHPC) were prepared by mixing appropriate amounts of either DMPC or DPPC powder and a DHPC chloroform solution to reach a DMPC/DHPC molar ratio $q = 2$, and a DPPC/DHPC molar ratio $q = 3.5$. After mixing the components, the chloroform was removed with a rotary evaporator and the systems were hydrated with water to reach 20% (w/v) of total lipid concentration. Bicelle solutions were prepared by subjecting the samples to several cycles of sonication and freezing until the samples became transparent.[1,21,22]

To prepare DHPC micelles (MicDHPC), a chloroform solution of this phospholipid was evaporated and the resulting DHPC was dissolved in water enough to reach a lipid concentration (c_L) of 10%. The formation of micelles took place spontaneously.

DMPC liposomes (LipDMPC) and DPPC liposomes (LipDPPC) were formed by removing the organic solvent by rotary evaporation from a chloroform solution containing each lipid. The lipid films formed were hydrated with water to obtain a $c_L = 20\%$ (w/v).

We also prepared vesicles (LipDMPC/DHPC and LipDPPC/DHPC) formed by the dilution of the DMPC/DHPC and DPPC/DHPC discoidal bicelles. The lipid content of these systems was reduced from $c_L = 20\%$ to $c_L = 5\%$, while q values remained the same.

Size and polydispersity of the samples were analyzed by dynamic light scattering (DLS). Measurements were performed at 20 °C, which is the temperature of the samples just before their application on the skin, and at 32 and 37 °C, which are the skin surface temperature and the physiological temperature of the human body, respectively. This procedure was carried out in order to evaluate the possible morphological changes in the structures induced by the corporal temperature. The samples were maintained under refrigeration (≈4 °C) during the 14 days of experiment. In order to evaluate the structural stability of the samples, size and polydispersity of the solutions were also analyzed by DLS 14 days after preparation. Freeze−fracture electron microscopy (FFEM) was used to obtain additional characterization features of some samples.

Dynamic Light Scattering (DLS). The hydrodynamic diameter (HD) and polydispersity index (PI) were determined by means of DLS using a Zetasizer Nano ZS (Malvern Systems, Southborough, MA).

The particle sizes were determined by detection and analysis of the scattered light from the 632 nm He/Ne laser beam. Noninvasive back scatter technology was used in order to minimize multiple scattering effects. The detection of the light scattered was performed at an angle of 173°.

Freeze−Fracture Electron Microscopy (FFEM). The experiments were done according to the procedure described by Egelhaaf.[23] About 1 μL of suspension was sandwiched between two copper platelets using a 400-mesh gold grid as spacer. Then, the samples were cryofixed by dipping into nitrogen-cooled liquid propane at −180 °C and fractured at −110 °C and 5×10^{-7} mbar in a BAF-060 freeze-etching apparatus (BAL-TEC, Liechtenstein). The replicas were obtained by unidirectional shadowing with 2 nm of Pt/C and 20 nm of C, and they were floated on distilled water and examined in a Hitachi H-600AB TEM at 75 kv.

***In Vivo* Study.** *Study Design.* Noninvasive biophysical parameters were measured in order to evaluate the effect of the different systems on the skin. Intraindividual comparison of the test areas on the volar forearm of volunteers was carried out. Then, BicDMPC/DHPC, BicDPPC/DHPC, MicDHPC, LipDMPC, LipDPPC, LipDMPC/DHPC, LipDPPC/DHPC, and control (nontreated) areas were randomized to the test sites on each subject. 10 μL of BicDMPC/DHPC, BicDPPC/DHPC, MicDHPC, LipDMPC and LipDPPC and 40 μL of LipDMPC/DHPC and LipDPPC/DHPC were applied daily over a period of 10 days, on 7 healthy Caucasian females (age 26−39) with no visible skin abnormalities. All volunteers gave their informed consent in written form for the study. The applied volume of LipDMPC/DHPC and LipDPPC/DHPC was 4-fold in order to apply the same lipid content as systems with $c_L = 20\%$. The subjects were not allowed to use moisturizers on the lower arm for one week prior to and during the days of the experiments. The volunteers were accommodated in an air-conditioned room at 21 ± 1 °C and 50 ± 5% relative humidity for 20 min prior to performance of measurements. The skin properties were measured prior to application of bicelles, on day 1. From then, ten measurements of the skin parameters were carried out (one by day) until one day after the last application (day 11). The measurements were carried out previously to the daily application of systems. In order to be able to make valuable statements on the effect of the

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systems, the results were doubly evaluated as a percentage of modification respect to the basal (initial value) and control zone with the following equation:[24]

$$\text{changes in skin property (\%)} = Q_{tv}/(Q_{0v} - 1) \times 100 \quad (1)$$

where Q_{tv} is the mean of the quotients of the measured values of treated and untreated skin area after application time t of all volunteers v, and Q_{0v} is the mean of quotients of the measured values of treated and untreated skin area before application time of all volunteers. Thus, the initial value for all the parameters corresponded to 100%. This value was compared with the average obtained at the end of the treatment with every system (final value, F).

Transepidermal Water Loss. The measurement of water evaporation rates with the Tewameter TM 300 (Courage and Khazaka, Electronic GmbH, Cologne, Germany) is based upon diffusion principles in an open cylinder system $dm/dt = -DA\ dp/dx$ (where A represents the surface in m^2, m the water transported in g, t the time in hours, D the diffusion constant 0.877 g/(m h mmHg), p the vapor pressure of the atmosphere in mmHg and x the distance from the skin surface to point of measurement in m) according to Fick's law.[25] The vapor density gradient is measured indirectly by two pairs of sensors (temperature and relative humidity) inside a hollow cylinder, and the resulting data are analyzed by a microprocessor. Measurement values are given in $g/m^2/h$.

Skin Hydration. The measurements of skin humidity were carried out by a Corneometer 825, which was mounted on a Multi Probe Adapter MPA5 (Courage and Khazaka, Electronic GmbH, Cologne, Germany). The measurement was performed by the capacitance method that uses the relatively high dielectric constant of water compared to the ones of other substances of the skin. Three measurements were performed in each testing area at different points of the volar forearms.

Melanin and Erythema Indices. Any kind of changes of color or modification of the skin surface was recorded. Thus, melanin and erythema indices were measured photometrically by a Mexameter 18 (Courage and Khazaka Electronic Gmbh, Cologne, Germany) based on the remission principle. The erythema index (EI) and the melanin index (MI) were calculated by the instrument according to the equations

$$\text{EI} = (500/\log 5)(\log(\text{red-reflection/green-reflection}) + \log 5) \quad (2)$$

$$\text{MI} = (500/\log 5)(\log(\text{infrared-reflection/red-reflection}) + \log 5) \quad (3)$$

Each measurement was performed in triplicate on different points of the volar forearms.

Data Treatment. The mean values of the different skin parameters and the corresponding standard deviations were calculated. The results were doubly evaluated as a percentage of modification with respect to the basal (initial value) and control zone. The mean of the initial values of skin before treatment are represented as the 100% values.

Dixon's test was used for detecting outliers, which were excluded from the data. The ANOVA variance analysis was used to determine significant differences between values obtained from different treatments (significance level accepted $*p < 0.05$) using the Statgraphics program.

***In Vitro* Study.** *Stratum Corneum Isolation.* Sections of human fresh skin excised for clinical reasons in the Department of Dermatology, University Hospital "Principes de España", Barcelona, Spain, were placed in water at 65 °C for 4−5 min, and the epidermis was scraped off in sheets. These sheets were placed in 100 mL of 0.5% trypsin in PBS (pH 7.4, 4 °C, overnight). The SC pieces were then collected, rinsed with distilled water, and suspended in a large volume of water. The pieces were transferred to a round flask with fresh trypsin/PBS solution, and the flask was rotated at 100 rpm (25 °C, 2 h) and washed with distilled water.[16] Afterward, the SC pieces were incubated with bicelles (DMPC/DHPC and DPPC/DHPC) and deionized water for 18 h at room temperature.

Freeze-Substitution Transmission Electron Microscopy Experiments. The SC was cut into small ribbons with a size of approximately 2 × 1 mm. The ribbons were fixed in 5% (w/v) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, and postfixed in 0.2% (w/v) RuO_4 in sodium cacodylate buffer, pH 6.8 with 0.25% (w/v) potassium ferrocyanide ($K_4Fe(CN)_6$). After 1 h, the RuO_4 solution was replaced by fresh RuO_4 in order to establish an optimal fixation. After rinsing in buffer, the tissue samples were cryofixed, by rapid freezing on a liquid nitrogen cooled metal mirror (Cryovacublock, Leica) at −196 °C prior to freeze-substitution.

The freeze-substitution procedure was carried out in an AFS (Automatic Freeze Substitution) system (Leica). The tissue samples were cryosubstituted at −90 °C for 48 h using 100% methanol, containing 1.0% (w/v) osmium tetroxide (OsO_4), 0.5% (w/v) uranyl acetate and 3.0% (w/v) glutaraldehyde. After the 48 h substitution period, the temperature was raised to −50 °C, the samples were washed 3 times in 100% methanol, and subsequently the methanol solution was gradually replaced by the embedding medium, Lowicryl HM20 (100%). This resin was replaced after 24 and 48 h by freshly made embedding medium. Finally the samples were transferred to a mold containing Lowicryl, and were incubated for 48 h at −50 °C under UVA-radiation, to allow polymerization. Ultrathin sections were cut (Ultracut UCT, Leica), transferred to Formvar-coated grids and examined

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Table 1. Size and Polydispersity Index (PI) Values Obtained by DLS for All Samples at 20 °C and 37 °C[a]

	20 °C		37 °C	
	HD (nm)	PI (au)	HD (nm)	PI (au)
BicDMPC/DHPC	13.5	0.181	103.2	0.411[b]
LipDMPC/DHPC	123.5	0.383[b]	208.9	0.263[b]
BicDPPC/DHPC	10.7	0.107	11.3	0.072
LipDPPC/DHPC	178.2	0.451[b]	112.6	0.342[b]
LipDMPC	135.0	0.180	130.5	0.165
LipDPPC	132.1	0.172	124.7	0.113
MicDHPC	4.2	0.117	4.3	0.103

[a] 24 h after preparation of the systems. [b] Distributions with PI higher than 0.200.

in a Hitachi 600 transmission electron microscope. For each sample, 10 overview and approximately 30−40 detail electron micrographs were taken.[20,26]

Results

Characterization of the Lipid Nanostructures: Effect of the Temperature. The size and polydispersity values were obtained by DLS for all samples at 20, 32, and 37 °C 24 h after preparation. Results obtained at 32 and 37 °C were very similar. This fact was expected because the lipids used did not experience any phase transition in the temperature range of 32−37 °C. Thus, only values of HD and PI at 20 and 37 °C are shown in Table 1 (PI values higher than 0.200 are noted in Table 1). The HD obtained by this technique corresponds to that of a hypothetical hard sphere that diffuses with the same speed as the particle under experiment. Therefore, considering that bicelles may display different sizes and morphologies, the values of particle size obtained with this technique were considered as an estimation of the structure dimension of our samples, representing a useful tool to study the particle size changes as a function of temperature and composition.

In most cases the samples showed low PI values and the HD was used for comparative purposes. For broader distributions, where the PI was over 0.200 (indicated in Table 1), a distribution analysis of the different peaks corresponding to different size populations was carried out. Table 2 shows the diameter and the proportion of scattered intensity corresponding to the different peaks of the size distribution curves in samples with high PI.

Liposome samples constituted by pure lipids (LipDMPC and LipDPPC) resulted in large structures, about 130 nm and low PI values, at both of the temperatures studied (see Table 1). The samples MicDHPC and BicDPPC/DHPC also showed low polydispersity and remained unaltered by effect of the temperature, although as it is expected the sizes were smaller than those of liposomes. Systems formed by dilution of the discoidal bicelles (LipDMPC/DHPC and LipDPPC/DHPC) resulted in two peaks corresponding with two size

Table 2. Diameter and Proportion of Scattered Intensity Corresponding to the Different Peaks of the Size Distribution Curves in Samples with High PI[a]

		peak 1		peak 2	
	temp (°C)	diam (nm)	prop. (%)	diam (nm)	prop. (%)
BicDMPC/DHPC	37	21.6	85	570.0	15
LipDMPC/DHPC	20	250.1	45	20.1	55
	37	219.2	94.5	34.7	5.5
LipDPPC/DHPC	20	203.0	86	25.8	14
	37	117.0	90	72.9	10

[a] Values 24 h after preparation of the systems.

Table 3. Size and Polydispersity Index (PI) Values Obtained by DLS for All Samples at 20 °C and 37 °C[a]

	20 °C		37 °C	
	HD (nm)	PI (au)	HD (nm)	PI (au)
BicDMPC/DHPC	13.7	0.175	100.2	0.501
LipDMPC/DHPC	125.5	0.362	210.1	0.199
BicDPPC/DHPC	12.1	0.111	11.5	0.080
LipDPPC/DHPC	181.2	0.463	115.5	0.430
LipDMPC	140.1	0.169	131.7	0.172
LipDPPC	137.2	0.171	128.5	0.115
MicDHPC	4.2	0.128	4.4	0.100

[a] 14 days after preparation of the systems.

distributions (see Table 2) and consequently PI resulted higher than 0.200 (Table 1). In both cases one peak centered in dimensions compatible with vesicles (peak 1) and another compatible with disks (peak 2) were detected. When the temperature rose from 20 to 37 °C the proportion of scattered intensity by each population almost did not experience changes for the LipDPPC/DHPC sample, whereas for the LipDMPC/DHPC one, the proportion scattered by large particles increased with respect to that for small particles. The temperature also affected the BicDMPC/DHPC systems, which showed one peak in the distribution curve at 20 °C, and two peaks at 37 °C. A control of the reproducibility of the DLS values 14 days after preparation was carried out. Values of HD and PI obtained are shown in Table 3. These data are very similar to those reported in Table 1 (values obtained 24 h after preparation) indicating the good structural stability of the samples for at least two weeks. This fact guarantees its integrity during the *in vivo* experiment.

The FFEM allowed us to characterize the morphology of the different structures present in the samples. Figure 1 shows BicDMPC/DHPC system at 20 °C (panel A) and at 37 °C (panel B).

In samples cryofixed from 20 °C, the image depicts small structures with diameters about 15−20 nm in which face-on (arrows) and edge-on (arrowhead) disks are visualized (Figure 1A). At 37 °C and in accordance with the DLS results two different structures were visualized, some large elongated aggregates and small discoidal structures with sizes between 20 and 25 nm.

Also corroborating the DLS results, Figure 2 shows the BicDPPC/DHPC system at 20 °C, in which small discoidal

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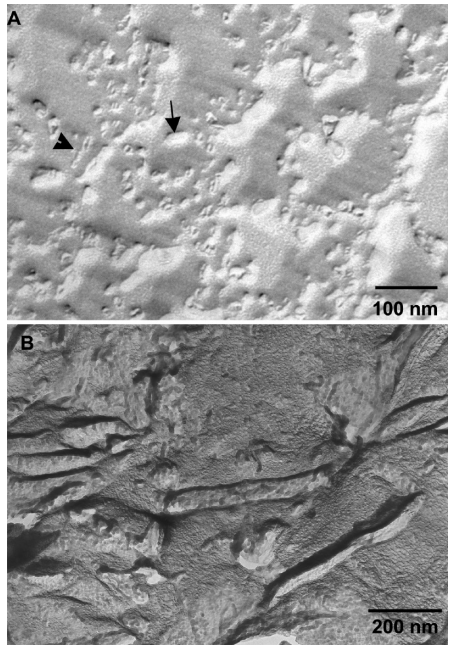

Figure 1. FFEM micrographs of DMPC/DHPC $q = 2$ bicelles. At 20 °C (panel A) the image depicts small structures in which face-on (arrows) and edge-on (arrowhead) disks are visualized. In panel B (sample at 37 °C) large elongated aggregates (arrow) and small discoidal structures (arrowhead) are shown.

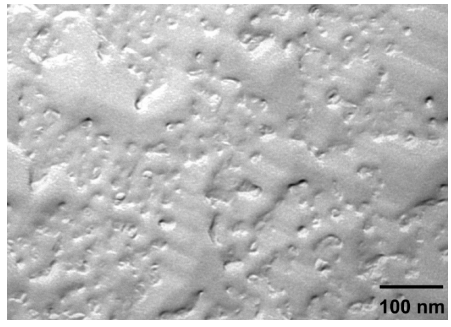

Figure 2. FFEM micrographs of DPPC/DHPC $q = 3.5$ bicelles at 20 °C showing small discoidal structures.

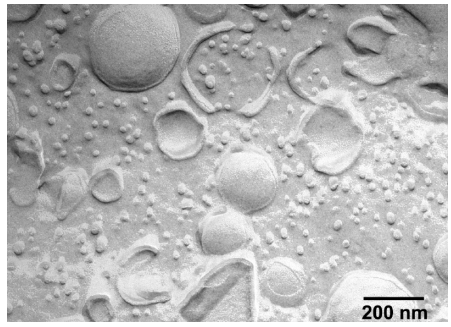

Figure 3. FFEM micrographs of LipDMPC/DHPC systems, in which vesicles and small structures are visualized.

structures were visualized. At 37 °C very similar objects were detected (data not shown).

Figures 3 and 4 depict LipDMPC/DHPC and LipDPPC/DHPC samples respectively.

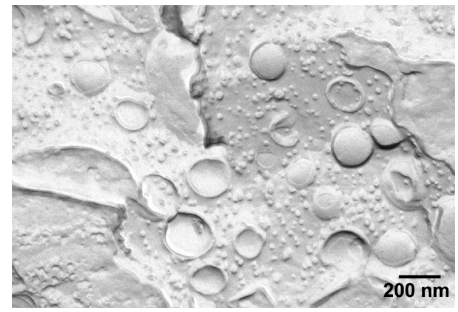

Figure 4. FFEM micrographs of LipDPPC/DHPC systems showing vesicles and small structures.

Given the similarity of these samples at different temperatures, only the samples cryofixed from 20 °C are shown. In both samples the presence of vesicles with diameters around 200 nm and also small structures with about 20 nm in size is appreciated. The main difference between these two samples is related to the proportion of large and small particles. The LipDPPC/DHPC sample at 20 °C contains a higher amount of large structures compared to the small ones. These results related to changes in the morphology of aggregates in DMPC/DHPC and DPPC/DHPC systems induced by temperature and dilution are in good agreement with those obtained by DLS and have also been described by different authors.[2,4,27] The two techniques used in this work are complementary in the sense that DLS offers a more statistical view whereas FFEM allows for the examination of the morphology of the structures. The detection of large and small structures with both techniques indicates that the transition from small discoidal bicelles to vesicles when bicellar systems were diluted was not complete in the experimental conditions.

Effect of the Lipid Nanostructures *in Vivo*: Relevance of the Lipid Assembly and Composition. Table 4 shows the skin biophysical parameter values after the *in vivo* treatment with the different phospholipid systems (F, final value). Considering eq 1, the initial value for all the parameters corresponded to 100%. Then, the variation of final values from 100 allows us to estimate the effect of each system on the barrier function. In addition, the standard deviation (SD) reflecting the interindividual variation is also shown in this table.

Consecutive application of samples containing bicelles on the skin increases TEWL. This effect was more drastic when the bicelles were formed by DMPC/DHPC $q = 2$. In all cases, samples containing DMPC promoted higher barrier impairment than samples containing DPPC with similar structure. Liposome or vesicular samples did not modify TEWL values to a great extent, with the exception of the system LipDMPC/DHPC that induced a remarkable increase in this parameter, and the system LipDPPC that decreased it. It is interesting to note the differences on TEWL values caused by samples with different self-assembly but the same lipid composition. Systems organized as discoidal bicelles

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Table 4. Skin Biophysical Parameter Values (*F*, Final Value) after the *in Vivo* Treatment with the Different Phospholipid Systems and Standard Deviation (SD) Reflecting the Interindividual Variation

	TEWL		hydration		melanin		erythema	
	F	SD	*F*	SD	*F*	SD	*F*	SD
BicDMPC/DHPC	161.84	27.20	71.70	9.62	100.54	0.41	100.88	0.72
LipDMPC/DHPC	118.13	30.83	92.18	21.20	94.87	0.31	101.31	0.68
BicDPPC/DHPC	115.81	31.44	87.18	21.14	100.68	0.97	100.30	1.38
LipDPPC/DHPC	102.17	29.06	110.23	26.46	100.07	0.39	99.49	1.41
LipDMPC	109.44	26.48	88.14	21.40	99.80	0.41	101.30	0.84
LipDPPC	93.59	20.01	90.96	15.30	100.16	0.59	99.22	1.67
MicDHPC	124.71	22.04	83.64	21.93	100.67	0.76	100.84	1.28

(BicDMPC/DHPC and BicDPPC/DHPC) increased the TEWL values, whereas if the organization was as vesicles this increase was minimized (in the case of LipDMPC/DHPC) and even countered (in the case of LipDPPC/DHPC). This result underlines the important role played by the self-assembly and the morphology of the lipid systems on its effects on the skin. Micelles formed exclusively by DHPC were applied on the skin *in vivo*. The effect of this sample was to increase the TEWL, as it could be expected given the hydrophilic character and the tendency of this molecule to favor regions with high curvature in lipid bilayers.[28] However, surprisingly, the increase in the TEWL induced by these nanostructures was lesser than that promoted by the system BicDMPC/DHPC, which contains the same amount of DHPC.

Hydration values decreased after treatment with whatever of the samples, with the only exception of the area treated with LipDPPC/DHPC, which shows a slight increase. The lowest values of hydration correspond to the areas in which the highest TEWL values had been detected. Given that TEWL is a measurement of the rate of water lost through the skin, determination of this parameter is also useful to estimate the ability of the skin to retain moisture. Thus, in some cases, the increase of TEWL correlates with the decrease of water content of the skin. This fact would explain the reduced capacitance values detected in our results when TEWL increases. It is notable that, as in the TEWL data, the hydration values also showed the different effect caused by systems with the same composition but different self-assembly (comparing BicDMPC/DHPC with LipDMPC/DHPC and BicDPPC/DHPC with LipDPPC/DHPC).

Ocular inspections of the treated areas did not report any irritant effect or color modification, and no volunteer perceived irritation or annoyances in the test zones. Additionally, the erythema and melanin indexes were evaluated photometrically using the Mexameter. The final values of these parameters are shown in Table 3. Considering 100 as initial value (before treatment) and taking into account data presented in Table 3, we can assume that no modification in these two parameters was promoted by the treatment with any of the nanostructures used. Values of standard deviation indicated a low variability between different individuals, as it could be expected by the scarce modification of the melanin and erythema indexes.

Effect of DMPC/DHPC and DPPC/DHPC Bicelles *in Vitro*. Pieces of SC were incubated *in vitro* with samples BicDMPC/DHPC and BicDPPC/DHPC. Micrographs of native SC and SC after treatment with BicDMPC/DHPC and with BicDPPC/DHPC are shown in Figure 5, panels A, B and C, respectively.

In these figures are clearly visualized skin corneocytes (C), flattened cells characterized by the absence of cell organelles and the presence of electron dense keratin filaments. In the intercellular spaces, lipid bilayers (L) and corneosomes (CD) (SC desmosomes representing contact areas between adjacent corneocytes) are visualized. These images are very similar to those previously published by different authors[26] for native SC. Comparison of micrographs of SC native (Figure 5A) and SC treated with BicDMPC/DHPC (Figure 5B) allows one to assume that no structural modifications in the SC were induced by the treatment with these bicelles. Figure 5C depicts SC sections after treatment with DPPC/DHPC bicelles. In a similar way to the native sample, the SC treated with bicelles showed corneocytes and corneosomes and, in the intercellular spaces, the lipid lamellae were also clearly visualized. The difference between this micrograph and that depicted in Figure 5, panels A and B, is the presence of vesicle-like structures (V) inside the tissue after treatment with the sample BicDPPC/DHPC.

Discussion

Considerable evidence in the present work supports that discoidal bicelles modify the skin barrier (TEWL and hydration values) to a greater extent than vesicular systems with the same composition (Table 4). This fact could be due to the self-assembly of lipids in the different nanostructures that results in different size and morphology of aggregates. The small size of bicelles in comparison with that of vesicles seems to be a relevant factor to modify the barrier. Dimensions of bicellar systems are small enough for penetrating through the narrow intercellular spaces of the SC. This

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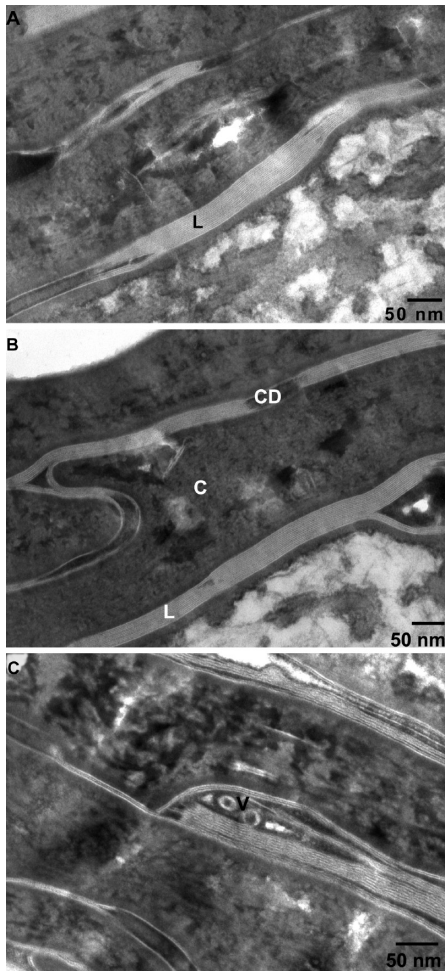

Figure 5. Samples of SC native (panel A) after treatment with the system BicDMPC/DHPC (panel B) and after treatment with the system BicDPPC/DHPC (panel C).

penetration allows lipids from the bicelles to interact with lipids from SC, and this interaction might induce the modification of the barrier function. In the case of vesicular systems (LipDMPC/DHPC, LipDPPC/DHPC, LipDMPC and LipDPPC), in which diameters of nanostructures are higher than 100 nm (Table 1 and Figures 3 and 4), the penetration is more difficult and the modifications of SC lipids and barrier function are less pronounced. Similar findings related to the low ability of intact vesicles to penetrate through the skin have been published in previous works.[20,29] It has been reported that lipid nanoparticles with small size can form a film on the skin surface inducing an occlusive effect.[30] This occlusion induced an increased skin hydration effect.[31] Our results only showed a slight increase in the hydration in one case (in the treatment with DPPC liposomes). Thus, although we can assume some occlusion effect, the influence of this occlusion in the penetration is not clear. In fact, when the penetration is driven by the osmotic gradient across the skin, occlusion could eliminate this osmotic gradient and, therefore, be detrimental for penetration.[32]

In any case, although argument of the size helps us to understand in part our results, it is obvious that other factors are involved, because systems with similar size as BicDMPC/DHPC and BicDPPC/DHPC induced different modifications on skin parameters. Moreover, after treatment with MicDHPC (the smallest structures studied in this work with no bilayer forming lipid), when the skin parameters should be more affected, the values obtained were more discrete than those obtained after treatment with bicelles. Probably, aside of the different morphology of the systems (rounded for micelles or discoidal for bicelles), the different organization of lipids in lipid mixtures or unicomponent samples also affects the skin barrier function. In fact, in a study performed with liposomes, Vrhovnik et al. reported that vesicles with a heterogeneous lipid composition, or in other words, with several coexisting domains of different fluidity characteristics, may induce modifications in the barrier function.[33]

Another interesting result to be discussed concerns the effect of the composition of the systems on the modification of skin parameters. In systems containing similar nanostructures, DMPC/DHPC systems disturbed more the barrier than those formed by DPPC/DHPC (comparison of BicDMPC/DHPC with BicDPPC/DHPC and LipDMPC/DHPC with LipDPPC/DHPC in Table 4). One possible cause of this behavior is the different molar ratio long-chain phospholipids/short-chain phospholipids (q) in the systems. Samples DMPC/DHPC were formed with a q value of 2 and samples DPPC/DHPC with a q value of 3.5. This involves a bigger amount of DHPC in DMPC/DHPC systems. It would be reasonable to expect a higher modification of the barrier when skin is treated with systems richer in DHPC (short-chain phospholipid that exhibits surfactant characteristics with tendency to form micelles[28]). That is, systems with lower q values would induce higher barrier modification, as indeed occurred in our experiments. Then, following this argument, sample MicDHPC, containing the same DHPC amount than BicDMPC/DHPC and also small in size, should promote similar TEWL and hydration alterations as the bicellar system. However, the modifications induced by micelles (MicDHPC) were quite smaller (Table 4). This fact makes one think that not only the difference of the DHPC amount in DMPC/DHPC and DPPC/DHPC systems is involved in

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the alteration of the barrier; again the self-assembly should be taken into account.

Other important factors to be considered are the different gel-to-liquid crystalline phase transition temperatures (T_m) of the two long-chain phospholipids used and the experimental temperature conditions. The T_m for DMPC is 23 °C, and the T_m for DPPC is 41.5 °C.[34] This involves that at the temperature of sample application (20 °C), both lipids were below their T_m, i.e., the lipids were in the gel state. In contact with the skin (*in vivo* and *in vitro*) the temperature rose, 32 °C at the skin surface and 37 °C in the human body. In any case, this increase induced the phase change from the gel to the liquid crystalline state only in DMPC systems; DPPC systems remained in the gel state.

According to different works, gel state lipids are less effective in increasing permeation across the skin than liquid ones. These authors claim that gel state vesicles can even inhibit drug permeation.[29] This inhibition is in agreement with the less capacity of DPPC systems to alter skin barrier function detected in the present work. Regarding micrographs from SC treated *in vitro*, it is interesting to note that precisely systems promoting higher modification *in vivo* (BicDMPC/DHPC) did not show any changes in the SC microstructre *in vitro* (Figure 5B). In contrast, tissue samples treated with BicDPPC/DHPC presented vesicular structures (Figure 5C) although the barrier function *in vivo* had been considerably preserved. BicDMPC/DHPC at 20 °C could penetrate through the skin, whereby the effect of the temperature increase would grow (Table 1 and Figure 1) and due to the phase transition gel-to-liquid crystalline could mix and modify the lateral organization of the SC lipids. This modification is imperceptible by the microscopy technique used in the present work, but would explain alteration in the barrier function. BicDPPC/DHPC preserve their small size, morphology and phase state at the temperatures studied (20 and 37 °C, Table 1 and Figure 2). Then, these small structures could penetrate through the SC, and although a possible interaction between lipids from bicelles and from SC cannot be disregarded, the gel state of these lipids would minimize the mixing with SC inhibiting the modification of the barrier function and allowing the transformation from discoidal structures to vesicles inside the SC. This transformation could be induced by the skin hydration gradient, in a similar way that it is produced in surfactant−lipid systems.[20] The growth from small structures to large vesicles by effect of dilution in DPPC/DHPC systems has been recently reported[21] and supposes a new insight in the use of bicellar systems for skin application.

Controlling the physicochemical properties of lipid systems is a key factor to optimize the application of these nanostructures in the pharmaceutical field. Thus, bicelles could be used as skin permeability enhancers of drugs or as systems for reinforcement of the SC lipid structures. The inclusion of different drugs and peptides in the bicellar systems is being studied in different laboratories.[13,14] The present work demonstrates that hydration and temperature determine the self-assembly (size, morphology, structure) and this self-assembly has a relevant impact on the effect of the systems on the skin. Of course, the selection of an adequate composition of the systems, for instance use of lipids with transition temperatures that guarantees a determinate state, is also a key factor in order to make more efficient the use of lipid nanostructures. However, our results indicated a stronger influence of the self-assembly in comparison with the lipid composition. In addition, the knowledge of the self-assembly behavior of lipid systems reported by the present work opens the possibility of an improved application of these nanostructures in fields different from the skin, as delivery by systemic and/or ocular routes. The modulator effect of permeability of some of these systems should be investigated on other biological barriers.

Abbreviations Used

Cer, ceramide; DHPC, dihexanoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; SC, stratum corneum; FFEM, freeze-fracture electron microscopy; FSTEM, freeze-substitution transmission electron microscopy; DLS, dynamic light scattering; TEWL, trans-epidermal water loss.

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