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Analysis of skin penetration of phytosphingosine by fluorescence detection and influence of the thermotropic behaviour of DPPC liposomes

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ABSTRACT

Phytosphingosine (PS) is a promising compound in skin formulations, considering its application in the treatment of acne and different inflammations as well as in the 'anti age' cosmetics. PS, as an active substance was incorporated in DPPC liposomes intended to standard diffusion experiments, where dermatomed porcine skin was mounted in FRANZ cells. The proved skin retention was about 5.5% (w/w) after 24 h and about 6.8% (w/w) after 48 h of the applied PS amount, whereas only about 0.05% (w/w) and about 0.07% (w/w) PS, respectively, could be observed in the acceptor medium. To increase analytical sensitivity PS was derivatised by o-phtalaldehyde (OPA) reagent and analysed by HPLC with fluorescence detection. The higher amount of PS within the skin symbolised an interaction with lipid structures in skin. Further evaluation of this interaction was accomplished by applying microDSC studies of PS with DPPC as a model membrane. For this purpose liposomes were prepared by increasing PS content. The characteristic endothermic peak observed for the single system was shifted to a slightly higher temperature and broadened as the mole fraction of PS increased. This might be the effect of mixing of PS with DPPC. An addition of 10 mol% PS resulted in more than double sized particles pointing to a possible change in the liposomal shape.

1. Introduction

The free sphingoid base phytosphingosine (PS) is naturally found in cell membranes of a human body and is present in small amounts in the epidermis (Thompson et al., 2006). PS is involved in keratinocytes differentiation and anti-inflammation (Schiemann et al., 2008). In several studies PS showed anti-microbial properties against probionibacterium acnes and staphylococcus aureus and therefore effectively reduced signs of acne. Furthermore, there are investigations confirming that PS reduced redness of inflamed skin (Yilmaz and Borchert, 2005, 2006; Kang et al., 2007). Its positive influence on the skin permeation of fludrocortisone acetate and flumethasone pivalate in nanoemulsions formulations was documented by our group (Hoeller et al., 2009). Recently an additionally preventive effect of PS on UV-induced decrease of pro-collagen was demonstrated in human dermal fibroblasts. Moreover, PS showed the inhibition of UV-induced IL-6 and COX2 gene expression. These results indicate that topically applied PS has anti-aging properties pointing to the potential use of PS as a therapeutic agent in the prevention and treatment of extrinsic aging (Cho et al., 2008). Due to all these studies, PS could be considered as an ideal active ingredient for different pharmaceutical and cosmetic applications whose biological activity can be enhanced by the use of an appropriate formulation. DPPC liposomes, spherical bilayer vehicles, have been chosen due to ability to encapsulate oil soluble components, increase their stability and maintain their activity in different environments (Laye et al., 2008). Moreover, they are able to increase concentration of the compound in the epidermis and in the deeper layers of the skin probably through the similarity of their lipid bilayer to that in the skin (Perugini et al., 2000).

In the first part the PS amount of skin penetration as well as skin retention from DPPC liposomes were investigated. For this purpose we performed diffusion studies on dermatomed porcine skin placed in FRANZ cells. The analytical method following derivatisation of PS involving HPLC was successful. It was simple, sensitive and exact, due to fluorescence detection.

In the second part DPPC liposomes were used as model membranes to gain more information about their interaction with PS. MicroDSC studies investigating the influence of PS on DPPC thermotropic behaviour were selected.

2. Materials and methods

2.1. Materials

1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) was purchased from Lipoid (Switzerland). The product was Lipoid PC 16:0/16:0. According to its specification the content of

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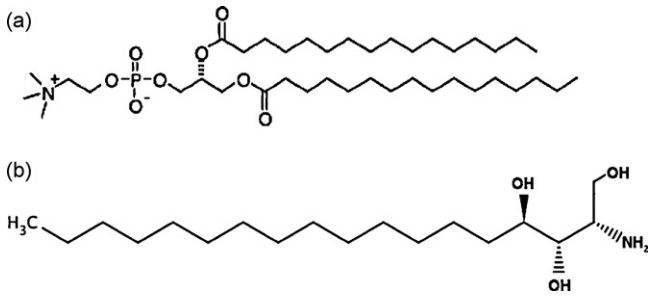

Fig. 1. (a) Chemical structure of DPPC and (b) chemical structure of PS.

phosphatidylcholine related to the dry weight was at least 99%. Phytosphingosine was kindly provided by Degussa (Cosmoferm BV, NL). All other chemicals used in this study were of analytical reagent grade and were used as received without any further purification.

The chemical structures of DPPC and PS are presented in Fig. 1.

2.2. Preparation of liposomes

DPPC liposomes were prepared according to a modified method of Thompson et al. (2006). Briefly, the phospholipid powder was dispersed in distilled water at 60 °C in an end concentration of 2.5% (w/w) and magnetically stirred until the powder was completely dissolved and thoroughly mixed using an ultra-Turrax (Omni 500). Afterwards the dispersion was homogenised with a high-pressure homogeniser (EmulsiFlex-C3, Avestin) for 16 times at approximately 1100 bar. The liposomes were kept at 4 °C over night and characterised the day after in terms of mean particles size and PDI.

Into these liposomes PS was incorporated directly in the following concentration: 2.5, 5 and 10 mol%, respectively, and mechanically stirred for 24 h at 60 °C. The resulting liposomes were used for microDSC studies.

For the diffusion experiments, liposomes loaded with 0.2% (w/w) PS were prepared in the same conditions.

2.3. Diffusion studies

In vitro permeation studies with porcine abdominal skin were performed as previously reported (Hoeller et al., 2008). To obtain optimal sink conditions the receptor compartment was filled with 2 ml of 0.02 M phosphate buffer adjusted with lactic acid to pH 5. Since the concentration of PS actually used in cosmetics is 0.05–0.2% (w/w), about 0.5–0.7 ml of 0.2% (w/w) PS loaded liposomes were applied to the skin surface. Application of the liposomes was defined as the starting point of the procedure. Two independent sets of experiment were performed: one for 24 and one set for 48 h. Samples were drawn, centrifuged at 17,586 × *g* for 6 min. Subsequently the supernatants were discarded and frozen at −20 °C. They were then freeze-dried under the following conditions: at −30 °C for 3 h and afterwards at −55 °C over night. The lyophilisates were resuspended in ethanol. Each batch was derivatised with OPA-reagent prior to quantification by HPLC.

Additionally experiments were performed in order to analyse the content of the permeated PS in the skin 24 and 48 h after application, respectively. At the end of each experiment sample-applied skin was wiped with paper than sliced and homogenised with a defined amount of ethanol, vortexed for 30 s and stirred over night. After centrifugation, supernatants were derivatised with OPA-reagent and quantified by HPLC as described in 2.4. Due to the naturally occurring PS in the epidermis we additionally carried out parallel experiments with PS-free liposomes. The measured PS concentration in the skin was about 0.1 μg ± 0.03/cm^2 and therefore negligible.

2.4. OPA-reagent derivatisation and HPLC analyses

The OPA-reagent derivatisation was accomplished as previously described by Min et al. Briefly, the sample solution was mixed with OPA-reagent (5 mg o-phtalaldehyde, 25 μl 2-mercaptoethanol, 0.5 ml ethanol and 50 ml borate buffer pH 10.5) in 1:1 ratio for 2 min and incubated at room temperature for 30 min. The eppendorf tube was light protected to prevent degradation of OPA-reagent (Min et al., 2002).

The HPLC analysis was performed using C-18 RP column (Nucleosil 100-5, 250 mm × 4 mm, Macherey-Nagel, Germany) at 1 ml/min flow rate, with acetonitrile/water 90/10. The fluorescence intensity of the eluate was monitored at a wavelength of 370/460 nm excitation/emission by fluorescence detector (LS40, PerkinElmer). Ethanol standard solutions of PS in the range from 0.3 to 100 μg/ml ($n = 7$) were derivatised with OPA-reagent and used to construct the calibration curve. Linearity was determined by the elaboration of three standard calibration curve (every fresh prepared). A very high correlation of 0.9998 ± 0.0002 was obtained. The deviations of all points of calibration curve of PS were in the range of 1.1–2.5% of the slope of the corresponding regression equation.

2.5. Particle size measurement of liposomes

The mean particle size and size distribution were determined by photon correlation spectroscopy with a Zetasizer Nano ZS (Malvern, UK) at 25 °C. The liposomes dispersion was diluted to the appropriate concentration with deionised water after 24 h of preparation. The size distribution was represented by the polydispersity index (PDI) values. The measurements were performed using a He–Ne laser at 633 nm.

2.6. Micro-differential scanning calorimetry (microDSC) studies

Differential thermal analysis was performed using a Setaram III micro-calorimeter. Samples of pure and PS loaded liposomes, prepared as described in Section 2.2 were scanned. The measurements conditions were: 1 °C/min for the scanning rate over the temperature range 15–65 °C, using purified water as reference.

Thermal transitions were calculated using Setsoft 2000 Setaram software. After baseline subtraction, raw power data were converted to molar heat capacity data. Baselines were fitted to the pre-transition and main transition regions using a linear baseline function so that transition temperatures and enthalpies of reaction could be calculated for each lipid concentration.

2.7. Statistical data analyses

Results of all studies are expressed as the means of at least three experiments ± S.D. Statistical data analysis was performed using *t*-test with $p < 0.05$ as minimal level of significance.

3. Results and discussion

Due to increasing number of atopic eczema and pruritic diseases, new dermal preparations with the anti-inflammatory PS are of a great interest. Recently, additional penetration enhancing effect on the skin permeation of fludrocortisone acetate and flumethasone pivalate was shown (Hoeller et al., 2009). Therefore, PS could have the extra benefit of reducing topical corticosteroids acting as a multifunctional drug.

In a first part of our research the amount of penetrated PS through porcine skin was measured. Due to the high lipophilicity of the PS (LogP 5.18), it could be expected that its main amount would be restored within the skin. In preliminary experiments it was seen that an HPLC analytic of PS with UV detection could not be used due

Table 1
Skin retention, cumulative permeated amount and amount in the donor compartment of PS in % of the applied amount after 24 h (one set of experiments, and after 48 h (one set of experiments) of diffusion (means ± S.D. of at least three experiments).

Time (h)	PS (donor) (%)	PS (retained) (%)	PS permeated amount (%)
24	94 ± 9	5.5 ± 2.4	0.005 ± 0.0002
48	93 ± 16	6.8 ± 0.8	0.007 ± 0.0002

to the very small PS amount remained under the detection limit. Therefore a more sensitive method using fluorescence detection was achieved. To gain a fluorescent detectability PS was labelled by o-phthalaldehyde (OPA) reagent. Previously this method was useful for measurement of different sphingoid bases in biological samples (Min et al., 2002). The derivatisation relied on chemical reaction between o-phthalaldehyde and the primer amine group of PS in the presence of reduced sulphur compound mercaptan. The obtained product, iso-indole, was analysed by HPLC using fluorescence detection. Diffusion experiments were run for 24 and 48 h, respectively, after which the skin was shredded and analysed for PS. The applied amount of liposomes corresponded to an infinite dose. As seen in Table 1 after 24 h about 5.5% (w/w) and after 48 h about 6.7% (w/w) of the applied PS amount was retained within the skin whereas only a small PS concentration of about 0.05% (w/w) and about 0.07% (w/w) could be detected in the receiver compartment after 24 and 48 h of diffusion, respectively. Interestingly no significant increase of PS in the acceptor medium can be achieved after 48 h compared to 24 h of diffusion. This result may indicate an interaction of PS with structures within the skin.

In order to obtain a better understanding of the mechanism of this interaction of PS, microDSC studies with DPPC as a model membrane were performed (El Maghraby et al., 2005, 2008). Although we are aware that the lipid composition of skin is completely different, DPPC liposomes, due to their, simplicity, were used as a model membrane for many other studies (Auner et al., 2005; Lucio et al., 2008; Panicker, 2008).

Since DPPC is a widely used model for lipid bilayers and its transition temperature T_{max} can be easily measured as it has a narrow main endothermic peak at 41.4 °C (gel-to-liquid crystalline phase $P\beta' \rightarrow L\alpha$), where the lipid acyl chains in an all-trans configuration undergoing a chain melting transition at a temperature, T_{max}, to liquid crystalline phase with the chains having both trans as well as gauche configurations (Wolka et al., 2004; El Maghraby et al., 2005; Panicker, 2008). A smaller pre-transition, which appears near 34.4 °C reflecting the lamellar gel to rippled gel ($L\beta' \rightarrow P\beta'$) transition in the gel phase (Wolka et al., 2004; Auner et al., 2005; El Maghraby et al., 2005; Krivanek et al., 2008; Panicker, 2008). The pre-transition has been generally attributed on the surface structure of the vehicle and is related to rotations of the phospholipid head groups or transformation in the lamellar structure and changes in the hydrocarbon chain packing (Auner et al., 2005). Fig. 2 gives the microDSC curves of the DPPC-PS loaded liposomes. As seen in Table 2, the characteristic endothermic peak observed for the single system is shifted to a slightly higher temperature and broadened as the mole fraction of PS increased. This effect on main transition endotherm may be explained as a result of mixing of PS with DPPC. This is in agreement with other studies investigating the similar structured stearylamine interacting with DPPC (Sakaia et al., 2008). In other words, the higher transition temperature observed from the compositions of DPPC and PS may indicate closer packing of the DPPC and PS molecules forming the lamellar bilayers.

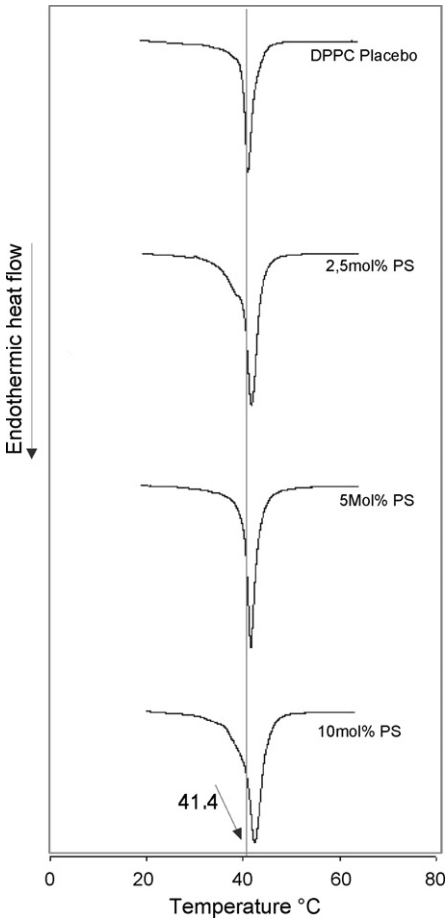

Fig. 2. Representative microDSC endotherms of DPPC liposomes containing 0–10 mol% PS respectively. T_{max} of DPPC is 41.4 °C, as indicated with an arrow.

On the one hand the preferential partition of the lipophilic compound, PS, into the lipid domains of the bilayer structure by incorporation is expected. On the other hand the disappearance of the pre-transition (Table 2) indicates an interaction with the polar region as well (Auner et al., 2005; Panicker, 2008). Moreover, an incorporation of 10 mol% PS was able to increase the liposome size more than twice (Table 3). This might be explained as a change of the shape of multilamellar vesicles (MLV) to large unilamellar vesicles (LUV) (Yokoyama et al., 2005; Biruss and Valenta, 2007) and due to the charge effect of PS (Hoeller et al., 2009). However, TEM microscopy studies have to be performed to confirm this hypothesis.

Table 2
Transition temperature and enthalpy values of DPPC liposomes with and without PS (means ± S.D. of at least three experiments).

mol% PS	T_{max} (°C)	Linear onset (°C)	Enthalpy (J/M)
0	34.4 ± 0.3 pre	33.5 ± 0.6 pre	30 ± 1 pre
0	41.4 ± 0.1 main	40.1 ± 0.1 main	30,600 ± 430 main
2.5	41.7 ± 0.1	39.8 ± 0.0	44,000 ± 2000
5	41.8 ± 0.0	40.3 ± 0.3	40,000 ± 15,000
10	42.4 ± 0.1	40.0 ± 0.2	88,000 ± 16,000

pre = pre-transition temperature; main = main transition temperature.

Table 3
The effect of incorporation of PS into 2.5% (w/w) DPPC liposomes on mean particle size, polydispersity index (PDI) and pH-values (means ± S.D. of at least five experiments).

mol% PS	Particle size (*d*, nm)	PDI	pH
0	81 ± 6	0.24–0.25	6.3 ± 0.2
2.5	152 ± 7	0.29–0.41	8.4 ± 0.0
5	166 ± 10	0.26–0.48	8.3 ± 0.1
10	178 ± 7	0.34–0.50	8.6 ± 0.0

4. Conclusion

In penetration studies with PS loaded DPPC liposomes it could be proven, that PS is retained in skin in relevant amounts. This seemed to be due to PS-lipid interactions seen in microDSC studies, indicating a closer packing of DPPC and PS in model membranes.

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