Stratum corneum hydration: Phase transformations and mobility in stratum corneum, extracted lipids and isolated corneocytes

C.L. Silva [a], D. Topgaard [b], V. Kocherbitov [c], J.J.S. Sousa [a], A.A.C.C. Pais [d], E. Sparr [b,*]

[a] *Faculdade de Farmácia, Universidade de Coimbra, 3004-535 Coimbra, Portugal*
[b] *Physical Chemistry 1, Center for Chemistry and Chemical Engineering, P.O. Box 124, Lund University, SE-22100 Lund, Sweden*
[c] *Biomedical Laboratory, Health and Society, Malmö University, SE-20506 Malmö, Sweden*
[d] *Departamento de Química, Universidade de Coimbra, 3004-535 Coimbra, Portugal*

Abstract

The outermost layer of skin, stratum corneum (SC), functions as the major barrier to diffusion. SC has the architecture of dead keratin filled cells embedded in a lipid matrix. This work presents a detailed study of the hydration process in extracted SC lipids, isolated corneocytes and intact SC. Using isothermal sorption microcalorimetry and relaxation and wideline ^{1}H NMR, we study these systems at varying degrees of hydration/relative humidities (RH) at 25 °C. The basic findings are (i) there is a substantial swelling both of SC lipids, the corneocytes and the intact SC at high RH. At low RHs corneocytes take up more water than SC lipids do, while at high RHs swelling of SC lipids is more pronounced than that of corneocytes. (ii) Lipids in a fluid state are present in both extracted SC lipids and in the intact SC. (iii) The fraction of fluid lipids is lower at 1.4% water content than at 15% but remains virtually constant as the water content is further increased. (iv) Three exothermic phase transitions are detected in the SC lipids at RH=91–94%, and we speculate that the lipid re-organization is responsible for the hydration-induced variations in SC permeability. (v) The hydration causes swelling in the corneocytes, while it does not affect the mobility of solid components (keratin filaments).
© 2007 Elsevier B.V. All rights reserved.

Keywords: SC; Sorption microcalorimetry; Wideline NMR; Phase behavior; Molecular mobility; Lipid; Corneocyte

1. Introduction

The skin is our largest organ and it comprises many important functions. It permits the terrestrial life in that it forms the barrier that protects body homeostasis. The prevention of uncontrolled water loss, uptake of hazardous chemicals from the environment, immune surveillance, thermoregulation, synthesis of vitamin D and mechanical protection are some of the functions that make the skin a vital organ. The most important function of the skin is probably its ability to serve as an efficient barrier to molecular diffusion, which is assured by the very outer epidermis layer, the stratum corneum (SC) [1]. It is however important to bear in mind that, even though SC has a very low permeability, it is not totally tight. As an example,

there is a non-negligible transepidermal water loss (TEWL) of about 100–150 ml per day and square meter of skin surface through the intact healthy skin [2].

The SC is exposed to large variations in the chemical surroundings, which are able to affect its structure and functions. Furthermore, the SC is subjected to several different gradients in, e.g. water activity, temperature and pH, which can also influence its function. Important examples are the observations of a non-linear response in SC permeability to variations in the degree of hydration, and that the barrier properties can be regulated by, e.g., the relative humidity (RH) of the environment [3–7]. In a theoretical model for transport in responding lipid membranes in the presence of a water gradient, this non-linearity was explained by structural transformations induced by this water gradient, which largely affects the overall permeability [8].

The normal water content in SC is about 30%±5% [9], it establishes the SC permeability [4,7], and is also a determinant

---

* Corresponding author. Fax: +46 46 222 44 13.
  E-mail address: emma.sparr@fkem1.lu.se (E. Sparr).

factor to other vital function of healthy skin in, e.g., its relation to the mechanical properties, the appearance and the enzymatic activity in SC [10,11]. This intimate coupling between structure, function and hydration of SC motivates the investigations of the SC ultrastructural organization and how it responds to variations in hydration. Several studies on the hydration of human SC indicate a swelling limit in the interval 22–33 wt.% [12–16]. The SC consists of 10–15 layers of dead flattened keratin filled cells (corneocytes) with about 0.5 μm thickness and 40 μm diameter. The corneocytes are embedded in a matrix of stacked lipid lamellae in an array similar to "bricks and mortar" [17]. The corneocytes exhibit a cell envelope that consists of cross-linked proteins and covalently bound long chain ceramides with important functions such as acting as a permeability barrier and as a template to orient intercellular lipid lamellae [18,19]. As the extracellular lipids constitute the sole continuous regions of the stratum corneum, the molecules that pass through the skin barrier must be mainly transported through them [6,20,21]. Here, the multi-lamellar arrangement of the lipids represents an almost ideal barrier towards strongly polar as well as non-polar substances. Due to its direct impact on the barrier properties, the organization and composition of these lipids has been extensively studied [22–25]. Most of these studies concern the phase behavior at various temperatures. However, when considering the skin system, it is equally relevant to consider the phase behavior at different RH/water contents under isothermal conditions, which is the aim of the present work.

The main components of the SC extracellular lipid system are ceramides, free fatty acids and cholesterol. The lipid composition differs considerably from most other biological membranes, having longer and more saturated lipids and basically no phospholipids [26]. At physiological temperatures, the SC lipids in human, pig and mouse SC are arranged in a lamellar structure with two typical repeating units, a long lamellar structure with a repeat period of ca. 134 Å and a short lamellar structure with a repeat period of ca. 60 Å [27,28]. The majority of the SC intercellular lipids are in a solid state at normal RH and ambient temperature [29–34]. However, there are several studies indicating that a small fraction of the lipids is in a fluid state [34,35]. The existence of fluid lipids could account for the non-negligible TEWL, which appears difficult to explain on basis only of the solid SC lipids. It could also allow for the high elasticity of the skin and for the enzymatic activity in the SC intercellular space that is unlikely to take place in a crystalline phase [36]. Several models that combine the structural information with the chemical and physical properties of the SC have been developed. According to the Domain Mosaic Model [31], the lipid phase can be envisioned as crystalline domains held together by lipids in a liquid crystalline state. The sandwich model [37] agrees on the coexistence of crystalline and liquid crystalline domains, while it describes a complex structure of connected bilayers where a discontinuous fluid phase is located in the central zone. In contrast to these models, the single gel phase model [38] proposes that the SC lipids form a "single and coherent lamellar gel phase".

Taken together, it is well recognized that the mobility (fluidity) of the different SC components as well as the SC hydration is very important to several aspects of the vital functions of SC. However, the actual mechanisms of the SC–water interaction, how it is related with the hydration of the individual building-blocks (lipids and corneocytes) and whether these components have independent or cooperative roles in the hydration of SC are still unresolved issues whose solution forms the goal of the present study. Extracted SC lipids, isolated corneocytes and whole SC were investigated at different RH/water contents by means of isothermal sorption microcalorimetry, and relaxation and wideline ^{1}H NMR. The sorption calorimetric technique allows for simultaneous measurement of the sorption isotherms and sorption enthalpies. The combination of the thermodynamic characterization of the hydration process and the structural information from the ^{1}H NMR measurements provides deeper molecular insight in the SC response to hydration. The characterization of this process is crucial to the understanding of skin structure and physiology, as well as for the development of new therapies for the prevention and correction of dermatological disorders related with low water content (e.g. eczema, psoriasis), and to the development of new pharmaceutical formulations for transdermal drug delivery and new cosmeceutics.

2. Materials and methods

2.1. Isolation of stratum corneum

The pig skin from two different animals was a kind gift from "Slakteriprodukter i Helsingborg AB". The hair was removed with an electric shaver and the dermatomed skin was placed dermal side down on filter paper soaked with a 0.2% trypsin (Sigma Chemical Company, St. Louis, MO) in phosphate buffered saline (PBS) solution, pH 7.4. Digestion occurred during the night [39]. In order to remove any traces of viable epidermal cells, the SC is rubbed and extensively rinsed with ultrapure water [Durapore (0.22 μm), Millipore, Bedford, MA], dried under vacuum and stored at −20 °C until used.

2.2. Extraction of SC lipids

The SC was rinsed with hexane to remove any lipids which might have contaminated the SC surface, such as sebaceous or subcutaneous fat [40]. For the actual extraction we have followed the procedure described in [41]. Briefly, the samples were sequentially immersed in three different HPLC-grade chloroform/methanol mixtures (2:1, 1:1, 1:2) for 2 h each at room temperature. The extractions were then repeated for 1 h each, and the sample was extracted overnight with methanol. The extraction in methanol is used to extract any polar lipids that are still remaining in the SC after the previous extraction steps [41]. All the extracts were combined and recovered by filtration through a filter paper. The final extract composed by the SC free lipids was dried under vacuum in a rotary evaporator and stored at −20 °C.

2.3. Isolation of corneocytes

The stratum corneum membranes recovered after extraction of SC lipids, were suspended in 1 M NaOH in 90% methanol and heated at 60 °C for 1 h in order to extract the covalently linked lipids of the cornified cell envelope. The mixture was acidified to pH 4 by addition of 2M HCl and agitated with chloroform [41]. After filtration, the remaining stratum corneum material was washed with chloroform to eliminate residual lipids. In order to eliminate NaCl resulting from the extraction procedure, isolated corneocytes were extensively rinsed with ultrapure water, dried under vacuum and stored at −20 °C until used. Isolated corneocytes with normal size and shape were recovered after this procedure and confirmed by optical

microscopy (data not shown). Earlier studies demonstrated that the bulk keratin conformation is not modified by the delipidation procedure [42] nor by the treatment with solutions with a pH < 12 [43].

2.4. Sample preparation

After isolation and freeze-drying, all samples (intact SC, extracted SC lipids and isolated corneocytes) were dried in vacuum at room temperature in contact with 3 Å molecular sieves during 24 h. This procedure was necessary to remove all traces of water and organic solvents as confirmed by the self-diffusion NMR experiments. The transfer of the samples to the calorimetric cell and to the NMR tubes took place in a dry nitrogen atmosphere. H_2O was added to each sample used in the NMR experiments after the samples being transferred into 4-mm diameter NMR tubes in N_2 atmosphere, in order to achieve the desired hydration. To avoid evaporation the sample tubes were flame-sealed. The samples were allowed to equilibrate for at least 1 week at constant agitation before the measurements. Condensation of water was never observed in the tubes in any of the samples.

2.5. Sorption microcalorimetry

A double twin isothermal microcalorimeter was used to study the water vapour sorption of the SC and its components. A detailed description of the instrument is presented elsewhere [44]. The method of sorption calorimetry was used to monitor the water activity a_w and the partial molar enthalpy of mixing of water, H_w^m. A two-chamber calorimetric cell (diameter 20 mm) with the sample chamber on the top and water chamber on the bottom was used. The calorimetric cell was inserted into the double-twin microcalorimeter [44]. Water evaporated in the bottom chamber diffused through the tube connecting the two chambers and was absorbed by the studied sample in the top chamber. The thermal powers corresponding to the evaporation of water in the vaporization chamber and to the sorption of water vapour in the sorption chamber were used to calculate the H_w^m with the sample. For the calculations of the H_w^m, the sorption calorimeter was calibrated using magnesium nitrate hexahydrate as a standard substance [45]. Water activity was calculated from the thermal power measured in the vaporization chamber as described in ref [46]. The experimental set-up could be looked upon as a continuous titration of an initially dry lipid with water vapour. The rate of water diffusion in the vapour is controlled by the geometry of the vessel and the boundary conditions. We have confirmed that sorption process takes place under quasi-equilibrium conditions by conducting separate experiments with samples of different size. The complete sorption calorimetry experiment in the present study took approximately 13 days for the SC lipid samples, 3 days for the corneocyte samples and 7 days for the intact SC.

2.6. NMR

^{1}H NMR spectra were obtained on samples of extracted SC lipids, isolated corneocytes and intact SC with different water contents. Wideline ^{1}H NMR measurements were performed on a Bruker DMX-200 spectrometer using a Bruker DIFF-25 gradient probe at a temperature of 25 ± 0.5 °C. The ^{1}H resonance frequency for this system is 200 MHz. The probe is equipped with a home made 5 mm saddle-coil RF insert with negligible ^{1}H background signal. Free induction decays (FIDs) were recorded after a 4-μs 90° pulse using a dwell time of 1 μs and a receiver dead time of 4.5 μs. The FIDs were both analysed in the time-domain, to extract solid/liquid ratios, and Fourier transformed to obtain frequency domain NMR spectra. Transverse relaxation time (T_2) measurements were performed with the spin echo pulse sequence (90° – t_E/2 – 180° – t_E/2 – acquire) using 64 logarithmically spaced echo times t_E between 0.1 ms and 0.5 s. For a single component the signal I decays according to $I = I_0 \exp(-R_2 t_E)$, where $R_2 = 1/T_2$ and I_0 is the signal at $t_E = 0$. Multicomponent signal decays can be deconvoluted to yield relaxation probability distributions $P(R_2)$ using an inverse Laplace transform algorithm [47]. 2D relaxation –chemical shift correlation spectra were obtained by Fourier transform in the chemical shift dimension, and subsequent inverse Laplace transform in the relaxation dimension in a manner analogous to the DOSY method for analysis of NMR diffusion experiments [48]. In this way overlapping peaks in the 1 D NMR spectrum can be separated according to their relaxation times.
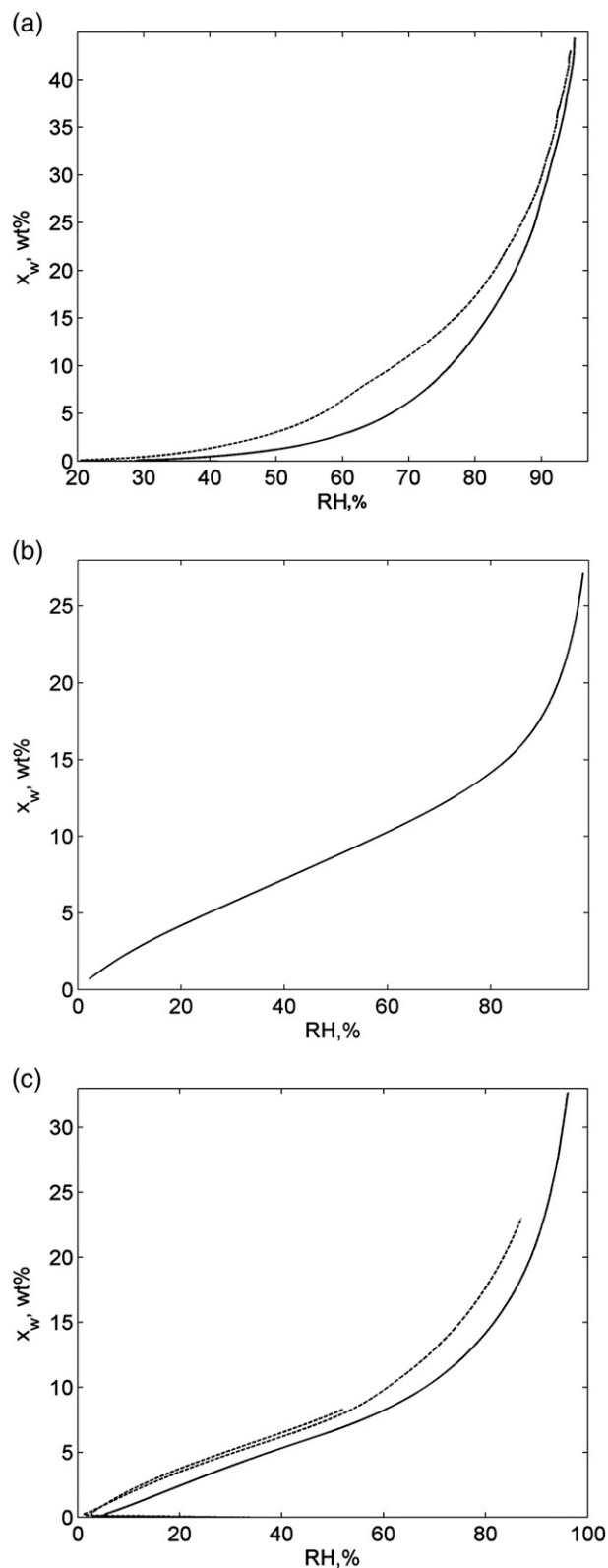

Fig. 1. Microcalorimetric sorption data (water content [wt.%] versus RH) at 25 °C for (a) extracted SC lipids, (b) isolated corneocytes and (c) SC. Key: dashed lines—sample 1; solid line—sample 2.

3. Results

Three independent properties related to the hydration of the SC, extracted SC lipids and isolated corneocytes were investigated: the water sorption, the partial molar enthalpy of mixing of water, and the molecular mobility. Samples obtained from two different animals (1 and 2) were investigated, and all measurements were performed at 25 °C. Below, we first present separate descriptions of the measured physical parameters. The results are then collected into a unified discussion on the hydration of SC and its components.

3.1. Sorption measurements

The calorimetric sorption measurement provides a relation between the water content and the water activity (a_w), which can also be expressed in terms of the relative humidity (RH= $a_w \cdot 100\%$) or the osmotic pressure $\Pi_{osm} = -RT/V_w \ln(a_w)$. The sorption isotherms (water content, wt.%, given as the mass of water divided by the mass of the whole system including the water, as a function of RH) are presented in Fig. 1. Data from sample 1 are shown as dashed lines and data from sample 2 as solid lines.

3.1.1. Extracted SC lipids

The sorption isotherms for the samples composed of extracted SC lipids are shown in Fig. 1a. The calorimetric measurements show a minor uptake of water until ca. 60–80% RH, followed by a more pronounced swelling at higher RH. In the latter region, three small steps at ca. 91%, 92% and 94% RH are visible in the isotherms. These are better shown in the magnification in Fig. 2 (lower curve, arrows), representing RH vs. water content. The steps, which can be interpreted as transitions in a fraction of the extracted SC lipids, are associated with a small uptake of ca. 1 wt.% water at almost constant RH,

where the smallest uptake is seen for the transition at 91% RH. There is a continuously increasing water uptake at RH >60%. When comparing the sorption data from the different animals, there is a similar response at high RH, while the water uptake is slightly higher in the lipids extracted from animal 1 compared to animal 2. Furthermore, a kink at ca. 60% RH is observed in the isotherms from sample 1, which was not observed in the sample from animal 2. The extracted lipid samples were prepared by drying in vacuum and freeze-drying without special precautions taken to ensure the formation of equilibrium crystals. The discrepancy between the sorption curves at low RH might therefore be related to the presence of different amorphous states in the dry lipids, as well as biological variation in the lipid composition.

3.1.2. Isolated corneocytes

The sorption curve for the isolated corneocytes [Fig. 1b] shows a gradual swelling over the whole range of RH without pronounced steps that would indicate phase transitions. The shape of the sorption isotherm is similar to that of hen egg lysozyme studied previously using the same calorimetric method [49], although corneocytes take up slightly less water than lysozyme. The sorption isotherm of corneocytes can be roughly divided into three regimes: the initial sorption below 20% RH, the regime between 20 and 70% RH that features almost linear sorption isotherm, and the final regime above 70% RH where water uptake increases.

3.1.3. Stratum corneum

Sorption data for SC are shown in Fig. 1c. The isotherms show a continuous uptake of water over the entire range of RH, and no phase transitions are detected. At RH <60%, there is an almost linear relation between the water uptake and RH. At higher RH, there is an increase in the slope of the isotherm, implying a higher uptake of water. Finally, at RH >90%, there is
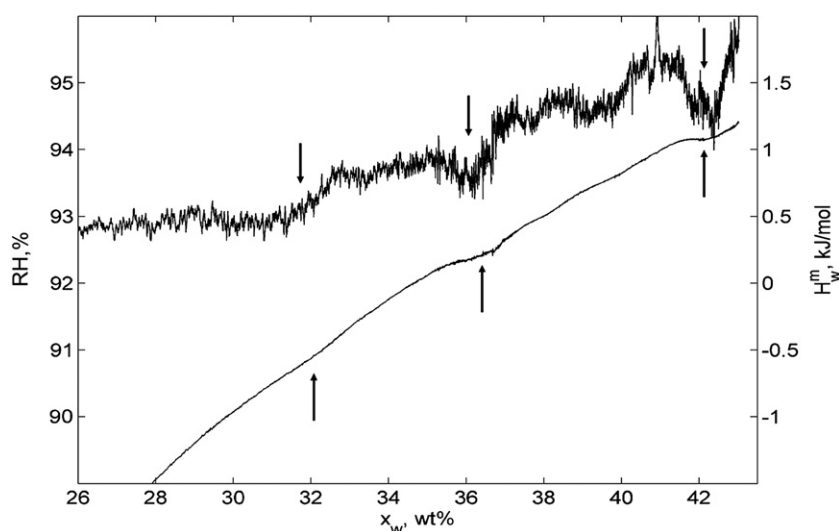

Fig. 2. Magnifications of both the enthalpy curve (upper line, right y-axis) and the sorption isotherm (lower line, left y-axis) obtained from the extracted SC lipids from animal 1. In this regime, the sorption data suggest the presence of three phase transitions that coincide with small exothermic peaks in the enthalpy curves at the same water contents (indicated by arrows).

again a large increase in water uptake. Fig. 1c shows three data sets obtained for SC from two different animals. There is a very good agreement between the data from the two pieces of SC from animal 1, and there is a qualitative agreement between the sorption isotherms from the samples from the two different animals.

3.2. Enthalpy of sorption

A great advantage of the double twin calorimeter system is the simultaneous monitoring of the water activity (RH) and the partial molar enthalpy of mixing of water (H_w^m) during the hydration process at constant temperature [50]. The enthalpy curves obtained at 25 °C for the three types of samples are shown in Fig. 3 (H_w^m as a function of water content).

3.2.1. Extracted SC lipids

The enthalpy data for the extracted SC lipids is shown in Fig. 3a. The values of enthalpy effects measured in experiments with two samples are close to zero in almost the whole concentration range studied. At very low water contents the enthalpy effect was slightly exothermic for the sample from animal 1, and slightly endothermic for the sample from animal 2. The enthalpy data obtained at higher water contents provide further information on the transitions observed in the sorption isotherms. Fig. 2 shows the magnifications of the enthalpy curve (upper curve) together with the corresponding sorption isotherm (lower curve) at high water contents. In this regime, the sorption data suggest the presence of three phase transitions. We see that these are all coinciding with small exothermic peaks in the enthalpy curves at the same water contents [Fig. 2, arrows]. The data shown in Fig. 2 were obtained from animal 1. The transitions indicated by arrows in the figure were also observed for the samples from animal 2, and are therefore judged as real and reproducible effects. Due to the quasi-equilibrium conditions in the experiments and the reproducibility of these transitions, it is unlikely that they arise from, e.g., heterogeneities in the sample. The low transition energies are consistent with the involvement of just a small fraction of the lipids and low enthalpy transformations.

3.2.2. Isolated corneocytes

The enthalpy curve obtained from the isolated corneocytes from SC from animal 2 is shown in Fig. 3b. The curve can be divided into four regimes: strongly exothermic regime with water contents 0–5 wt.%, two moderately exothermic regimes with water contents 5–11 wt.% and 11–17 wt.% and the last regime (endothermic) with water contents above 17 wt.%. The shape of the curve and the values of the enthalpy of mixing H_w^m are close to those observed in the sorption calorimetric study of hen egg lysozyme [49].

3.2.3. Stratum corneum

Fig. 3c shows the enthalpy data obtained for the complete SC of the different animals at 25 °C. There is a very good agreement between the enthalpy curves at water contents for which comparisons can be made. At low water contents, H_w^m is
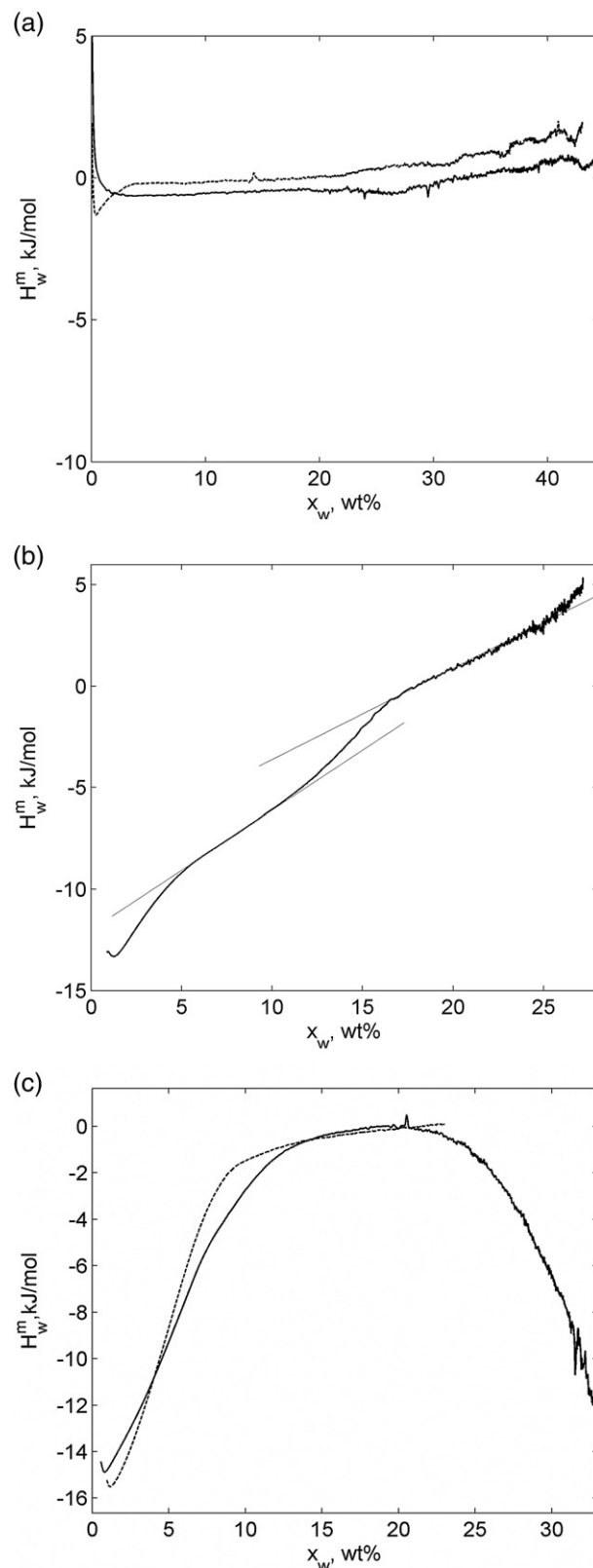

Fig. 3. The partial molar enthalpy of mixing of water at 25 °C measured by sorption microcalorimetry. (a) Extracted SC lipids (b) isolated corneocytes (c) SC. Key: dashed curves—sample 1; solid curves—sample 2.
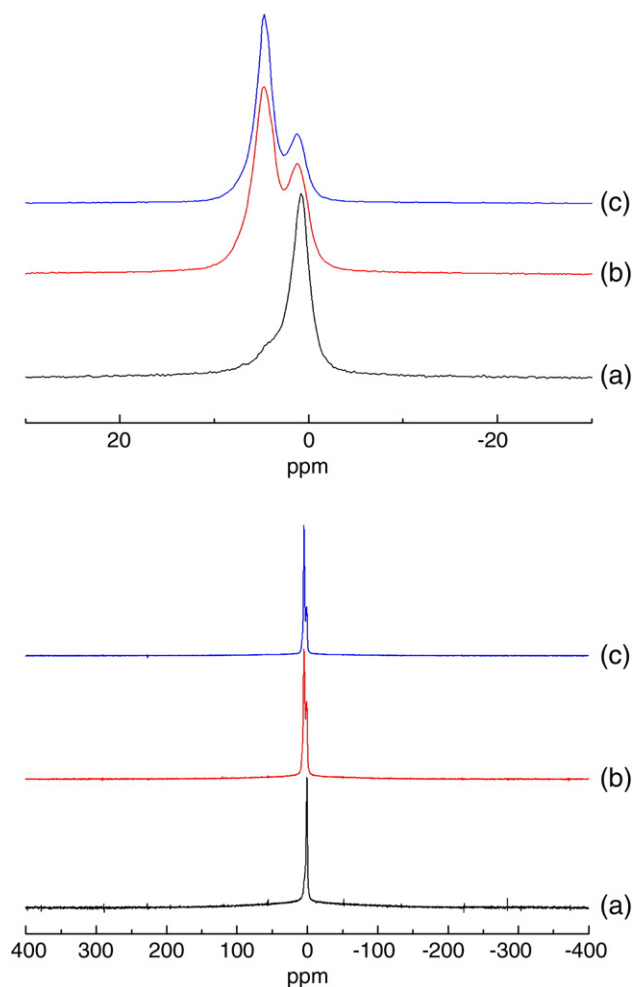
Fig. 4. Wideline ^{1}H NMR spectra for the extracted SC lipids with (a) 1.4 wt.%, (b) 29.2 wt.% and (c) 37.3 wt.% water at 25 °C (sample 2).

negative, implying an exothermic primary hydration of the SC. At higher water contents, H_w^m is small and negative and it increases towards zero when approaching a water content of 20 wt.%. Finally, when the water content exceeds 20 wt.%, there is again a large exothermic enthalpy. The latter effect was only observed for SC from animal 2, as the experiment for SC from animal 1 was interrupted at lower water contents, and therefore the reproducibility of this exothermic effect at high water contents was not studied.

3.3. NMR measurements

The mobility in different fractions of the SC as well as in the extracted lipids and the isolated corneocytes was investigated by means of relaxation and wideline ^{1}H NMR. Static dipolar interactions for molecules located in a solid environment result in fast T_2 relaxation and broad ^{1}H resonance lines—on the order of 10 kHz [51]. The dipolar interactions are averaged by molecular motions in a liquid environment leading to slow ^{1}H NMR relaxation and narrow resonance lines. Thus, NMR is a sensitive method to estimate if molecules are located in a solid or liquid environment. With sufficiently sharp resonance lines, different fluid components can be resolved in the chemical shift

dimension. Even without chemical shift resolution, different components can be resolved utilizing their different relaxation rates. For microheterogeneous systems containing both solid and liquid domains, the ratio between these domains can be determined from the FID as described e.g., in ref. [52]. The terms "fluid" and "solid" used for the description of the NMR data should be interpreted in terms of the degree of averaging of the dipolar interactions. Molecular rotation and translational diffusion averages the couplings in a liquid crystal. If the system is anisotropic, such as for a hexagonal or a lamellar phase, the averaging of the intramolecular couplings is not complete, leading to the characteristic super-Lorentzian lineshape of the ^{1}H NMR spectrum [53]. The NMR data shown are all obtained for samples from animal 2.

3.3.1. Extracted SC lipids

The extracted SC lipids were studied at different water contents. Fig. 4 shows the ^{1}H NMR spectra for the extracted SC lipids with (a) 1.4 wt.%, (b) 29.2 wt.% and (c) 37.3 wt.% water. The ^{1}H NMR spectra contain two liquid-like components with chemical shifts corresponding to water and methylene groups in a hydrocarbon chain. The spectrum is too broad to observe individual peaks originating from other parts of the lipids, such as the headgroups and the methyl at the end of the hydrocarbon chain. Nevertheless, these peaks make non-resolved contributions to the liquid-like part of the spectrum. The liquid peaks are located on top of a broad peak originating from solid material. This latter component is more easily observed in the FID data (Fig. 5) as a component with fast decay. The more slowly decaying part of the FID arises from mobile protons. Extrapolation of the components to the time origin (the center of the excitation pulse) gives the ratio between the number of protons in liquid and solid environments [52]. The extrapolation was performed by fitting a bi-exponential function to the data as shown in Fig. 5. Monte Carlo error estimation was applied to assess the uncertainty in the analysis, including the noise contribution from the extrapolation. It is more difficult to get an estimate of the error originating from the choice of functional form for the signal decay. However, it should be noted that a
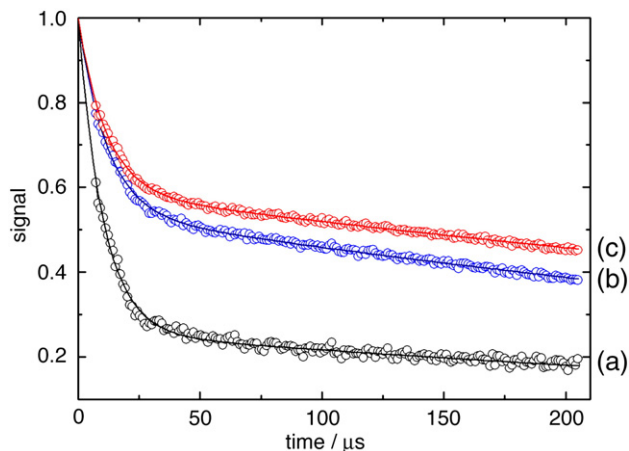

Fig. 5. Free induction decay for the extracted SC lipids with (a) 1.4 wt.%, (b) 29.2 wt.% and (c) 37.3 wt.% water at 25 °C (sample 2).
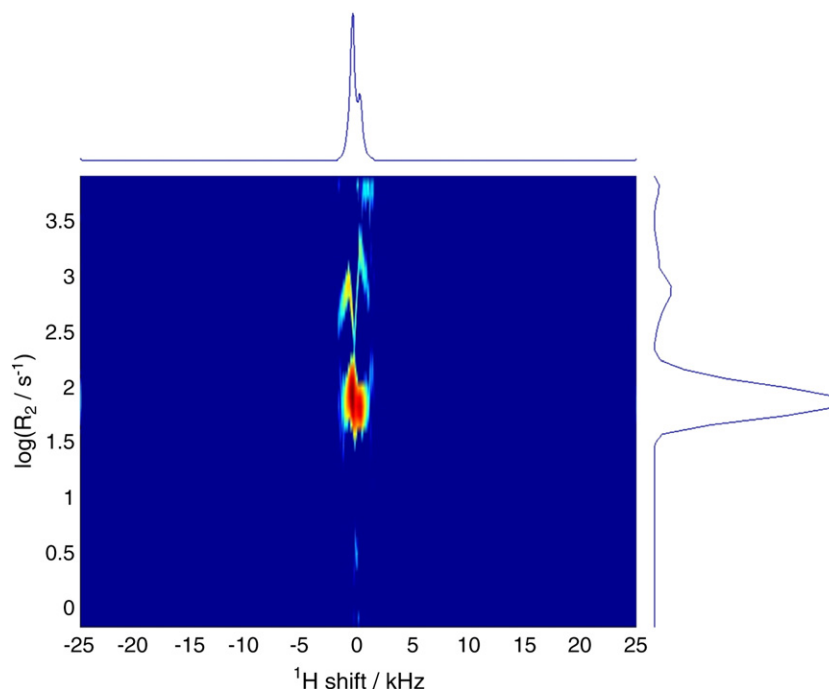
Fig. 6. 2D relaxation–chemical shift correlation spectra for extracted SC lipids with 37.3 wt.% water at 25 °C (sample 2).

sum of a Gaussian and an exponential decay produced a significantly low quality fit. Both solid and fluid lipids are detected in the lipid mixtures at all water contents investigated. T_2 relaxation experiments (log $R_2 =$ log$1/T_2$) were performed with the purpose of improving the resolution between the liquid components and getting further information about the environment in which the molecules are located. The T_2 distribution plot of the mobile protons in the hydrated samples is multicomponent, but it is not possible to distinguish aqueous protons from non-aqueous protons (see Fig. 6, for 37.3 wt.% water). If present, excess bulk water would be detected as a component with a T_2 of about 1 s. This was not the case for any of the studied samples. Since the water contents of the samples are known, it is possible to make an estimate of the fraction of the non-aqueous protons that are mobile from the NMR FID experiments, assuming an approximate proton content in lipids of 11.9 g H/100 g dry weight [54]. The calculated values of the fraction of fluid lipids in the extracted SC lipids at different water contents are summarized in Table 1. For very low water content the value of the fluid lipid fraction is small. In the range 14.9–43.7 wt.% water, the fraction of fluid lipids is clearly higher and no variation in fluid fraction with hydration could be detected within the resolution of the measurements.

Table 1
Estimate of the fraction of non-aqueous protons arising from lipids in the mobile state ($n_{non-aq\ mobile}/n_{non-aq\ total}$), as derived from NMR FID experiments

Water content (%)	$n_{non-aq\ mobile}/n_{non-aq\ total}$
1.4	0.24
14.9	0.36
29.2	0.35
37.3	0.35
43.7	0.38

Finally, we note that the ^{1}H NMR spectra do not exhibit the characteristic lineshape of an anisotropic liquid crystalline phase [53]. This could be explained by molecular exchange between regions with different orientation of the lamellar director occurring on a time scale that is short with respect to the inverse NMR line width in the absence of exchange (app. 0.1 ms). Alternatively, the environment of the fluid lipids are much more disordered and dynamic than in a typical bilayer, resulting in almost complete averaging of the anisotropic spin interactions.

3.3.2. Isolated corneocytes

The isolated corneocytes were investigated at different degrees of hydration. The ^{1}H NMR spectra and T_2 relaxation experiments indicate the presence of only one liquid-like component while the major part of the sample is solid. With increasing water content, there is a continuous decrease in the fraction of the solid component (from 0.91 until 0.36) and an increase in the value of T_2 for the liquid component (from ca. 0.13 ms to a maximum value of 10 ms) — an indication that the mobile protons are those of water. An estimate of the fraction of mobile protons arising from the non-aqueous part of the sample based on the NMR FID experiments, assuming a proton content in keratin of 5.8 g H/100 g of dry weight [54] shows that the fraction of the fluid component in the non-aqueous part of the sample is zero and that it is not affected by the water content. The ^{1}H spectra shown in Fig. 7 further indicate that no significant change of the mobility of the solid component occurs upon hydration.

3.3.3. Stratum corneum

Samples of intact SC were investigated at different water contents. For all compositions, both fluid and solid material is
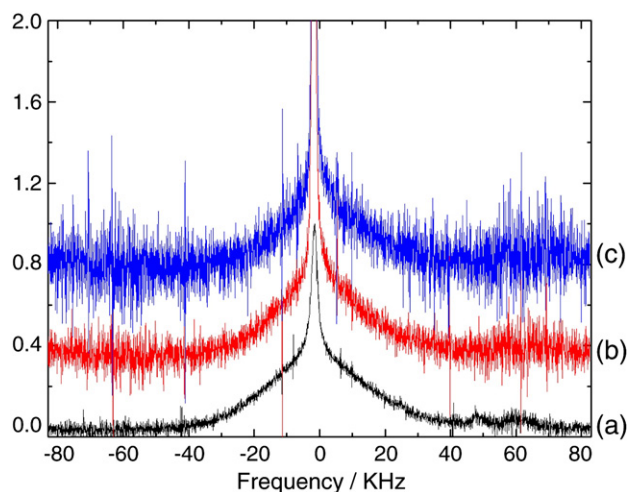
Fig. 7. Wideline ^{1}H NMR for the isolated corneocytes with (a) 5.8 wt.%, (b) 15.1 wt.% and (c) 31.2 wt.% at 25 °C (sample 2).

present. Only one liquid-like peak can be observed in the chemical shift dimension. Using the relaxation-chemical shift correlation experiment, the liquid-like peak is resolved into two components as shown in Fig. 8 for 12.8 wt.% water. In contrast to the case of extracted lipids the aqueous and non-aqueous fluid components cannot be resolved in the chemical shift dimension (Fig. 6) for the intact SC, presumably because of peak broadening originating from magnetic susceptibility differences between the different domains in the microheterogeneous system. The intensity of the peak with higher T_2 value did not change with different water contents, therefore it is probably related with the non-aqueous mobile component of the sample and the lipid hydrocarbon tails. This result shows that lipids in a fluid state are also present in the intact SC and not

only in the isolated lipids. The rather fast T_2 relaxation of the water is typical for water in close proximity (<1 nm) to solid components [55]. Upon hydration the value of T_2 is continuously increasing. This fact can be explained by fast exchange occurring between a perturbed surface layer with fast relaxation and a layer of slowly relaxing free water without direct contact with the solid surface. At 44 wt.% water content a considerable part of the sample remains solid. As was also the case for the extracted SC lipids and the isolated corneocytes, excess bulk water was not detected in the investigated samples.

4. Discussion

The data presented in this work are obtained from measurements with two complementary techniques, NMR and sorption microcalorimetry. NMR is a very powerful tool to detect minor fractions of fluid components in complex mixtures, which is more difficult to reach with, e.g., X-ray diffraction techniques. NMR has also some advantages over, e.g., fluorescent techniques and ESR, in that it does not require any labeling of the molecules or the presence of fluorescent probes that might affect the (local) phase equilibria. The sorption microcalorimetry measurements provide almost complete thermodynamic description of the hydration process in the different systems. By combining these techniques, the thermodynamic events can be related to the local mobility, and thereby molecular interpretations on the process of SC hydration can be made.

4.1. Solid and fluid SC lipids

It is well established that the extracellular SC lipids form a lamellar structure [28,34,56,57]. Still, the molecular organization of the SC lipids within this lipid lamellar matrix
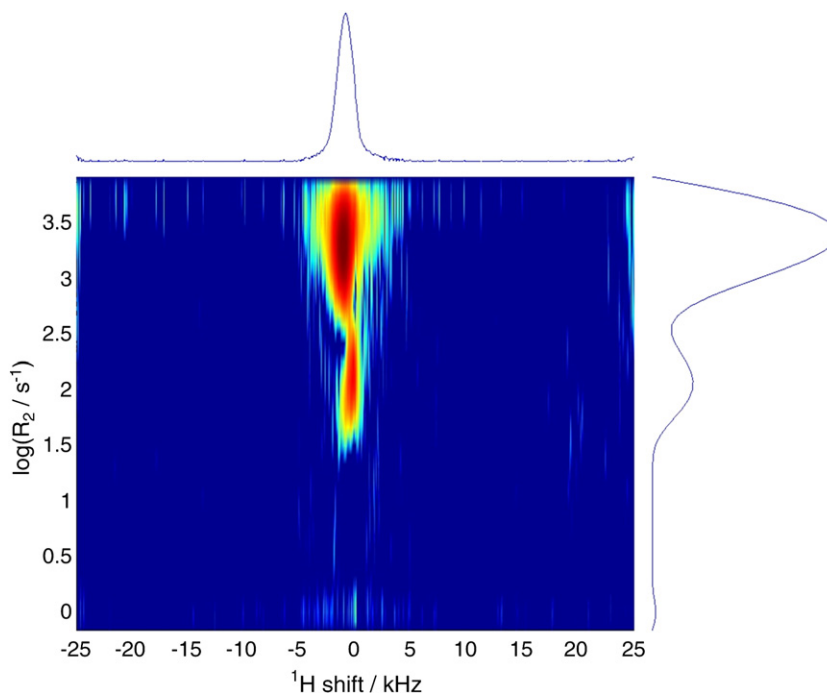

Fig. 8. 2D relaxation–chemical shift correlation spectra for SC with 12.8 wt.% water at 25 °C (sample 2).

is not fully understood. Several models based on large amount of experimental data have been proposed, including structures of connected bilayers [37,58,59] and the formation of domains within the bilayers [31]. These models take into account the coexistence of fluid and solid lipids, although the relative amounts have not been quantified experimentally. The NMR data in the present study clearly show such coexistence of fluid and solid lipids. It is also shown that a small fraction of the lipids remain in the fluid state at water contents as low as 1.4 wt.% water. The existence of fluid lipids is considered crucial to the barrier properties of the SC because these are lipids likely to constitute a major transport route. Presumably, water and other small molecules that penetrate the SC diffuse through the fluid lipid regions, as the permeability is considerably higher in the fluid phase than in the solid phase.

From values of the fraction of fluid lipids estimated from the NMR FID experiments (Table 1), we conclude that a rather substantial fraction of the lipids are in the liquid state. This confirms previous results pointing to the existence of fluid SC lipids at ambient temperatures [34,35,60–64], and it is the first time that a numerical value is assigned. The amount of fluid lipids is significantly lower at a water content of 1.4% than at water contents of 15% or higher. Within the resolution of our method, we are not able to demonstrate any variation within the fraction of fluid lipids at 25 °C and water contents above 14.9 wt.% of water. Previous IR studies have shown that the acyl-chain order in the intercellular lipids increases with hydration at low water contents, while it is independent of the degree of hydration at higher water contents [61,65]. On the other hand, ESR studies have shown that, at a slightly higher temperature, there is an increase in the membrane fluidity with increasing water content up to the fully hydrated state [5,60]. Taken together, this implies that both temperature and hydration influence the SC phase behavior. It is also likely that, e.g., pH affect the SC lipid phase behavior. The hydration process can effect the degree of ionization of the fatty acids [66] in the SC lipids, and it is possible that the proton concentration between the lamellae can vary between the swollen and the dry sample. However, it is not possible to control pH in the sorption calorimetry measurements.

From the sorption data we conclude that there is a substantial swelling of the extracted SC lipids upon hydration. At RH approaching 100%, the lipid phase contains more than 40 wt.% water (Fig. 1). This is consistent with the presence of liquid crystalline lipids, as solid lipids generally have a much lower ability to take up water. The sorption isotherm in Fig. 1a can be analysed in terms of interlamellar forces in bilayer systems because the osmotic pressure of water is equal to the interbilayer force in a lamellar system. At RH>65%, the sorption data show an exponential relation between the osmotic pressure and water content, which is typical for the swelling of lamellar lipid systems [67]. There exists a debate in the literature on whether the SC lipids are able to swell in water or not. This discussion is mainly based on data obtained from SAXS measurements on SC and SC lipid models that in fact, have shown somewhat contradictory results. In some of these studies, no swelling was

detected in human and mouse SC [28,56,68], while minor swelling has been reported for the lipid bilayers in pig SC [27] and SC lipid models [69], and a rather pronounced swelling was shown for the short lamellar repeat distance structure in the SC of hairless mouse from 5.8 nm at 12 wt.% water to 6.6 nm at 50 wt.% water [57]. More recently, also neutron scattering results [70] indicated swelling of the bilayer regions of human SC. An explanation for why the swelling was not observed in some of the studies might lie in the inherent limitations of the X-ray techniques, e.g. the second order peak for the long repeat distance lies very close to the first peak of the short one, which might lead to overlapping. In fact, the most clear observation [57] of swelling in the short lamellar repeat distance was detected for SC from hairless mouse, which apparently gives sharper diffraction peaks than that from human or pig SC. The previous data displaying swelling of the short lamellar phase [27,57], together with the present NMR and sorption data, indicate that swelling fluid lipids are present in the short lamellar structure of the SC lipids. From the present data, we cannot judge whether fluid lipids are also present in the non-swelling long repeat distance lamellar structure, which has been previously suggested [37,59].

The NMR data show a significant increase in the fraction of fluid lipids between 1.4 wt.% and 14.9 wt.% water. In the calorimetric sorption measurements we did not observe pronounced phase transitions between solid and fluid lipids during the hydration process of the SC lipids at 25 °C, but the narrow endothermic regime seen for the sample from animal 2 may indicate the melting of some ordered domains at low water contents. We also note that the initial hydration of the SC lipids from animal 1 features exothermic heat effect, which is typical for the hydration of glassy materials [71]. The glassy materials are disordered like liquids but exhibit solid-like dynamic properties. The increase of the fraction of the lipids in the mobile state can thus be caused by melting or by a glass transition in a fraction of the lipids. The difference between the values of enthalpies of hydration of two samples of SC lipids at very low water contents can be explained by biological variations and by effects by the preparation procedure. Even small differences in the drying procedure can lead to different degrees of crystallinity of the dry lipid samples. However, after the uptake of the first water molecules, the hydration process is very similar for the samples from the different animals, and the possible variations in the degree of crystallinity in the dry sample does not appear to affect the hydration process at water contents above 4 wt.%.

The calorimetric data demonstrate three exothermic phase transitions in the extracted lipids at high RH [Fig. 2]. These transitions cannot be associated with chain melting, as that would give rise to an endothermic heat effect, and the molecular explanations for the observed transitions are not fully understood. The exothermic transition is compatible with a transition between different liquid crystalline phases, e.g. from a phase with lower curvature to a phase with a higher curvature, has been observed for other lipid systems [72]. However, there are no evidences in the literature of non-lamellar structures in the SC at ambient temperatures, although there are indications of a

reversed hexagonal phase in ceramide mixtures at high temperatures [73,74]. The exothermic heat effect could also be related to an increase in the local curvature at the boundaries between the domains of different lamellar structures. Previous SAXS data showed that the swelling limit of the short lamellar structure coincides with the water content (ca. 50 wt.%) where two repeating units seems to match the repeat unit of the long lamellar structure [57], and it was suggested that further swelling is constrained due to the structural restriction put up by the domains of the non-swelling long lamellar structure. The curvature at the domain interface would then go from a negative value to zero, which could give rise to an exothermic heat effect, in accordance to the discussion above [72]. A related explanation for the exothermic transitions at high RH lies in the reorganization of the lipid domains within the lamellar structure, e.g., fusion of fluid domains at increasing the water content. It should be noted that the domain reorganization and domain swelling are not to be considered as phase transitions from a thermodynamic point of view. Still, it could give rise to the type of enthalpy effects detected in the calorimetric measurements. The proposed explanations for the exothermic phase transition have in common that they are not expected to give rise to any large enthalpy effects. They are also consistent with the very minor uptake of water associated with the transitions, while much larger effect would be expected for a transition between a solid and a fluid phase. However, it is hard to estimate the relative amount of lipids that are involved in the transitions, which also means that we cannot judge exactly how large these effects really are.

Finally, we recall that the properties of the extracellular SC lipids are crucial to the barrier properties of the skin, as these lipids constitute the only continuous route for molecular transport. It is therefore important to relate the lipid structure to barrier properties. In fact, the exothermic transitions at RH=91–94% coincide with the region in RH where previous studies shown on a distinct change in water permeability of the SC [5,7]. We therefore speculate that the hydration-induced lipid re-organization observed could be responsible for the alteration in SC permeability, and thereby partly explain the non-linear transport behavior of the SC.

4.2. Swelling of the isolated corneocytes

The major components of isolated corneocytes are the keratin filaments [75], and it is reasonable to assume that measured properties are related to the hydration of these. The wideline ^{1}H spectra (Fig. 7) do not exhibit any changes in the mobility of the non-aqueous components of the corneocytes when increasing the water content, leading to the conclusion that the keratin filaments remains solid throughout the whole hydration process. The increase in the value of T_2 for the aqueous component with hydration, and the gradually increasing component in the sorption isotherm, are both consistent with a continuous swelling of the solid keratin filament with hydration without any major structural rearrangements. There is evidence in the literature of unspecified protein conformation change induced by hydration [12], and both α and β forms have

been identified as predominant secondary structures in SC proteins [76,77]. The present measurements cannot distinguish between these different protein conformations, although the different rigid conformations are both consistent with the wideline ^{1}H NMR measurements.

As was pointed out above, the sorption isotherm of corneocytes has similar shape to that of lysozyme. This reflects the fact that both substances consist of amino acid residues. Different amino acids have different hydrophilicities, the most hydrophilic ones hydrate first, the most hydrophobic ones hydrate after, which gives rise to a smooth sorption isotherm. The observation that lysozyme takes up more water at the same relative humidity can reflect difference in the structures of the two protein materials (globular vs. fibrillar) as well as the difference in their amino acid compositions. The gradual swelling profile is in good agreement with previously reported sorption data for SC samples depleted of intercellular lipids from sorption microbalance measurements [13,78]. The enthalpy measurements show a strongly exothermic enthalpy effect at low water contents and endothermic effect at high water contents. [Fig. 3b]. The observed effects indicate that in the beginning of sorption the material is in the glassy state, which is typical for proteins at low water contents [49]. We also note that previous studies have also demonstrated a brittle to ductile transition in rat SC [75] upon hydration, which was explained by a glass transition in the keratin molecules. The exact position of the glass transition in corneocytes is difficult to determine because in proteins this transition can be stretched over a wide range of compositions and temperatures [49]. We suggest that the glass transition occurs in the third regime on the curve of enthalpy (i.e. between 11 and 17 wt.% of water). The straight lines in Fig. 3b correspond then to the second glassy regime (5–11 wt.%) and to the elastic regime (above 17 wt.%).

4.3. Hydration of stratum corneum

A major finding in the present study is the presence of fluid lipids in the intact SC. This is considered crucial to the barrier properties of SC as the fluid lipids likely constitute a major transport route for molecular diffusion. The fact that fluid lipids are detected in both the extracted lipids and in the intact SC further strengthens the link between the findings for the SC components to the complete SC. By reducing the complexity and study the different components separately, it is possible to achieve more detailed information that would not be accessible for the complex system. One example of this is the transitions detected for the extracted SC lipids at high RH, which are not observed for the intact SC. The lipids constitute only a small fraction of the complete SC (ca. 15%) and the exothermic transitions are difficult to detect even in the sample composed exclusively by lipids (Fig. 2). Due to the low signal we cannot expect to detect these transitions in the sorption calorimetry data for the intact SC. Still, the observed transitions might have important implications to the non-linear transport properties of the SC as discussed above.

The sorption isotherms of intact SC are similar to previous observations for human, porcine or neonatal rat SC [3,5,7,12,16],
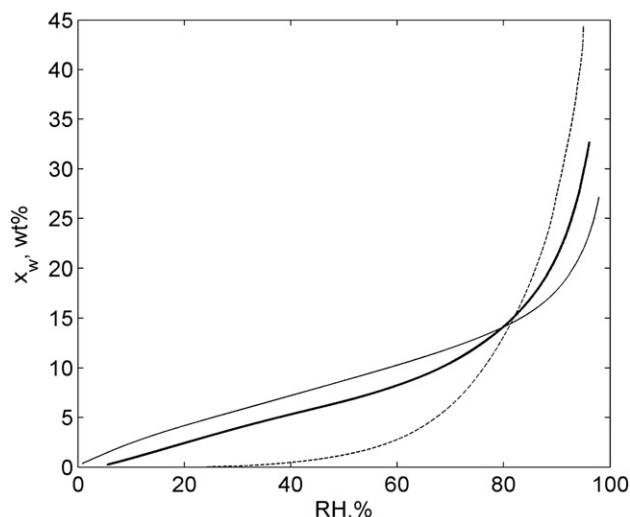
Fig. 9. Sorption isotherms of extracted SC lipids (dashed curve), isolated corneocytes (thin solid curve) and stratum corneum (thick solid curve).

although we were able to provide a more accurate description of the SC sorption behavior, especially at high RH. The sorption data are also accompanied by the thermodynamic description of the whole hydration process. This value is higher than the swelling limit of SC previously reported for human SC (22–33 wt.%) [12–16]. The enthalpy data for intact SC also show a large exothermic heat effect at the end of hydration process [Fig. 3c]. Such exothermic heat effects at high water contents has not previously been observed in any other materials studied by the method of sorption calorimetry. We suggest that the exothermic heat effect is a kinetic effect related to "delayed" hydration of the SC lipids and corneocytes in the glassy states due to very slow hydration (water diffusion) of the extracellular SC lipids and the protective corneocyte envelope [79]. Still, as the time of the experiment evolves and RH increases, water penetrates through the lipids and the cornified envelope and hydrates the sample, thus producing a "delayed" exothermic effect. Note that the beginning of the final exothermic effect also corresponds to a large increase in water uptake by SC at ca 90 % RH [Fig. 1c].

The capacity of SC to take up water has been attributed to swelling of the corneocytes and to the formation of water-pools in the extracellular SC lipids [27,80] rather then swelling of the extracellular SC lipids. The formation of water-pools indicates excess solution conditions, or in other words, water contents above the swelling limit (100% RH), and this is not considered relevant to the present experiments (RH < 100%). The combination of the presented calorimetric data for intact SC and its components at varying water contents can be used to further explore the different mechanisms of SC swelling. In Fig. 9 we present combined data on sorption isotherms of SC and its components. This plot shows that the sorption isotherms are approximately additive, i.e. the sorption isotherm of SC lies between sorption isotherms of its components and may roughly be approximated as their weighted sum. All three sorption isotherms cross at RH slightly higher than 80%. Below 80% RH, the hydration of corneocytes is more pronounced than that

of lipids, while at high RHs, the lipids take up more water than corneocytes do. This is also consistent with previous observations [81,82], and this implies that the swelling and the water holding capacity of the SC lipids cannot be ignored. In this comparison one should, however, be aware that the sorption isotherm for the intact SC might include non-equilibrium effects due to a "delayed" hydration, which might complicate the analysis. Still, we believe that the results presented here reflect the general trends of hydration behavior of stratum corneum and its components.

5. Conclusions

The SC is exposed to large variations in the chemical surroundings that can affect its structure, and thereby also its function. An important example is that the transport properties can be regulated by the water content in SC, which is related to the RH of the environment. Furthermore, the water content has profound influence on other vital functions of the SC, e.g., the mechanical properties and the enzymatic activity. In this study, we explore the process of hydration in intact SC as well as in extracted SC lipids and isolated corneocytes, and we conclude that:

- There is a substantial swelling of SC as well as of its components at high RH. At low RHs corneocytes take up more water than SC lipids do, while at high RHs swelling of SC lipids is more pronounced than that of corneocytes. This implies that uptake of water in SC is strongly dependent on the hydration of both the lipids and the corneocytes.
- Lipids in a fluid state are present in both extracted SC lipids and in the intact SC.
- At water contents ranging from 1.5 to 40 wt.%, there is a coexistence of fluid and solid SC lipids. This coexistence is considered crucial to the barrier properties of the SC, as these phases have totally different diffusion characteristics.
- There is an increase in the fraction of the fluid lipids at water contents below 15 wt.%, whereas the fraction of fluid lipids remains virtually constant when the water content is further increased.
- Three exothermic phase transitions are detected in the SC lipids at RH = 91–94%. These transitions coincide with the region in RH where previous studies have shown a distinct change in water permeability of the intact SC, and it is possible that this hydration-induced lipid re-organization is partially responsible for non-linear transport behavior of the SC.
- The hydration causes swelling of the corneocytes, while it does not affect the mobility of solid components (keratin filaments).

References

[1] Y. Kalia, F. Pirot, R. Guy, Homogeneous transport in a heterogeneous membrane: water diffusion across human stratum corneum in vivo, Biophys. J. 71 (1996) 2692–2700.

[2] R. Marks, The stratum corneum barrier: the final frontier, J. Nutr. 134 (2004) 2017S–2021S.

[3] J. Pieper, G. Charalambopoulou, T. Steriotis, S. Vasenkov, A. Desmedt, R.E. Lechner, Water diffusion in fully hydrated porcine stratum corneum, Chem. Phys. 292 (2003) 465–476.

[4] M. Denda, J. Sato, Y. Masuda, T. Tsuchiya, J. Koyama, M. Kuramoto, P.M. Elias, K.R. Feingold, Exposure to a dry environment enhances epidermal permeability barrier function, J. Invest. Dermatol. 111 (1998) 858–863.

[5] A. Alonso, N.C. Meirelles, V.E. Yushmanov, M. Tabak, Water increases fluidity of intercellular membranes of stratum corneum: correlation with water permeability, elastic, and electrical resistance properties, J. Invest. Dermatol. 106 (1996) 1058–1063.

[6] R.O. Potts, M.L. Francoeur, Lipid biophysics of water loss through the skin, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 3871–3873.

[7] I.H. Blank, J. Moloney, A.G.E.I. Simon, C. Apt, The diffusion of water across the stratum corneum as a function of its water content, J. Invest. Dermatol. 82 (1984) 188–194.

[8] E. Sparr, H. Wennerström, Responding phospholipid membranes—Interplay between hydration and permeability, Biophys. J. 81 (2001) 1014–1028.

[9] P.J. Caspers, G.W. Lucassen, E.A. Carter, H.A. Bruining, G.J. Puppels, In vivo confocal Raman microspectroscopy of the skin: noninvasive determination of molecular concentration profiles, JID 116 (2001) 434–442.

[10] C.R. Harding, A. Watkinson, A.V. Rawlings, Dry skin, moisturization and corneodesmolysis, Int. J. Cosmet. Sci. 22 (2000) 21–52.

[11] A.V. Rawlings, C.R. Harding, Moisturization and skin barrier function, Dermatol. Ther. 17 (2004) 43–48.

[12] R.L. Anderson, J.M. Cassidy, J.R. Hansen, W. Yellin, Hydration of stratum corneum, Biopolymers 12 (1973) 2789–2802.

[13] M. Hey, D. Taylor, W. Derbyshire, Water sorption by human callus, Biochim. Biophys. Acta 540 (1978) 518–533.

[14] G. Imokawa, H. Kuno, M. Kawai, Stratum corneum lipids serve as a bound-water modulator, J. Invest. Dermatol. 96 (1991) 845–851.

[15] T. Inoue, K. Tsujii, K. Okamoto, K. Toda, Differential scanning calorimetric studies on the melting behavior of water in stratum corneum, J. Invest. Dermatol. 86 (1986) 689–693.

[16] G.B. Kasting, N.D. Barai, Equilibrium water sorption in human stratum corneum, J. Pharm. Sci. 92 (2003) 1624–1631.

[17] P. Elias, Epidermal lipids, barrier function, and desquamation, J. Invest. Dermatol. 80 (1983) 44–49.

[18] A.E. Kalinin, A.V. Kajava, P.M. Steinert, Epithelial barrier function: assembly and structural features of the cornified cell envelope, BioEssays 24 (2002) 789–800.

[19] P.W. Wertz, D.C. Swartzendruber, D.J. Kitko, K.C. Madison, D.T. Downing, The role of the corneocyte lipid envelopes in cohesion of the stratum corneum, J. Invest. Dermatol. 93 (1989) 169–172.

[20] H. Boddé, M. Kruithof, J. Brussee, H. Koerten, Visualisation of normal and enhanced HgCl2 transport through human skin in vitro, Int. J. Pharm. 53 (1989) 13–24.

[21] J.A. Bouwstra, P.L. Honeywell-Nguyen, G.S. Gooris, M. Ponec, Structure of the skin barrier and its modulation by vesicular formulations, Prog. Lipid Res. 42 (2003) 1–36.

[22] F. Bonté, A. Saunois, P. Pinguet, A. Meybeck, Existence of a lipid gradient in the upper stratum corneum and its possible biological significance, Arch. Dermatol. Res. 289 (1997) 78–82.

[23] C.L. Silva, S.C.C. Nunes, M.E.S. Eusébio, A.A.C.C. Pais, J.J.S. Sousa, Study of human stratum corneum and extracted lipids by thermomicroscopy and DSC, Chem. Phys. Lipids 140 (2006) 36–47.

[24] C.L. Silva, S.C.C. Nunes, M.E.S. Eusébio, A.A.C.C. Pais, J.J.S. Sousa, Thermal behavior of human stratum corneum. A differential scanning calorimetry study at high scanning rates, Skin Pharmacol. Physiol. 19 (2006) 132–139.

[25] P. Wertz, L. Norlén, "Confidence Intervals" for the "True" Lipid Composition of the Human Skin Barrier?, in Skin, Hair and Nails: Structure and Function, Marcel Dekker Inc., 2004.

[26] M. Ponec, A. Weeheim, P. Lankhorst, P. Wertz, New acylceramide in native and reconstructed epidermis, J. Invest. Dermatol. 120 (2003) 581–588.

[27] J.A. Bouwstra, G. Gooris, W. Bras, D. Downing, Lipid organization in pig stratum corneum, J. Lipid Res. 36 (1995) 685–695.

[28] J.A. Bouwstra, G.S. Gooris, J.A.V.D. Spek, W. Bras, Structural investigations of human stratum corneum by small-angle X-ray scattering, J. Invest. Dermatol. 97 (1991) 1005–1012.

[29] J.A. Bouwstra, G.S. Gooris, F.E.R. Dubbelaar, A.M. Weerheim, A.P. Ijzerman, M. Ponec, Role of ceramide 1 in the molecular organization of stratum corneum lipids, J. Lipid Res. 39 (1998) 186–196.

[30] D.B. Fenske, J.L. Thewalt, M. Bloom, N. Kitson, Model of stratum corneum intercellular membranes: 2H NMR of macroscopically oriented multilayers, Biophys. J. 67 (1994) 1562–1573.

[31] B. Forslind, A domain mosaic model of the skin barrier, Acta Derm. Venereol. 74 (1994) 1–6.

[32] D.J. Moore, M.E. Rerek, R. Mendelsohn, Lipid domains and orthorhombic phases in model stratum corneum: evidence from Fourier Transform Infrared Spectroscopy studies, Biochem. Biophys. Res. Commun. 231 (1997) 797–801.

[33] B. Ongpipattanakul, M.L. Francoeur, R.O. Potts, Polymorphism in stratum corneum lipids, Biochim. Biophys. Acta 1190 (1994) 115–122.

[34] S.H. White, D. Mirejovsky, G.I. King, Structure of lamellar lipid domains and corneocyte envelopes of murine stratum corneum. An X-ray diffraction study, Biochemistry 27 (1988) 3725–3732.

[35] J.A. Bouwstra, G.S. Gooris, F.E.R. Dubbelaar, M. Ponec, Phase behaviour of lipid mixtures based on human ceramides: coexistence of crystalline and liquid phases, J. Lipid Res. 42 (2001) 1759–1770.

[36] A.V. Rawlings, Trends in stratum corneum research and the management of dry skin conditions, Int. J. Cosmet. Sci. 25 (2003) 63–95.

[37] J.A. Bouwstra, F.E.R. Dubbelaar, G.S. Gooris, M. Ponec, The lipid organisation in the skin barrier, Acta Derm. Venereol., Suppl. 208 (2000) 23–30.

[38] L. Norlén, Skin barrier structure and function: the single gel phase model, J. Invest. Dermatol. 117 (2001) 830–836.

[39] J.A. Bouwstra, L.J.C. Peschier, J. Brussee, H.E. Boddé, Effect of n-alkyl-azocycloheptan-2-ones including azone on thermal behaviour of human stratum corneum, Int. J. Pharm. 52 (1989) 47–54.

[40] B.F.V. Duzee, Thermal analysis of human stratum corneum, J. Invest. Dermatol. 75 (1975) 404–408.

[41] P.W. Wertz, D.T. Downing, Covalently bound w-hydroxiacylsphingosine in the stratum corneum, Biochim. Biophys. Acta 917 (1987) 108–111.

[42] A. Alonso, J. Silva, M. Tabak, Stratum corneum protein mobility as evaluated by a spin label maleimide derivative, BBA 1478 (2000) 89–101.

[43] M. Sznitowska, S. Janicki, A. Williams, S. Lau, A. Stolyhwo, pH-Induced modifications to stratum corneum lipids investigated using thermal, spectroscopic, and chromatographic techniques, J. Pharm. Sci. 92 (2003) 173–179.

[44] I. Wadsö, L. Wadsö, A new method for determination of vapour sorption isotherms using a twin double microcalorimeter, Thermochim. Acta 271 (1996) 179–187.

[45] V. Kocherbitov, Salt-saturated salt solution as a standard system for sorption calorimetry, Thermochim. Acta 421 (2004) 105–110.

[46] V. Kocherbitov, A new formula for accurate calculation of water activity in sorption calorimetric experiments, Thermochim. Acta 414 (2004) 43–45.

[47] K. Whittal, A. MacKay, Quantitative interpretation of NMR relaxation data, J. Magn. Reson. 84 (1989) 134–152.

[48] C.J. Johnson Jr., Diffusion ordered nuclear magnetic resonance spectroscopy: principles and applications, Prog. Nucl. Magn. Reson. Spectrosc. 34 (1999) 203–256.

[49] V. Kocherbitov, T. Arnebrant, O. Söderman, Lysozyme–water interactions studied by sorption calorimetry, J. Phys. Chem., B 108 (2004) 19036–19042.

[50] L. Wadsö, N. Markova, A method to simultaneously determine sorption isotherms and sorption enthalpies with a double twin microcalorimeter, Rev. Sci. Instrum. 73 (2002) 2743–2754.

[51] M.H. Levitt, Spin Dynamics: Basics of Nuclear Magnetic Resonance, Chichester, 2001.

[52] D. Topgaard, O. Söderman, Self-diffusion of nonfreezing water in porous carbohydrate polymer systems studied with NMR, Biophys. J. 83 (2002) 3596–3606.

[53] H. Wennerström, Proton nuclear magnetic resonance lineshapes in lamellar liquid crystals, Chem. Phys. Lett. 18 (1973) 41–44.

[54] K.J. Packer, T.C. Sellwood, Proton magnetic resonance studies of hydrated stratum corneum. Part 1.—Spin-lattice and transverse relaxation, J. Chem. Soc., Faraday Trans. 2 (74) (1978) 1579–1591.

[55] Y. Xu, C. Araujo, A. MacKay, K. Whittal, Proton spin-lattice relaxation in wood—T1 related to local specific gravity using a fast-exchange model, J. Magn. Reson., Ser. B 110 (1996) 55–64.

[56] J.A. Bouwstra, G.S. Gooris, M.A.S. Vries, J.A.V. Spek, W. Bras, Structure of human stratum corneum as a function of temperature and hydration: a wide-angle X-ray diffraction study, Int. J. Pharm. 84 (1992) 205–216.

[57] N. Ohta, S. Ban, H. Tanaka, S. Nakata, I. Hatta, Swelling of intercellular lipid lamellar structure with short repeat distance in hairless mouse stratum corneum as studied by X-ray diffraction, Chem. Phys. Lipids 123 (2003) 1–8.

[58] J.R. Hill, P.W. Wertz, Molecular models of the intercellular lipid lamellae from epidermal stratum corneum, Biochim. Biophys. Acta 1616 (2003) 121–126.

[59] T.J. McIntosh, Organization of skin stratum corneum extracellular lamellae: diffraction evidence for asymmetric distribution of cholesterol, Biophys. J. 85 (2003) 1675–1681.

[60] A. Alonso, N.C. Meirelles, M. Tabak, Effect of hydration upon the fluidity of intercellular membranes of stratum corneum: an EPR study, Biochim. Biophys. Acta 1237 (1995) 6–15.

[61] C.L. Gay, R.H. Guy, G.M. Golden, V.H.W. Mak, M.L. Francoeur, Characterization of low-temperature (i.e. <65 °C) lipid transitions in human stratum corneum, J. Invest. Dermatol. 103 (1994) 233–239.

[62] M.E. Hatcher, W.Z. Plachy, Dioxygen diffusion in the stratum corneum: an EPR spin label study, Biochim. Biophys. Acta 1149 (1993) 73–78.

[63] D. Parrott, J. Turner, Mesophase formation by ceramides and cholesterol: a model for stratum corneum lipid packing? Biochim. Biophys. Acta 1147 (1993) 273–276.

[64] S. Rehfeld, W. Plachy, S. Hou, P. Elias, Localization of lipid microdomains and thermal phenomena in murine stratum corneum and isolated membrane complexes: an electron spin resonance study, J. Invest. Dermatol. 95 (1990) 217–223.

[65] V. Mak, R. Potts, R. Guy, Does hydration affect intercellular lipid organization in the stratum corneum? Pharm. Res. 8 (1991) 1064–1065.

[66] B. Ninham, V. Parsegian, Electrostatic potential between surfaces bearing ionizable groups in ionic equilibrium with physiologic saline solution, J. Theor. Biol. 31 (1971) 405–428.

[67] J. Israelachvili, Intermolecular and Surface Forces, 2nd ed., Academic Press Limited, London, 1992.

[68] J. Bouwstra, G. Gooris, J.v.d. Spek, S. Lavrijsen, W. Bras, The lipid and protein structure of mouse stratum corneum: a wide and small angle diffraction study, Biochim. Biophys. Acta 1212 (1994) 183–192.

[69] T.J. McIntosh, M.E. Stewart, D.T. Downing, X-ray diffraction analysis of isolated skin lipids: reconstitution of intercellular lipid domains, Biochemistry 35 (1996) 3649–3653.

[70] G. Charalambopoulou, T.A. Steriotis, T. Hauss, A.K. Stubos, N.K. Kanellopoulos, Structural alterations of fully hydrated human stratum corneum, Physica, B 350 (2004) e603–e606.

[71] V. Kocherbitov, O. Söderman, Glassy crystalline state and water sorption of alkyl maltosides, Langmuir 20 (2004) 3056–3061.

[72] V. Kocherbitov, Driving forces of phase transitions in surfactant and lipid systems, J. Phys. Chem., B 109 (2005) 6430–6435.

[73] W. Abraham, D.T. Downing, Deuterium NMR investigation of polymorphism in stratum corneum lipids, Biochim. Biophys. Acta 1068 (1991) 189–194.

[74] S. Raudenkolb, S. Wartewig, G. Brezesinski, S. Funari, R. Neubert, Hydration properties of n-(alpha-hydroxyacyl)-sphingosine: X-ray powder diffraction and FT-Raman spectroscopic studies, Chem. Phys. Lipids 136 (2005) 13–22.

[75] Y.S. Papir, K. Hsu, R.H. Wildnauer, The mechanical properties of water and ambient temperature on the tensile properties of newborn rat stratum corneum, Biochim. Biophys. Acta 399 (1975) 170–180.

[76] H.P. Baden, A.L.A. Goldsmith, L. Bonar, Conformational changes in the α-fibrous protein of epidermis, J. Invest. Dermatol. 60 (1973) 215–218.

[77] J. Garson, J. Doucet, J. Lévêque, G. Tsoucaris, Oriented structure in human stratum corneum revealed by X-ray diffraction, J. Invest. Dermatol. 96 (1991) 43–49.

[78] P. Elsner, E. Berardesca, H. Maibach, Bioengineering of the Skin: Water and Stratum Corneum, CRC Press, Switzerland, 1994.

[79] M.A. Kiselev, N.Y. Ryabova, A.M. Balagurov, S. Dante, T. Hauss, J. Zbytovska, S. Wartewig, R.H.H. Neubert, New insights into the structure and hydration of a stratum corneum lipid model membrane by neutron diffraction, Eur. Biophys. J. 34 (2005) 1030–1040.

[80] J.A. Bouwstra, A. Graaff, G.S. Gooris, J. Nijsse, J.W. Wiechers, A.C. Aelst, Water distribution and related morphology in human stratum corneum at different hydration levels, J. Invest. Dermatol. 120 (2003) 750–758.

[81] S.E. Friberg, I. Kayali, L.D. Rhein, F.A. Simion, R.H. Cagan, The importance of lipids for water uptake in stratum corneum, Int. J. Cosmet. Sci. 12 (1990) 5–12.

[82] L. Norlén, A. Emilson, B. Forslind, Stratum corneum swelling. Biophysical and computer assisted quantitative assessments, Arch. Dermatol. Res. 289 (1997) 506–513.