Research Article

TOWARDS A SIMPLIFIED MODEL MEMBRANE OF SKIN LIPIDS: PREPARATION AND CHARACTERISATION OF A TERNARY LIPID MIXTURE

[1,2]*HASSAN M. GHONAIM, [1]NAGARAJAN PERIASAMY, [3]MASSIMO G. NORO,[1,4]JAMSHED ANWAR

[1]Computational Chemistry group, Institute of Life Sciences Research, University of Bradford, Bradford, W. Yorkshire BD7 1DP U.K., [2]Department of Pharmaceutics, Faculty of Pharmacy, Suez Canal University, Ismailia, Egypt, [3]Physical and Chemical Insights Group, Unilever Port Sunlight, U.K., [4]Dept of Chemistry, Lancaster University, Bailrigg, Lancaster, LA1 [4]YB, UK.
Email: hassan_ghonaim@pharm.suez.edu.eg

ABSTRACT

Objective: To develop a simple but a robust model membrane comprising the key generic components of the skin lipid matrix.

Methods: We prepared a model membrane comprising an equimolar mixture of ceramide [NS], palmitic acid, and cholesterol. The membrane was deposited on a solid porous substrate (50 nm porous polycarbonate filter) as a continuous lamellar layer. The membrane's biophysical properties were characterized and the permeability of the membrane to both benzoic acid and caffeine evaluated.

Results: The deposited membrane's biophysical properties were characterized using a variety of methods including Laurdan fluorescence spectroscopy, SEM, and Raman scattering. The permeability co-efficients (kp) of the model membrane to benzoic acid and the hydrophilic molecule caffeine were determined to be of the same order of magnitude as that through real stratum corneum.

Conclusions: A simple but robust model membrane for skin lipids has been developed that can be deposited to give a continuous coverage on a porous substrate. It's permeability to caffeine and benzoic acid has been found to be of the same order as that of real stratum corneum. The model membrane will be invaluable for fundamental mechanistic studies (e.g. effect of penetration enhancers) that can be linked with molecular simulations.

Keywords: Ceramide; Stratum corneum substitute; Caffeine; Benzoic acid, Skin permeability; *In vitro* permeation; Model membrane.

INTRODUCTION

The unique barrier property of skin is due to the outermost layer of the skin known as the stratum corneum (SC). The SC comprises several layers of overlapping corneocytes embedded in a matrix of lipid lamellae. The lipid matrix appears to be an important determinant of the physico-chemical properties of the SC layer [1-3]. For example, it is the primary barrier to water loss from the skin and is the main route by which molecules may permeate into or through the skin in topical and transdermal drug delivery. The lipid matrix is composed mainly of ceramides (~50%), cholesterol (~25%), cholesterol sulfate (~5%), fatty acids (10-15%) and a small amount of cholesterol esters, with little or no phospholipids[4]. Recent reports indicate that there are 12 classes of ceramides consisting of a base of varying alkyl chain lengths which can be either a dihydrosphingosine [DS], a sphingosine [S], a phytosphingosine [P], or 6-hydroxysphingosine [H], linked to either a nonhydroxy fatty acid [N], α-hydroxy fatty acid [A] or esterified ω-hydroxy fatty acid [EO] of varying alkyl chain lengths[5-7].Whilst the chemical composition of the SC is fairly well characterised, the physical organization of the lipid molecules is still not entirely resolved [1, 8, 9]. The lipids form lamellar structures that are considered to exist either as bilayers or as three-layer 'sandwich'-type structures comprising a softer, more fluid inner layer surrounded by condensed outer layers[10]. A recent report involving high resolution cryo-electron microscopy of vitreous skin section (CEMOVIS) suggests that the lipids are stacked bilayers of fully-extended ceramides with cholesterol molecules associated with the ceramidesphingoid moiety[9, 11]. Either way, the phases are considered to be essentially solid-like with the lateral packing of the alkyl chains being orthorhombic, though some looser-packed hexagonal gel phase has also been observed, being confined to the topmost layers of the SC [12-15].

There is considerable interest in developing *in vitro* models of the SC lipids, notably in areas of skin care and drug delivery [16-18]. There are two motivations, firstly the *in vitro* models could minimise the use of human and animal tissues – the use of skin for the testing of finished cosmetic products and cosmetic ingredients on animalsis banned in the EU [http://ec.europa.eu/enterprise/sectors/cosmetics/animal-testing/]. Secondly, the reductionist approach involved in the development of *in*

vitro models, which involves physical characterization of varying lipid compositions, has the potential to increase our knowledge of the structure and function of the SC lipids in their native biological state, and aid the identification of the key components that determine the overall physico-chemical and mechanical properties of the SC lipids [19]. For example, the known ~ 6 nm and ~13 nm periodicities observed for native SC lamellar phase structures can be reproduced by in-vitro mixtures comprising cholesterol, free fatty acids, and specific ceramides [13, 20].

Whilst there has been some recent success in developing an *in vitro* model for percutaneous penetration studies [20], the systems investigated are complex involving a cocktail (although well defined) of differing ceramides and free fatty acids. The approach adopted in such studies is top down i.e. are duction in the complexity of the native SC whilst retaining the objective of reproducing structural features and the barrier properties of skin to molecules. In contrast, our reported study here takes the bottom-up minimalist approach, using only a ternary system comprising a ceramide, cholesterol and a selected fatty acid. Such systems are much more amenable to characterisation and endeavour to capture the essential physics, a particular advantage being that they are accessible to molecular simulation that can yield atomistic-level resolution as well as estimates of dynamical and thermodynamic quantities [8, 19, 21-23]. Molecular simulation is a powerful tool that complements experiments and which is increasingly enhancing our understanding of simple ceramide-based model lipid membranes [24-27]. Meaningful simulations currently tend to utilize at most 2-3 molecular components making comparison with complex multi-component systems difficult. A simplified model membrane of skin lipids will serve to bridge the gap between real complex systems and molecular simulation. In developing a model membrane for transport studies, the essential challenge is to be able to deposit a continuous layer with a length scale in the centimetre range, which persists when exposed to an aqueous environment in a diffusion cell. Deposited lipid films invariably fragment forming patches and/or detach from the substrate and to form vesicles on hydration [28-33]. Furthermore, gross defects in the meso-structure (exhibiting the least barrier) could dominate the transport properties, and may serve as sites for initiating membrane

lifting from the substrate. We report here our success of depositing a robust, continuous layer of a simplified model membrane for skin lipids comprising an equimolar mixture of synthetic ceramide [N(16) S(18)] (ceramide containing only a nonhydroxylated fatty acid (C 16:0) and sphingosine (C18)),palmitic acid and cholesterol(chemical structures are given in Fig. 1).Ceramide [NS] is one of the most abundant ceramide in the SC with varying chain length of C_{16-30} N and C_{16-26} S [7]. We thought it prudent to avoid the complexity of asymmetric acyl chains at this stage, though we note that the alkyl chain asymmetry of the skin ceramides might be an important characteristic (along with ceramide EOS) determining the molecular organization of the skin lipids [34]. Palmitic acid was chosen to match the acyl chain length of ceramide [NS]. The proposed ternary model, whilst not entirely employing specific skin ceramides, is a significant step up from phospholipids [35] and yet could serve as a generic ceramide-based membrane for molecular level studies. The phase behaviour and structure of the ternary mixture has been characterised using various biophysical techniques including Laurdan fluorescence spectroscopy, SEM and Raman scattering. Further, the barrier property of this model membrane has been investigated using caffeine and benzoic acid as permeants in a Franz type diffusion cell. The study reveals that the chosen, rather simple, ternary composition is able to form a coherent membrane on porous supports and has permeability characteristics of the same order as SC.

METHODS AND MATERIALS

Materials

Ceramide [N (C16:0) S (C18)] was purchased from Quest, C16 fatty acid palmitic acid (PA), cholesterol (Chol), caffeine and benzoic acid (BA) were purchased from Sigma. Nucleopore polycarbonate filter disks (pore size 50 nm, 25 mm diameter) were purchased from Whatman (Kent, UK). Laurdan (6-dodecanoyl-2-dimethyl aminonapthalene) was purchased from Molecular Probes Inc (Eugene, OR). All chemicals were used without further purification. The Evolution Silverline airbrush utilized for preparing a membrane on a porous support was purchased from Artimagination Limited, UK. All organic solvents used were of analytical grade and purchased from VWR international.

Laurdan Fluorescence Spectroscopy

Ceramide [NS], palmitic acid and cholesterol stock solutions were prepared in chloroform. Laurdan was dissolved in chloroform at a concentration of 1 mmol/L. Vesicles containing an equal molar ratio of CER [NS], PA and Chol were prepared together with the Laurdanfluorophore. After co-dissolving the lipids with the fluorophore the solvent chloroform was removed by a flowing stream of nitrogen gas. The samples were then dried under high vacuum for several hours to completely remove the remaining solvent. The residual dry film was then re-suspended in sodium citrate buffer pH 5.5, vortexed and sonicated for one hour (with a 5 minute inactive interval every 15 minutes) in a bath-type sonicator (Bandelin SONOREX RK100SH). Finally, several freeze–thaw cycles were applied to enhance the homogeneity of the vesicle preparation. The final concentration of the lipid vesicles in the samples used for the fluorescence measurements was 5.2 mmol/L and that of the fluorescent probe was 10.4 μmol/L. The final vesicle solution contained a 1:500 fluorophore to lipid ratio on a molecular basis. The fluorescence spectroscopic measurements were performed using a K2 multifrequency phase and a modulation fluorometer with photon counting mode equipment (ISS Inc., Champaign, Il). The temperature was controlled to ±0.1 °C by a circulating water bath.

Model membrane preparation by airbrushing

Model membranes comprising of CER [NS], PA and Chol were deposited using a manual airbrush technique. Required amounts of each lipid, CER [NS], PA and Chol, to get an equimolar mixture were dissolved in n-hexane: ethyl alcohol (2:1) solvent mixture and mixed.

The overall lipid concentration of the mixture was 4.5 mg/mL. The airbrush was connected to an oxygen free gaseous nitrogen cylinder and used to spray the lipid solution on to the top of a polycarbonate filter support. We followed the procedure developed by Bouwstra

[36]. The total amount of solvent used to deposit the model membrane on a particular filter support was 2mL. After each spray, the organic solvent was slowly evaporated using a gentle nitrogen flow. The lipid-coated polycarbonate filters were then equilibrated at 80 °C for 10 min and gradually (over 1 hour) cooled down to room temperature.

Scanning electron microscopy (SEM)

The surface structure of the lipid coated polycarbonate membrane was characterised using a scanning electron microscope (SEM) Quanta 400 FEI (FEI Inc.) with a tungsten electron emitter, operating at 20 KV under high vacuum.

Permeation studies

The permeability of the model membrane was investigated by means of Franz type diffusion cells (diffusion area of 1.00 cm^2, donor compartment 2 mL and receptor compartment 8 mL) using the model permeants caffeine and benzoic acid. The permeation profiles of both these molecules through the skin SC is well characterised [20, 37]. The model membranes coated on polycarbonate filter supports were mounted in the diffusion cells and hydrated with sodium citrate buffer (pH 5.5) for caffeine and PBS (pH 7.4) for benzoic acid prior to the experiment. The diffusion cells were placed on a hot plate with the temperature being maintained at 32 °C throughout the experiment.

The donor compartment was filled with 1.5 ml of caffeine solution in citrate buffer (pH 5.5) at a 25 mg/ml or benzoic acid solution in PBS (pH 7.4) at a 3.0 mg/ml concentration. The receptor compartment was completely filled with the respective buffer (8 ml) and gently stirred using a magnetic stirrer throughout the experiment. The donor compartment was covered with a plastic paraffin film (parafilm) to avoid evaporation of the solution. Sample fractions were collected from the receptor compartment at 1 hour intervals for 8 hours. The total volume of the receptor compartment was maintained by replenishing with fresh buffer.

The amount of drug permeated through the model membrane was determined by UV analysis in standard quartz cells after suitable dilution using a Perkin Elmer UV-Vis spectrometer at λ = 273 nm for caffeine and at λ = 225 for benzoic acid. A control experiment was also conducted using an uncoated polycarbonate support sprayed with only the solvent mixture i.e. without lipids.

Raman spectroscopy

Raman spectroscopy was performed on the membrane-coated polycarbonate supports as well as the individual lipid components at room temperature using a Renishawin Via Raman Microscope (Renishaw plc, Gluocestershire, UK) and the accompanying Reinshaw Wire® and GRAMS©AI version 8 (Thermo Electron Corp, MA, USA) software. The samples were excited using a 785 nm near-IR diode laser (Renishaw HPNIR laser) and a 50x objective lens giving a laser spot diameter of 5 μm. Spectral scans were carried out between 3200 cm^{-1} and 100 cm^{-1}using an exposure time of 10s and each spectrum being collected using 9 accumulations with 100 % laser power.
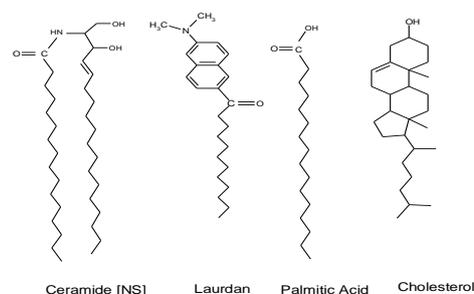

Fig. 1: Chemical structures of ceramide [NS], Laurdan, palmitic acid and cholesterol.

RESULTS AND DISCUSSION

The objective was to derive a simple, ternary lipid mixture that would form a continuous membrane in the centimetre range which is robust to hydration, and to characterise its physical properties including

permeability to a couple of model compounds. We looked at various combinations of lipids and a variety of methods for deposition including spinning, soaking the membrane with CER [NS]:PA:Chol mixture as well as airbrushing. The factorial space here is large and not just a matter of identify an appropriate lipid composition. There are numerous additional variables associated with the experimental protocols that include choice of solvent from which the lipids are being deposited, temperature of deposition, conditions of annealing, and whether the membrane support material is hydrophobic or hydrophilic as well as its pore size. Whilst this experience is worthy of being reported (we intend to do so after some further rationalization), we restrict the scope of the current report to the particular ternary system which reproducibly yielded a continuous membrane.

Laurdan fluorescence spectroscopy enables the characterisation of the phase behaviour of the model lipid membrane. The environmentally sensitive fluorophore Laurdan is virtually non-fluorescent in aqueous environments with a fluorescence lifetime of less than 100 ps, while in organic solvents and in membranes it displays a strong fluorescence signal with an average lifetime of about 4–8 ns, depending on the solvent [38]. The spectral changes of the emission spectrum of Laurdan are generally quantified by the so-called generalized polarization function, which is defined as $GP = (I_B-I_R)/(I_B+I_R)$, where I_B and I_R are the fluorescence intensities at 440 nm (characteristic for an ordered, gel phase state environment) and 490 nm (characteristic for a fluid, liquid-crystalline lipid state), respectively [38, 39]. Hence, GP values range from −1 to +1. In phase coexistence regions, the GP values exhibit values typical for gel and fluid domains. Hence, the measured GP values of our system reflect the overall phase behaviour and fluidity of the membrane as a function of temperature.

The generalised polarization (GP) values of the system CER/PA/Chol (1:1:1), measured over a temperature range of 15 to 90 °C are shown in Fig. 2. As can be clearly seen from the figure, the GP values gradually decrease with increasing temperature, with a sigmoidal profile in the temperature interval between 40 and 90 °C. The observed smooth behaviour is distinct from single-component lipid bilayer systems that tend to exhibit several sharp gel-to-gel and gel-to-fluid phase transitions in the temperature range covered [40, 41].The equimolar mixture of CER [NS]/PA/Chol exhibits relatively high GP values even at high temperature (> 60°C) when compared to a ternary system composed of phospholipids, sphingomyelin and cholesterol [42, 43]. This suggests that the lipid-lipid interactions, particularly between the acyl chains, in the ceramide mixture are stronger (hence maintain a gel-phase type structure even at high temperatures) than observed in similar phospholipid systems. The acyl chain interactions are probably enhanced due to the very small ceramide head group that allows for the chains to be sterically closer to each other [19, 44]. With increase in temperature, the lipid structure shows a more or less continuous increase in fluidity and conformational disorder, but the GP value does not reach the typical value for a pure fluid phase even at temperatures of up to ~90 °C [45].
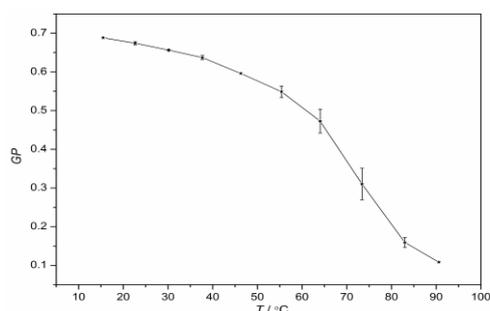
Fig. 2: Temperature dependence of Laurdan GP values of the lipid mixture CER [NS]/PA/Chol (1:1:1). Error bars represent the standard deviation (n=3).

SEM images of both the original and the lipid-covered polycarbonate substrate (with 50 nm pores) are shown in Fig. 3A–C. The pores in the original substrate are clearly visible as dark spots (Fig.3A). The sprayed lipid membrane shows particulate material on a continuous background (Fig. 3B),

confirming the deposition of a continuous lipid layer. The cross-section of the membrane deposited on the substrate is shown in Fig. 3D, which gives an indication of the thickness. The thickness of the lipid layer showed variation ranging from 16 to 26 um. We believe the particulate material results from spray droplets that are small and which dry just before or immediately on impact, and hence are unable to coalesce and become a part of the continuous layer. Such behaviour is well known in spray coating [46, 47]. An alternative explanation is that the particulate material was composed of crystallites of the individual lipids resulting from phase separation. However we can disregard this hypothesis, as we note from Raman scattering (see below) that these particles have essentially the same composition as the background lipid mixture.
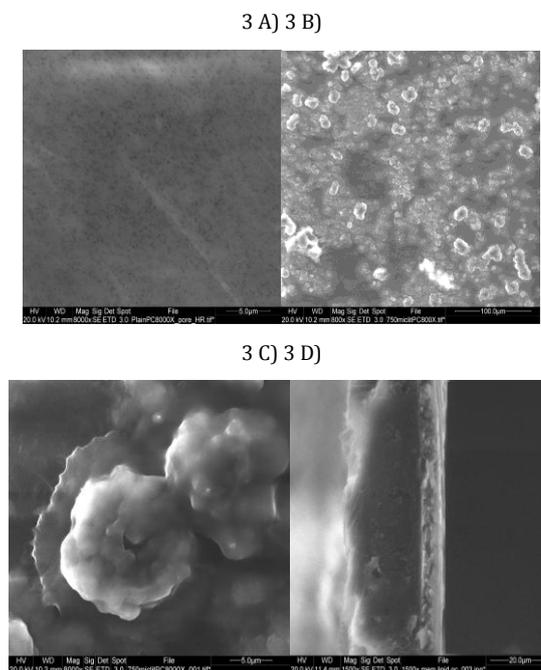
3 A) 3 B)

3 C) 3 D)

Fig. 3: SEM image of (A) original, (B) lipid-coated polycarbonate membrane at 800X magnification, (C) lipid-coated polycarbonate membrane at 8000X magnification showing a close up of the solid particulate material and (D) a cross section of the lipid-coated membrane at 1500X revealing the deposited lipid layer (on the left) on the substrate.

The permeation profiles (cumulative amount permeated as a function of time) of caffeine and benzoic acid through the deposited lipid mixture are shown in Fig. 4 and 5 respectively. The steady state flux (J_{ss}) is determined from the linear portion of these curves. The permeability co-efficient (kp) was calculated using the equation kp = J_{ss}/ C_d, where C_d is the donor concentration of the solution (in micrograms per cubic centimetre- $\mu g/cm^3$). The steady state flux (J_{ss}) for caffeine was determined to be 11.0 ± 2.2 $\mu g/(cm^2hr)$ (n=6), which gives a permeability coefficient kpof 4.4 x 10^{-4} cm/hr for the model membrane.

This kp value lies within the range ofkp values for caffeine in real stratum corneum, namely 0.7 to 4.6 x 10^{-4} cm/hr[48, 49].The broad range in the literature values is attributed to the intra and inter subject variation in skin lipid content, appendageal density, imperfections such as pore and cracks and the hydrophilic nature of caffeine [48]. For benzoic acid (Fig 5), the steady state flux (J_{ss}) was determined to be 254.9 ± 10.5 $\mu g/(cm^2hr)$ (n=6) yielding a kp value of8.5x 10^{-2} cm/hr. This kp value is close to that for benzoic acid in real stratum corneum, which ranges from 9 to 10 x 10^{-2} cm/hr[49, 50]. For comparison the permeability coefficient for both molecules across the polycarbonate substrate alone (control - solvent treated but without lipids) was in the range 0.2–0.3 cm/hr, which as expected is significantly higher than the kp values for caffeine and benzoic acid.
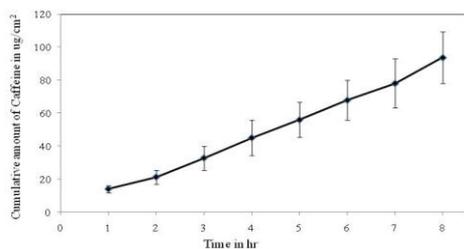
Fig. 4: Permeation profile of caffeine through the simplified model lipid membrane. Error bars refer to standard deviations (n=6).
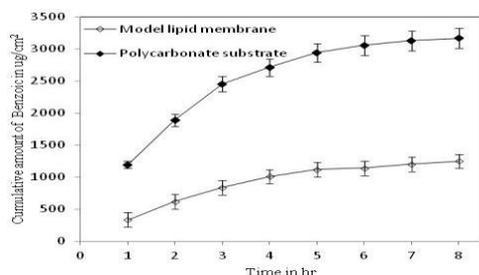

Fig. 5: Permeation profile of benzoic acid through the simplified model lipid membrane and the uncoated polycarbonate substrate. Error bars refer to standard deviations (n=6).

The Raman spectra of the three individual lipid components and the air sprayed mixture are shown in Fig. 6. The objective here was to ascertain whether the entire layer was a homogeneous phase (as expected from the Laurdan fluorescence spectroscopy results) or consisted of demixed domains of individual lipids. The constraint here was the resolution of the Raman beam cross-section which was 5 µm, the significance being that domain sizes lower than 5 µm cannot be resolved. For the individual lipids, the region from 400 to 1800 cm^{-1} exhibits the characteristic fingerprints. The Raman spectrum of the ceramide [NS] (Fig. 6a) shows several sharp bands from 400 to 1800 cm^{-1} including the characteristic ceramide back-bone peak at 1635 cm^{-1} and 888, 1060, 1127, 1294 and 1436 cm^{-1} corresponding to the acyl chains. Owing to the presence of similar vibrational groups, the Raman spectrum of palmitic acid (Fig. 6b) qualitatively coincides with the ceramide [NS] spectrum, showing bands at 890, 1061, 1126, 1294 and 1436 cm^{-1}. The Raman spectrum of cholesterol (Fig. 6c) shows several sharp bands with most intensive ones at 427, 544, 604, 698, 1117, 1437 and 1671 cm^{-1}. The observed Raman scattering values for cholesterol and palmitic acid are in good agreement with literature [51, 52]. To the best of our knowledge this is the first experimental data for the ceramide [NS] using Raman scattering. The observed ceramide [NS] band values are similar to CER3 and CER6 [52]. For the deposited equimolar mixture of Cer[NS]:PA:Chol (Fig. 6d), the Raman scans did not reveal regions or domains of pure individual lipids but rather yielded spectra composed of all the main bands that correspond to the three components of the lipid mixture namely, 698, 1061, 1177, 1294, 1436, 1635 and 2881 cm^{-1}. The wavelength region 2700 to 3200 cm^{-1} originates from the stretching vibrations of CH, NH and OH groups which strongly overlap and is therefore less informative [51]. We also employed Raman to determine the composition of the solid particulate matter on the surface of the membrane, which was determined to be the same as the underlying membrane and not pure crystallized lipid components.

In summary we have developed a simplified but robust, model membrane for skin lipids using an equimolar mixture of ceramide [NS], palmitic acid and cholesterol on a solid surface. The lipid mixture gives a continuous coverage on a porous substrate, enabling permeability studies to be conducted. The structure appears to be in gel-phase and is rather robust even at high temperatures. In vitro permeability experiments using caffeine and benzoic acid as model permeants reveal that the barrier properties of this simple model membrane are of the same order as that of real stratum corneum. The simplified model is expected to be invaluable for studying the mechanisms of penetration enhancers and linking the results with molecular-resolution simulations.
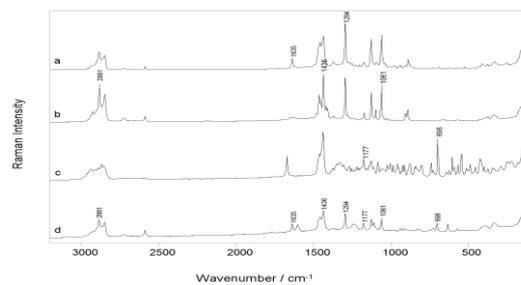

Fig. 6: Raman spectra from 100 to 3200 cm^{-1} of (a) pure ceramide [NS], (b) palmitic acid, (c) cholesterol and (d) equimolar mixture of Cer [NS], PA, Chol sprayed on a poly carbonate membrane

REFERENCES

1. J.A. Bouwstraand M. Ponec. The skin barrier in healthy and diseased state. Biochim Biophys Acta. 2006; 1758: 2080-2095.
2. L. Norlen, I.P. Gil, A. Simonsen, and P. Descouts. Human stratum corneum lipid organization as observed by atomic force microscopy on Langmuir-Blodgett films. J Struct Biol. 2007; 158: 386-400.
3. P.W. Wertzand D.T. Downing. Stratum Corneum: biological and biochemical considerations, in: J. Hadgraft, R.H. Guy (Eds.), Transdermal Drug Delivery: Developmental Issues and Research Initiatives, Marcel Dekker, New York, 1989.
4. W. Philip W. The nature of the epidermal barrier: biochemical aspects. Adv Drug Deliver Rev. 1996; 18: 283-294.
5. C.R. Harding. The stratum corneum: structure and function in health and disease. Dermatol Ther. 2004; 17 Suppl 1: 6-15.
6. K.J. Robson, M.E. Stewart, S. Michelsen, N.D. Lazo, and D.T. Downing. 6-Hydroxy-4-sphingenine in human epidermal ceramides. J Lipid Res. 1994; 35: 2060-2068.
7. Y. Masukawa, H. Narita, E. Shimizu, N. Kondo, Y. Sugai, T. Oba,... K. Kita. Characterization of overall ceramide species in human stratum corneum. J Lipid Res. 2008; 49: 1466-1476.
8. D. Kessner, A. Ruettinger, M.A. Kiselev, S. Wartewig, and R.H. Neubert. Properties of ceramides and their impact on the stratum corneum structure. Part 2: stratum corneum lipid model systems. Skin Pharmacol Physiol. 2008; 21: 58-74.
9. I. Iwai, H.M. Han, S. Svensson, L.-G. Öfverstedt, J. Anwar, J. Brewer, L. Norlén. The Human Skin Barrier is Organized as Stacked Bilayers of Fully-extended Ceramides With Cholesterol Molecules Associated With the Ceramide Sphingoid Moiety. J Invest Dermatol. 2012; In press.
10. J.A. Bouwstra, F.E. Dubbelaar, G.S. Gooris, and M. Ponec. The lipid organisation in the skin barrier. Acta Derm Venereol Suppl (Stockh). 2000; 208: 23-30.
11. R. Notmanand J. Anwar. Breaching the Skin Barrier – Insights from Molecular Simulation of Model Membranes. Adv Drug Deliver Rev2012; In press.
12. J.A. Bouwstra, G.S. Gooris, M.A.S.-d. Vries, J.A. van der Spek, and W. Bras. Structure of human stratum corneum as a function of temperature and hydration: A wide-angle X-ray diffraction study. Int J Pharm. 1992; 84: 205-216.
13. G.S. Pilgram, A.M. Engelsma-van Pelt, J.A. Bouwstra, and H.K. Koerten. Electron diffraction provides new information on

human stratum corneum lipid organization studied in relation to depth and temperature. J Invest Dermatol. 1999; 113: 403-409.

14. D. Bommannan, R.O. Potts, and R.H. Guy. Examination of stratum corneum barrier function in vivo by infrared spectroscopy. J Invest Dermatol. 1990; 95: 403-408.

15. B. Ongpipattanakul, M.L. Francoeur, and R.O. Potts. Polymorphism in stratum corneum lipids. Biochim Biophys Acta. 1994; 1190: 115-122.

16. J. Houkand R.H. Guy. Membrane models for skin penetration studies. Chem Rev. 1988; 88: 455-472.

17. A. El-Kattan, C.S. Asbill, and S. Haidar. Transdermal testing: practical aspects and methods. Pharm Sci Technolo Today. 2000; 3: 426-430.

18. J. Hadgraftand M.E. Lane. Skin permeation: the years of enlightenment. Int J Pharm. 2005; 305: 2-12.

19. D.J. Moore, M.E. Rerek, and R. Mendelsohn. Role of ceramides 2 and 5 in the structure of the stratum corneum lipid barrier. Int J Cosmet Sci. 1999; 21: 353-368.

20. D. Groen, G.S. Gooris, M. Ponec, and J.A. Bouwstra. Two new methods for preparing a unique stratum corneum substitute. BBA-Biomembranes. 2008; 1778: 2421-2429.

21. V. Velkovaand M. Lafleur. Influence of the lipid composition on the organization of skin lipid model mixtures: an infrared spectroscopy investigation. Chem Phys Lipids. 2002; 117: 63-74.

22. S. Wartewigand R.H. Neubert. Properties of ceramides and their impact on the stratum corneum structure: a review. Part 1: ceramides. Skin Pharmacol Physiol. 2007; 20: 220-229.

23. D.B. Fenske, J.L. Thewalt, M. Bloom, and N. Kitson. Models of stratum corneum intercellular membranes: 2H NMR of macroscopically oriented multilayers. Biophys J. 1994; 67: 1562-1573.

24. C. Das, M.G. Noro, and P.D. Olmsted. Simulation studies of stratum corneum lipid mixtures. Biophys J. 2009; 97: 1941-1951.

25. C. Das, P.D. Olmsted, and M.G. Noro. Water permeation through stratum corneum lipid bilayers from atomistic simulations. Soft Matter. 2009; 5: 4549-4555.

26. R. Notman, J. Anwar, W.J. Briels, M.G. Noro, and W.K. den Otter. Simulations of skin barrier function: free energies of hydrophobic and hydrophilic transmembrane pores in ceramide bilayers. Biophys J. 2008; 95: 4763-4771.

27. R. Notman, W.K. den Otter, M.G. Noro, W.J. Briels, and J. Anwar. The permeability enhancing mechanism of DMSO in ceramide bilayers simulated by molecular dynamics. Biophys J. 2007; 93: 2056-2068.

28. A.A. Brianand H.M. McConnell. Allogeneic stimulation of cytotoxic T cells by supported planar membranes. Proc Natl Acad Sci U S A. 1984; 81: 6159-6163.

29. A.J. Markvoort, P. Spijker, A.F. Smeijers, K. Pieterse, R.A. van Santen, and P.A. Hilbers. Vesicle deformation by draining: geometrical and topological shape changes. J Phys Chem B. 2009; 113: 8731-8737.

30. C. Naumann, T. Brumm, and T.M. Bayerl. Phase transition behavior of single phosphatidylcholine bilayers on a solid spherical support studied by DSC, NMR and FT-IR. Biophys J. 1992; 63: 1314-1319.

31. P. Nollert, H. Kiefer, and F. Jahnig. Lipid vesicle adsorption versus formation of planar bilayers on solid surfaces. Biophys J. 1995; 69: 1447-1455.

32. R. Richter, A. Mukhopadhyay, and A. Brisson. Pathways of lipid vesicle deposition on solid surfaces: a combined QCM-D and AFM study. Biophys J. 2003; 85: 3035-3047.

33. H.M. Seeger, G. Marino, A. Alessandrini, and P. Facci. Effect of physical parameters on the main phase transition of supported lipid bilayers. Biophys J. 2009; 97: 1067-1076.

34. M.W. de Jager, G.S. Gooris, M. Ponec, and J.A. Bouwstra. Lipid mixtures prepared with well-defined synthetic ceramides closely mimic the unique stratum corneum lipid phase behavior. J Lipid Res. 2005; 46: 2649-2656.

35. X. Chen, Z. Huang, W. Hua, H. Castada, and H.C. Allen. Reorganization and caging of DPPC, DPPE, DPPG, and DPPS monolayers caused by dimethylsulfoxide observed using Brewster angle microscopy. Langmuir. 2010; 26: 18902-18908.

36. M. de Jager, W. Groenink, J. van der Spek, C. Janmaat, G. Gooris, M. Ponec, and J. Bouwstra. Preparation and characterization of a stratum corneum substitute for in vitro percutaneous penetration studies. Biochim Biophys Acta. 2006; 1758: 636-644.

37. M. Dias, A. Farinha, E. Faustino, J. Hadgraft, J. Pais, and C. Toscano. Topical delivery of caffeine from some commercial formulations. Int J Pharm. 1999; 182: 41-47.

38. T. Parasassi, G. De Stasio, A. d'Ubaldo, and E. Gratton. Phase fluctuation in phospholipid membranes revealed by Laurdan fluorescence. Biophys J. 1990; 57: 1179-1186.

39. L.A. Bagatolli, B. Maggio, F. Aguilar, C.P. Sotomayor, and G.D. Fidelio. Laurdan properties in glycosphingolipid-phospholipid mixtures: a comparative fluorescence and calorimetric study. Biochim Biophys Acta. 1997; 1325: 80-90.

40. G.Cevc. Phospholipids Handbook, Marcel Dekker, New York, 1993.

41. O.G. Mouritsen. Life as a Matter of Fat. The emerging science of lipidomics, Springer-Verlag, Heidelberg, 2005.

42. C. Nicolini, J. Kraineva, M. Khurana, N. Periasamy, S.S. Funari, and R. Winter. Temperature and pressure effects on structural and conformational properties of POPC/SM/cholesterol model raft mixtures--a FT-IR, SAXS, DSC, PPC and Laurdan fluorescence spectroscopy study. Biochim Biophys Acta. 2006; 1758: 248-258.

43. N. Periasamyand R. Winter. The effects of temperature, pressure and peptide incorporation on ternary model raft mixtures--a Laurdan fluorescence spectroscopy study. Biochim Biophys Acta. 2006; 1764: 398-404.

44. D.J. Mooreand M.E. Rerek. Insights into the molecular organization of lipids in the skin barrier from infrared spectroscopy studies of stratum corneum lipid models. Acta Derm Venereol Suppl (Stockh). 2000; 208: 16-22.

45. L. Norlen, I. Plasencia, and L. Bagatolli. Stratum corneum lipid organization as observed by atomic force, confocal and two-photon excitation fluorescence microscopy. Int J Cosmet Sci. 2008; 30: 391-411.

46. K. Dewettinckand A.e. Huyghebaert. Top-spray fluidized bed coating : Effect of process variables on coating efficiency. Lebensmittel-Wissenschaft und Technologie. 1998; 31: 568–575.

47. D. Jones. Factors to consider in fluid-bed processing. Pharmaceutical Technology. 1985; 9: 50–62.

48. F.K. Akomeah, G.P. Martin, and M.B. Brown. Variability in human skin permeability in vitro: comparing penetrants with different physicochemical properties. J Pharm Sci. 2007; 96: 824-834.

49. M. Dias, J. Hadgraft, and M.E. Lane. Influence of membrane-solvent-solute interactions on solute permeation in skin. Int J Pharm. 2007; 340: 65-70.

50. S. Kitagawaand H. Li. Effects of removal of stratum corneum, delipidization and addition of enhancers, ethanol and l-menthol, on skin permeation of benzoic acid and its 4-n-alkyl substituents in excised guinea pig dorsal skin. Chem Pharm Bull (Tokyo). 1999; 47: 44-47.

51. C. Krafft, L. Neudert, T. Simat, and R. Salzer. Near infrared Raman spectra of human brain lipids. Spectrochim Acta A Mol Biomol Spectrosc. 2005; 61: 1529-1535.

52. S. Raudenkolb, S. Wartewig, and R.H. Neubert. Polymorphism of ceramide 6: a vibrational spectroscopic and X-ray powder diffraction investigation of the diastereomers of N-(alpha-hydroxyoctadecanoyl)-phytosphingosine. Chem Phys Lipids. 2005; 133: 89-102.