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Extraction of natural moisturizing factor from the stratum corneum and its implication on skin molecular mobility

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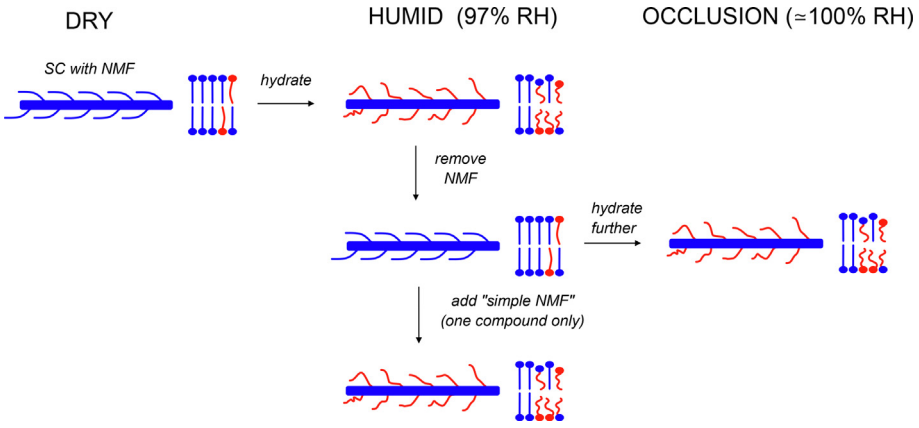
GRAPHICAL ABSTRACT



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ABSTRACT

The natural moisturizing factor (NMF) is a mixture of small water-soluble compounds present in the upper layer of the skin, stratum corneum (SC). Soaking of SC in water leads to extraction of the NMF molecules, which may influence the SC molecular properties and lead to brittle and dry skin. In this study, we investigate how the molecular dynamics in SC lipid and protein components are affected by the removal of the NMF compounds. We then explore whether the changes in SC components caused by NMF removal can be reversed by a subsequent addition of one single NMF component: urea, pyrrolidone carboxylic acid (PCA) or potassium lactate. Samples of intact SC were investigated using NMR, X-ray diffraction, infrared spectroscopy and sorption microbalance. It is shown that the removal of NMF leads to reduced molecular mobility in keratin filaments and SC lipids compared to untreated SC. When the complex NMF mixture is replaced by one single NMF component, the molecular mobility in both keratin filaments and lipids is regained. From this we propose a general relation between the molecular mobility in SC and the amount of polar solutes which does not appear specific to the precise chemical identify of the NMF compounds.

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1. Introduction

The stratum corneum (SC) is the outer most layer of the epidermis in skin with the main function to prevent from desiccation and protect the body against the uptake of hazardous chemicals [1,2]. The healthy SC should also be soft and pliable, and tolerates deformation from physical strain and stress. Taken together, the complex biomaterial that is the SC membrane has to fulfill several essentially different requirements.

SC consists of anucleated dead epidermal cells, also known as corneocytes, embedded in an extracellular multi-lamellar lipid matrix [3,4]. The corneocytes are filled with keratin arranged as coiled coils, which associates into filaments classified as intermediate filaments due to their size in diameter corresponding to approximately 7–10 nm [5]. These filaments are further embedded in a protein matrix and enclosed by a cornified envelope consisting of crosslinked proteins, including involucrin, loricrin and small proline-rich proteins, together with ceramide lipids [6]. The corneocytes with their keratin filaments and cornified envelope is what gives the skin its mechanical properties of being simultaneously strong and elastic [6]. As depicted in Fig. 1a, the corneocytes are embedded in a multi-lamellar lipid matrix composed of long-chain ceramides and fatty acids together with cholesterol in an close to equimolar mixture [7,8]. The chemical structures of ceramide and cholesterol are shown in Fig. 1b. The SC lipid composition is widely different from most other biological membranes in that it contains almost no phospholipids, and that the major part of the lipids are saturated [9]. The SC composite structure with hydrophilic corneocytes embedded in a hydrophobic lipid matrix ensures its barrier properties. The water content in SC exposed to air with ambient humidity is relatively low (around 20 wt% water [10]). However, the water concentration will typically vary between the different SC regions and locally be high enough to maintain enzymatic activity, which is important for processes such as desquamation and skin regeneration [11].

SC is regularly exposed to varying conditions in its surroundings in terms of relative humidity (RH), direct exposure to liquid water and moisturizing or occluding skin formulations [12]. Such changes in the water activity surrounding the skin will naturally also influence the water activity in the SC itself. Changes in hydration conditions will not only change the water content in SC but can also lead to melting of a small portion of the extracellular SC lipids, and an increased mobility in the terminal amino acids of the keratin filaments inside the corneocytes [13–15]. Mobility is in this case defined by the reorientational dynamics of the C-H bonds of the different components in SC [16]. The response in the molecular properties of SC lipids and proteins to the changes in hydration may in turn strongly affect the macroscopic SC material properties, including both barrier function [17] and mechanical properties [18,19].

One way to dampen the molecular responses to changes in hydration conditions, and to protect the SC membrane from severe drying is to introduce osmolytes, i.e. small polar compounds reducing the chemical potential of the water. In the healthy skin, osmolytes are naturally present and commonly referred to as the natural moisturizing factor (NMF). The NMF consists of a combination of small polar compounds derived from the protein filaggrin present in the corneocytes, sweat constituents and triglyceride turnover occurring in the sebaceous glands [20–22]. The NMF mixture can make up for up to 10% of the dry weight of the corneocytes and consists of different amino acids and amino acid derivatives, such as pyrrolidone carboxylic acid (PCA) and urocanic acid (UCA), as well as lactic acid, sugars, urea, glycerol and a variety of ions [23–25]. The complete role of NMF in SC is still not fully understood, but skin diseases such as winter xerosis, atopic dermatitis and severe skin dryness have been associated with a decreased level of NMF [26,27]. In addition, the osmolytes with low vapor pressure may in an almost ideal way replace the water in drying conditions and thereby prevent dehydration-induced changes in the lipid and protein components of SC [28]. The NMF compounds have also been shown to influence the macroscopic properties of SC. As an example, the SC elasticity was shown to decrease upon removal of NMF and then be regained through the addition of either basic and neutral amino acids [29], and the SC permeability was shown to increase with the addition of osmolytes such as urea or glycerol as compared to neat SC at the same humidity conditions [30].

When subjecting the skin to frequent washing and soaking in water, such as showering and bathing, a removal of water-soluble NMF compounds can take place [19,29,31–35]. In addition, lipid components (i.e. ceramides, free fatty acids and cholesterol) may also be extracted to the excess aqueous solution [32]. The removal of these components from SC has been associated with alterations in the macroscopic properties of SC, but molecular details about the consequences from the extraction during soaking of SC in water have still not been resolved.

In the present study, we aim to characterize the compounds removed from SC when soaked in water and assess how the extraction of these water-soluble compounds influences the molecular properties of the SC lipid and protein components. We thereafter investigate whether the molecular changes caused by the water extraction and removal of NMF compounds may be reversed by simply replacing the complex NMF with one single NMF component–urea, PCA or lactate (Fig. 1c)–or by the addition of excess of

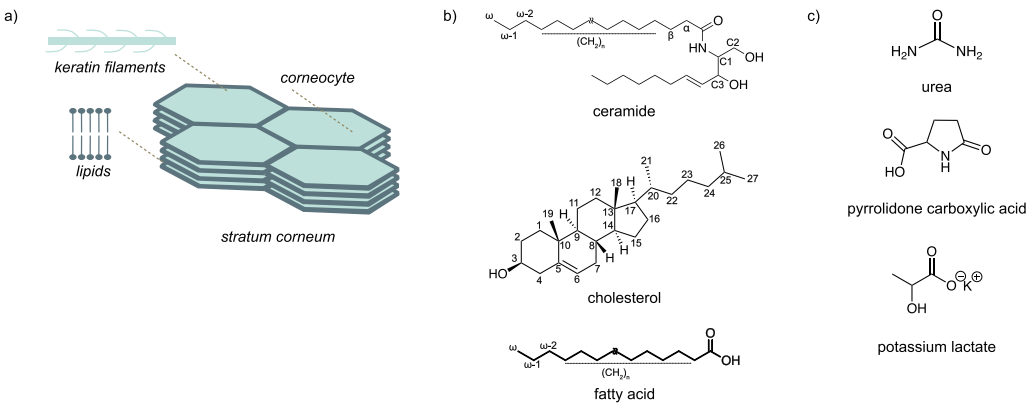

Fig. 1. Schematic representation of a) SC with corneocytes containing keratin filaments and surrounded by lipids. The chemical structures of b) lipid components ceramide and cholesterol and c) the NMF components urea, pyrrolidone carboxylic acid (PCA) and potassium lactate.

water. We take a combined experimental approach based on natural abundance ^{13}C nuclear magnetic resonance (NMR) spectroscopy, attenuated total reflectance infrared (ATR-FTIR) spectroscopy, small and wide-angle X-ray diffraction (SWAXD) and sorption microbalance. The NMR experiments provide information about the molecular dynamics of both lipid and protein segments in SC with close to atomic resolution while ATR-FTIR and SWAXD give information on the molecular organization. In combination with the water uptake profile recorded by sorption microbalance, a detailed picture of how the SC is affected on both molecular and macroscopic level is obtained.

2. Experimental section

2.1. Chemicals

Bovine pancrease trypsin (type III), urea, (-)-2-pyrrolidone-5-carboxylic acid (PCA), potassium lactate, chloroform, methanol and D_2O was purchased from Sigma–Aldrich Chemie GmbH (Schnelldorf, Germany). NaCl, $Na_2HPO_4.2H_2O$, KH_2PO_4 and K_2SO_4 were purchased from Merck. MilliQ water was prepared using a MilliQ water filtration system with a resistivity of 18 MΩ·cm and used for the hydration of SC and corneocytes as well as the preparation of PBS solution. All chemicals were used without further purification.

2.2. SC isolation

Pig ears, bought at a local slaughterhouse, were washed in cold tap water, dried and shaved with a trimmer to remove the hairs. Small slices of the inside of the ears were dermatomed to a thickness of approx. 500 μm and put on filter paper soaked in trypsin solution (0.2% (w/v) trypsin in PBS buffer). The filter papers were then wrapped in aluminum foil and stored at 4°C for 24 h. Subsequently, SC was peeled off with forceps and washed in MilliQ water five times to remove remaining trypsin. Finally, SC was dried *in vacuo*, disintegrated into a powder with pestle and mortar and stored at −20°C until further sample preparation. We have previously shown that SC powder and sheets give identical experimental results in solid-state NMR [36].

2.3. Sample preparation

Dry SC was soaked in MilliQ water at a ratio of 1:40 w/w at 32°C for 10 min, 60 min or 24 h. The sample was then separated into a solid and liquid fraction through filtration. The solid fraction was again dried *in vacuo* while the extracted components in the liquid fraction was isolated using a rotary evaporator. Two batches for each extraction time were prepared for comparison and validation of the induced effects, which were analyzed using the below mentioned techniques.

The same procedure for sample preparation was applied for corneocytes, which had been isolated according to the method described in [37]. In short, corneocytes were extracted from SC using chloroform:methanol mixtures at ratios of 2:1, 1:1 and 1:2 v/v. Each extraction step was performed for 2 h in room temperature with gentle shaking. The extraction sequence was repeated once more for 30 min in each extraction mixture before filtration. Lastly, the extracted corneocytes were placed in methanol over night before repeated washing in MilliQ water and drying *in vacuo*. The isolated corneocytes were thereafter stored at −20°C until further use.

For the solid-state NMR measurements, approximately 20 mg of dry SC and roughly 40 wt% of MilliQ water was added to a vial and conditioned at 97% RH in a desiccator with a saturated solution of K_2SO_4 at 32°C for 24 h. Samples with reintroduction of an NMF molecule were prepared by also adding 20 wt% of urea, PCA or potassium lactate based on the dry weight of SC. The sample representing full hydration was prepared by adding 70 wt% (extrapolated from the sorption measurements of untreated SC) of MilliQ water to SC and placing the sample in a sealed vial at 32°C for 24 h. The sample was then quickly transferred to an NMR insert (Bruker) before starting the measurements. The same hydration approach was used for the ATR-FTIR and SWAXD measurements but with a lower amount of sample (approximately 5 mg) and a different sample holder.

The liquid fraction was characterized using solution NMR by dissolving the extracted components in D_2O and transferring the solution into a 3 mm NMR tube.

2.4. NMR spectroscopy

^1H, ^{13}C, ^1H-^{13}C HSQC [38–43] and ^1H-^{13}C HMBC [44] solution NMR was recorded on a 700 MHz NMR spectrometer equipped with a Bruker Avance III console and a 5 mm QCI cryoprobe. All samples were performed in D_2O at 25°C.

Solid-state NMR measurements were performed on a 500 MHz NMR spectrometer equipped with a Bruker NEO console and Bruker HX CP-MAS 4 mm probe. The MAS was operating at the frequency of 5 kHz and the temperature, which was calibrated using methanol, was set to 32°C to maintain physiological skin temperature. The following setup was used for the solid-state NMR experiments: spectral width of 250 ppm, acquisition time 0.05 s, recycling delay of 5 s and number of scans of 2832. A combination of three experiments were recorded for all samples: DP (direct polarization) [45], CP (cross-polarization) [46] and INEPT (insensitive nuclei enhanced by polarization transfer) [47–49]. All spectra were recorded under 68 kHz two-pulse phase modulation [50] (TPPM) ^1H decoupling and the ^1H and ^{13}C hard pulses were given at $\omega_1^{H/C}/2\pi$ = 80 kHz. In the CP experiments, the ^{13}C nutation frequency was 80 kHz while the ^1H nutation frequency was linearly ramped from 72 to 88 kHz during 1 ms contact time. In the INEPT experiment, delay times of τ = 1.8 ms and $\tau\prime$ = 1.2 ms were used. The recorded time-domain data were then processed using an in-house MATLAB code partially derived from matNMR [51]. Line broadening of 10 Hz, zero-filling from 3124 to 8192 time-domain points, Fourier Transform, phase and baseline correction were used for all spectra. The ^{13}C peak assignment was made in comparison to the data published by Björklund et al. [15]. Selected samples, due to time constrains, were measured multiple times to confirm the repeatability of the measurements.

2.5. Attenuated total reflectance FTIR (ATR-FTIR) spectroscopy

ATR-FTIR spectroscopy was measured at 25°C instead of 32°C, due to technical constraints, using a PerkinElmer Spectrum One FT-IR spectrometer equipped with Universal ATR Accessory (ZrSe crystal) and deuterated triglycine sulphate (DTGS) detector. Untreated and water-extracted SC, both in dry and hydrated condition (using D_2O), were characterized by recording 120 scans with a spectral resolution of 4 cm^{-1}. For the hydrated samples, SC was placed on top of a piece of paper soaked in D_2O and let to absorb for 24 h at 32°C prior to the measurement.

2.6. X-ray diffraction

SAXD and WAXD patterns of untreated and treated SC were measured using an in-house X-ray setup, GANESHA 300 XL SAXD system (JJ-Xray, Denmark). Approximately 5 mg of sample, which had been hydrated at 97% RH, was sealed between Kapton films in a screw-tight sandwich cell to avoid dehydration. The samples

were then mounted in a sample holder and diffraction data was recorded between 30 and 120 min at 32°C. Diffraction intensities (I) were measured as a function of diffraction vector q ($Å^{-1}$) defined as:

$$q = \frac{(4\pi \sin\theta)}{\lambda} \quad (1)$$

where θ is the diffraction angle and λ is the wavelength of the incident beam, which for this setup was 1.54 Å. Based on the selected q-range, the distance between the sample and collector was adjusted and the d-spacing was calculated from the different positions of q with:

$$d = \frac{2\pi}{q} \quad (2)$$

2.7. Water sorption measurements

The water uptake at varying relative humidities (RH) was measured at 32°C for untreated SC, water-extracted SC and water-extracted SC with addition of 20 wt% urea (based on the dry weight of SC) using a DVS sorption microbalance. To ensure that the urea mixed properly with the SC, urea was dissolved in water and added to the water-extracted SC to give a final concentration of 20 wt% urea with respect to the dry weight of SC. The sample was then placed in a desiccator at 75% RH to avoid precipitation of the urea.

The DVS sorption microbalance measurements were performed by placing the sample on a pan attached to a scale and subject the sample to a stream of N_2 to control the RH. The samples uptake of H_2O was measured from 75% to 97% RH by weighing until equilibrium was reached in each RH step. The final equilibrium is defined by the condition where the rate of change in mass is less than 10^{-4}%/min. After finishing the highest RH step, the sample was dried out at close to 0% RH until equilibrium to record the dry weight. The sorption isotherm is plotted in terms of H_2O content (wt%) as a function of RH (%). The H_2O content (wt%) is calculated according to Eq. (3) where m_s is the total mass of the sample including water at a specific time point and $m_{s,dry}$ is the mass of the dry sample.

$$H_2O(\%) = \frac{(m_s - m_{s,dry})}{m_s} \quad (3)$$

3. Results

In this study, we explore how the removal of the complex mixture of small water-soluble NMF compounds influences the molecular properties of SC components, and whether the properties of the original system can be regained by the addition of one single NMF compound. The molecular changes are also correlated to changes in how much water that is taken up in SC at a given humidity condition, as determined from a constant relative humidity (RH) in the surrounding air. We use porcine SC, which has been found to resemble the human SC in many chemical and physical aspects [52]. The SC samples were placed in aqueous solutions for a short (10 min), intermediate (60 min) and long (24 h) period of time to extract water-soluble NMF components. In order to compare the water-extracted and the non-treated samples, we then place the pieces of SC in an atmosphere with controlled humidity (97% RH) and temperature (32°C) in order to equilibriate with respect to the water content, which gives a condition similar to when a formulation is applied on the skin. Next, we add back one single NMF component to the NMF-depleted SC and investigate whether the effects caused by removal of the complex NMF mixture can be reversed by adding back a single NMF component. The latter experiments were performed with urea, pyrrolidone carboxylic acid (PCA) and potassium lactate, at one concentration (20 wt% with respect to the dry weight of the sample). All samples were characterized in terms of molecular dynamics, structure and water uptake using several complementary techniques, including NMR, ATR-FTIR, SWAXD and water sorption measurements.

A schematic summary of the results from the NMR measurements in Figs. 4 and 5 is shown in Fig. 2 and this figure will be used as a guide for presentation of the NMR data in Section 3.2,3.3. The colors in the figures represents the molecular mobility where blue signifies rigid segments and red signifies mobile segments. Rigid segments exhibit slow rotational correlation time $\tau_c > 0.1$ ms and/or anisotropic reorientation with an order parameter $|S_{CH}| > 0.5$, whereas mobile segments are characterized by having $\tau_c < 10$ ns and isotropic reorientation $|S_{CH}| < 0.01$ according to the relations defined in Table 1 [16]. Fig. 2a shows how the mobility in SC at 97% RH is influenced when NMF is removed and again re-added as a single component or excess water corresponding to occluded conditions. A more detailed summary is shown in Fig. 2b, which illustrates how the mobility changes in the different components in SC, namely fatty acids (FA), ceramide (Cer), cholesterol (CHOL) and keratin filaments (Keratin), for the different treatments.

3.1. Chemical composition of the extracted mixtures

We first characterize what components that are removed from SC upon soaking in water. The composition of the aqueous solution obtained after the short, intermediate and long term water extraction of SC was analyzed using solution NMR spectroscopy. The benefit of this method is that the analysis is performed simultaneously on all extracted compounds without any additional need for prior separation of, for example, different amino acids, carbohydrates and lipids. ^1H-^{13}C HSQC and ^1H-^{13}C HMBC measurements give rise to a coupling pattern for carbons with covalently attached protons or protons on neighbouring carbons, respectively. This combination of measurements is therefore useful for the assignment of individual molecules in a mixed solution.

Fig. 3 shows the ^1H-^{13}C HSQC spectra recorded for the aqueous solutions collected from the water extraction of SC. A detailed description of the chemical composition of all solutions and how it varies with extraction time is presented in the SI. In summary, all filtrates contain large amounts of extracted molecules. The solutions obtained from the short (10 min) and intermediate (60 min) extractions showed very similar peak patterns, while the long (24 h) extraction time resulted in an increased complexity of extraction solution. The amino acids alanine, proline, leucine, glutamic acid, glycine, serine, arginine, citrulline, ornithine, histidine and threonine could be assigned in the spectra from all three solutions according to the work by Lewis et al. [53]. These amino acids have previously been assigned as part of the NMF in SC [54]. Moreover, PCA and lactate, which is present at high amount in NMF, were also detected and assigned [55]. Urea is not detected due to the lack of protons covalently bonded to a carbon and because of the <1 ms time-scale chemical exchange of hydrogen atoms between the amine groups and the water. In addition, the solution from the long extraction was shown to contain both peptide fragments and lipids, plausibly short-chain fatty acids.

3.2. Molecular dynamics in SC lipid and protein components after water extraction

With the knowledge of what is removed during the soaking of SC in water, the molecular dynamics of the different components in untreated and water-extracted SC were investigated. Polarization transfer solid-state NMR (PT ssNMR) on natural abundance

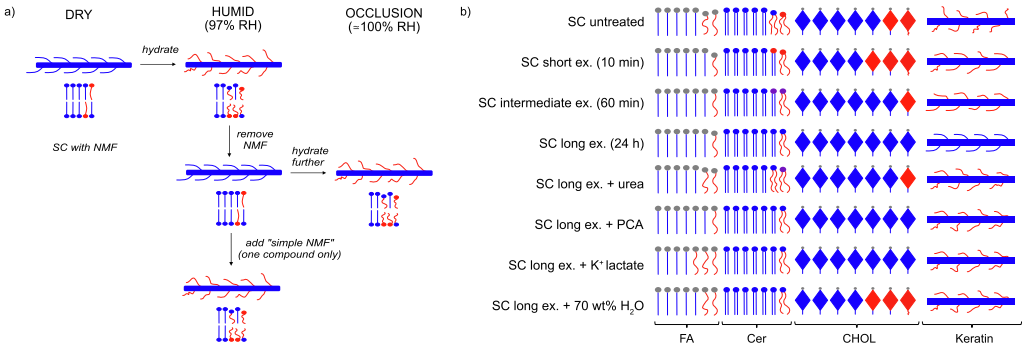

Fig. 2. a) Schematic summary of how the molecular mobility in SC changes from dry to humid condition (97% RH) and upon removal of NMF. When the NMF is removed from SC the mobility resembles that in dry condition but can be regained with the addition of a simple NMF compound at 97% RH or through only occlusion (≃100%) with water. b) Detailed summary of the molecular mobility in the different components in untreated SC, short water-extracted (10 min), intermediate water-extracted (60 min) or long water-extracted (24 h) SC. The untreated and water-extracted SC were dried and subsequently conditioned at 97% RH prior to the mobility measurement using NMR spectroscopy. The colors in the FA (fatty acids), Cer (ceramides), CHOL (cholesterol) and keratin filaments (Keratin) indicates the molecular mobility according to the quantitative definitions in Table 1 where blue and red signifies rigid and mobile, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Theoretical CP and INEPT signal intensity relationships for a CH_2 segment in dynamic regimes with different correlation times (τ_c) and order parameter (S_{CH}) at 11.72 T magnetic field and 5 kHz MAS [16]. The theoretical values are calculated with input parameters equal to the experimental settings

Dynamic regime	τ_c	$\lvert S_{CH}\rvert$	Signal intensity
Fast	< 10 ns	< 0.01	INEPT $\gg$CP = 0
		≈ 0.1	INEPT = CP
		> 0.5	CP $\gg$INEPT = 0
Fast-intermediate	≈ 0.1 μs		CP $\gg$INEPT = 0
Intermediate	≈ 1 μs		CP = INEPT = 0
Slow	> 0.1 ms		CP $\gg$INEPT = 0

^{13}C provides information close to atomic resolution about the molecular dynamics in the lipid and protein components in SC. In these experiments, we can discriminate between mobile and rigid segments within the very same molecule and also compare how the mobility varies for different components of the material. Although the NMR method is not truly quantitative, we can draw conclusions from the relative intensities of the peaks in the spectra and systematically follow how peaks originating from the same molecule, i.e. keratin, cholesterol, ceramide headgroups or lipid chains, increase or decrease upon the water extraction or the addition of single NMF compounds. A detailed description of the method and its application to SC is given in [15,56]. In brief, the method consists of three combined experiments; DP, CP and INEPT. The DP spectrum provides signal from all ^{13}C segments present in

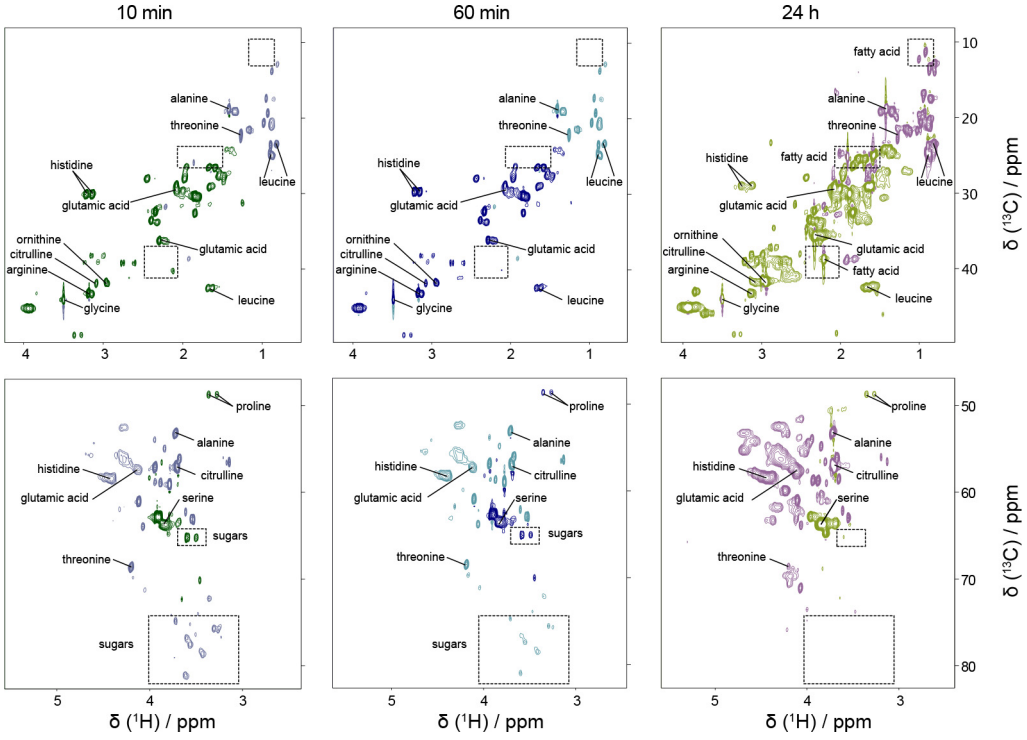

Fig. 3. ^{1}H-^{13}C HSQC of extracted compounds from SC during 10 min, 60 min and 24 h. Significant differences between the samples are highlighted with dashed lines. The experimental setup provides different colors for CH_3/CH (gray, turquoise and purple) and CH_2 (dark green, dark blue and light green) groups. All measurements were performed in D_2O at 25 °C. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the sample and serve as a reference. The CP scheme is used to boost the signal for rigid segments [46], whereas the INEPT spectrum shows signal for mobile segments [57]. The magnitude of the CP and INEPT signals can vary depending on the rotational correlation time (τ_c) and C-H bond order parameter ($|S_{CH}|$), which quantify the rate and anisotropy of C-H bond reorientation (Table 1). When comparing the signals from CP and INEPT with respect to DP, atomically resolved information regarding dynamics of different molecular segments can be obtained [56]. For simplicity, we will use the terms 'rigid' when only CP signal is detected and 'mobile' when INEPT signal also is observed.

Fig. 4 shows the spectra for untreated SC and water-extracted SC during a short, intermediate or long period of time. After the extraction, the samples were dried and conditioned at 97% RH, and thereafter compared to the untreated SC. In general, all spectra are dominated by the CP signal (blue), for most of the spectral range. This implies that the majority of the SC lipid and protein components are rigid under all conditions investigated. We also note the large and broad peak around 32 ppm corresponding to an all-trans (AT) conformation of the lipids chains. The untreated SC (Fig. 4a), used as reference in the evaluation of the extracted samples, shows INEPT signal (red spectrum) in both lipid and protein components, which indicates mobility in these segments. A detailed inspection of the spectra shows mobility in the lipid acyl chain (20–35 ppm) as well as in cholesterol (42 and 51 ppm (C4 and C9)) and the ceramide headgroup (56 ppm (C1)). In addition, mobility is detected in the amino acids Ser and Gly (44 ppm (Gly C_α), 57 ppm (Ser C_α) and 62 ppm (Ser C_β)), which are enriched in the terminal segments of the keratin filaments [6]. The core of the keratin filaments is enriched in leucine and lysine (Leu/Lys) and can be observed around 40 ppm together with an overlapping peak originating from cholesterol (C12/C14).

Extraction of water-soluble components from SC is shown to have a strong effect on the mobility of both SC lipid and protein components. The precise effects further depend on how long SC was soaked in water (Fig. 4b-d), which can be related to the extraction of different molecular mixtures as shown in the previous section. The mobility in the keratin amino acids was shown to decrease after the water extraction, and the strongest reduction is observed for the long extraction time. After the intermediate extraction time, only a small INEPT peak from the Ser C_β could be detected (Fig. 4c), while no amino acid mobility was detected after the long extraction as inferred from the lack of INEPT signal at the relevant chemical shifts. In addition, the overall CP intensity in the spectral regime 50–65 ppm, corresponding to the keratin amino acids, was shown to decrease as compared to the untreated SC. This decrease in signal intensity in the CP spectrum for the keratin amino acids regime may be explained by that for the extracted sample, the large amount of NMF is removed which contains a significant amount of free amino acids that appears in the same regime of the spectra and therefore contributes to the signal intensity when present. In addition, the filtrate collected after the long water extraction showed presence of peptide fragments which, when present in the untreated SC, also contributes to the intensity of the CP spectrum. Alternatively the lipids become more rigid after the water extraction which gives an increase in intensity for the AT(CH_2) peak and, hence, a decrease in the keratin region since the spectra are normalized against the AT(CH_2) peak.

Next we look at the effect on the SC lipids when removing the water-soluble compounds. Fig. 4 shows that the mobility in the lipid acyl chains as well as the cholesterol decreased after the water extraction, and that no INEPT signal from cholesterol segments could be detected after the long extraction in water. Even though the main trend is that the molecular mobility of most SC components is reduced due to the extractions, there are some exceptions to this trend. We here note that the INEPT peaks at 57.4 and 61.3 ppm are not present in the untreated SC but only appear after the short and intermediate times of water extraction (10 min and 60 min, Fig. 4b-c). These peaks were assigned to cholesterol (C14, C17) and the ceramide headgroup (C2), respectively and indicates increased mobility in these segments.

3.3. Addition of single NMF components to water-extracted SC

In the next step, we investigate whether the effects observed upon water extraction may be reversed by adding back one single NMF component. Experiments were performed for three different small polar molecules, urea, PCA or potassium lactate, which are all naturally found as part of the NMF [58], but also common components in medical and cosmetic skin care products [59,60]. Urea and PCA have been reported to be present at concentrations of 6 and 12%, respectively, of the total NMF, while the concentration of lactate with different counterions has been measured to 12% [24]. Experiments were here performed on SC samples that were first soaked in water for 24 h (Fig. 4d) and then either urea, lactate or PCA was added at a concentration of 20 wt% (relative to the dry weight of the sample). The concentrations of single added NMF compounds were chosen to enable direct comparisons with previ-

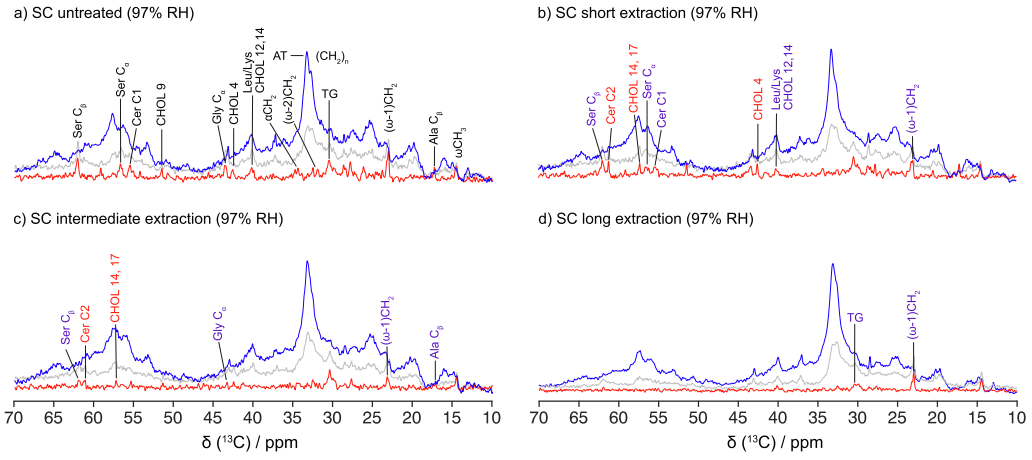

Fig. 4. ^{13}C MAS NMR spectra (DP; grey, CP; blue and INEPT; red) of a) SC untreated, b) SC short extraction (10 min), c) SC intermediate extraction (60 min) and d) SC long extraction (24 h). After the extraction, all materials were dried before subsequent hydration at 97% RH. All measurements were performed at 32°C. The red and purple labelled peaks indicates the increase or decrease, respectively, in mobility as compared to the untreated SC. The spectra are normalized with respect to the highest intensity in the CP spectrum. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

ous published studies [30,28,61]. This concentration is also relevant for typical NMF compounds (humectants) in skin formulations [60,62]. The samples were then conditioned at 97% RH for 24 h and again studied by means of NMR (Fig. 5). These data are compared to the reference sample of untreated SC at the same RH, and to the long water-extracted sample in Fig. 4a and d, respectively. The overall result from these experiments is that the molecular mobility in both lipids and amino acids is more or less regained upon the addition of a single NMF compound. It is here notable that the addition of a polar molecule actually leads to increased mobility in the hydrophobic lipids. This effect on lipids is stronger for urea and potassium lactate than for PCA. Furthermore, the addition of any of these single polar NMF components, which are not amino acids, leads to an increased INEPT signal for all the signature amino acids of the keratin filaments (Leu/Lys, Gly C_α and Ser C_α/C_β). Urea, in particular, has a strong effect on increasing the molecular mobility in both cholesterol (C9) and the lipid acyl chains (ωCH_3, $(\omega\text{-}1)CH_2$ and TG), and to some extent the carbons close to the lipid headgroup (αCH_2). For PCA, on the other hand, we were not able to resolve any profound effect on lipids, but cannot exclude that there is some effect since there are partial overlapping peaks between PCA and the lipid chains and small changes can therefore not be resolved. The addition of potassium lactate showed an effect similar to urea, but with even more pronounced increase in the mobility of the TG segment of the lipids.

The effects on SC by adding single NMF compounds were finally compared to the situation where we add an excess of water instead of adding a polar solute. More specifically, water was added at a concentration of 70 wt%, corresponding to close to full hydration conditions in SC, as inferred from extrapolation of the sorption isotherm for untreated SC towards the limit of 100% RH (Fig. 7). As shown in Fig. 5d, the molecular mobility was again regained in the SC that had been previously soaked in water, and the resulting spectra show large similarities with those obtained for untreated SC at a lower water content (Fig. 4a. Interestingly, the addition of 70 wt% water also lead to increased mobility in some of the lipid components, such as C4, C12/14 and C15 in cholesterol, which was not observed upon the addition of the single NMF compounds.

The combined results shown in Figs. 4 and 5, and the knowledge that a significant amount of compounds, including amino acids, are removed from SC when soaked in water (Fig. 3), leads to the conclusion that the mobile amino acids detected in the untreated SC (Fig. 4a) mainly originate from amino acids that are part of the keratin filaments and not free amino acids. This follows from the fact that the free amino acids are actually removed in the extraction step and therefore not present in the sample with regained mobility (Fig. 5a-c). This conclusion was further strengthened by the results on isolated corneocytes, which prior to the long water extraction were isolated from SC by removing the lipids through extraction with chloroform/methanol mixtures. Fig. 6a-b shows the NMR spectra for corneocytes hydrated at 97% RH before and after the long water extraction. Again, the water extraction resulted in a complete loss of mobility in the amino acids Gly $C\alpha$ and Ser C_α/C_β in the corneocytes (Fig. 6b). However, the addition of urea again lead to a regain in the mobility of the amino acids Val C_β, Leu/Lys, Gly C_β and Ser C_α/C_β in the water-extracted corneocytes (Fig. 6c). Notably, the INEPT peaks for the corneocytes with addition of urea were much higher in intensity compared to the corneocytes that had not be subjected to extraction in water.

Structural information regarding the ordered components in SC and how they are affected by the water extraction can be investigated using ATR-FTIR spectroscopy and small and wide-angle X-ray diffraction (SWAXD). The combination of these methods was used in the present study but only minor changes could be observed for the water-extracted SC in comparison to the untreated SC. A detailed description of the results and conclusions from the measurements is presented in the SI.

3.4. Water uptake in SC

Changes in the molecular properties of SC components as well as changes in NMF content may influence the ability for SC sample to take up water at varying humidity conditions [61,63,31,64]. Therefore, we further examine whether the ability of SC to swell in water correlates with the changes in the SC molecular dynamics. The water uptake was measured using a sorption microbalance, which monitors the weight increase of the sample during ramped RH at a constant temperature of 32°C. Water sorption isotherms were recorded for the water-extracted SC samples (short, intermediate and long extraction) and compared to the untreated SC reference sample that still contains its original mixture of NMF components. In addition, the water sorption isotherm was also measured for the intermediate water-extracted SC sample supplemented with 20 wt% urea. Fig. 7a shows the sorption isotherms plotted as amount of absorbed water (wt%) as a function of RH (%). Regardless of the extraction time, the water uptake is clearly lower in the water-extracted SC samples in comparison to the

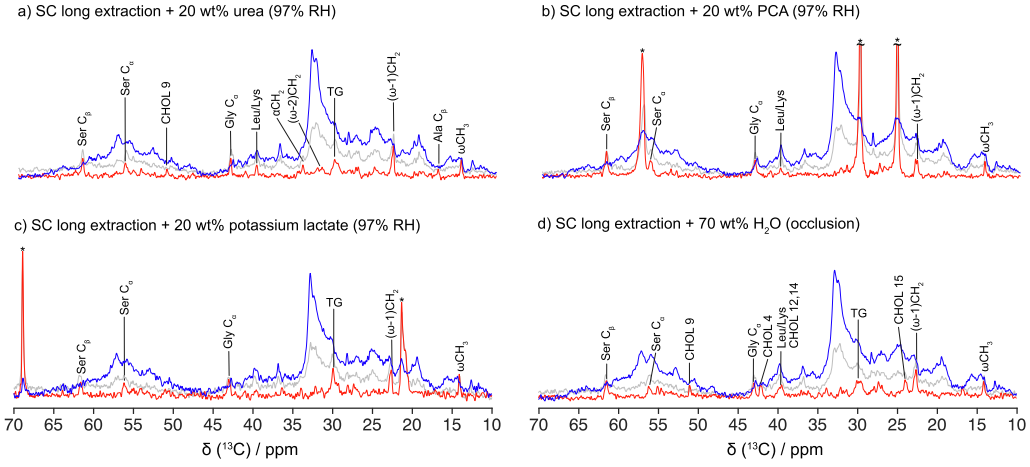

Fig. 5. ^{13}C MAS NMR spectra (DP; grey, CP; blue and INEPT; red) of SC long extraction (24 h) with addition of a) 20 wt% urea, b) 20 wt% PCA, c) 20 wt% potassium lactate and d) 70 wt% H_2O. After the extraction, sample a)-c) were dried before subsequent addition of chemical and hydration at 97% RH. Sample d) was dried before subsequent addition of water and sealing, which represents an occlusive state. All measurements were performed at 32 °C. Peaks marked with ∗ originate from the chemicals. The spectra are normalized with respect to the highest intensity in the CP spectrum.

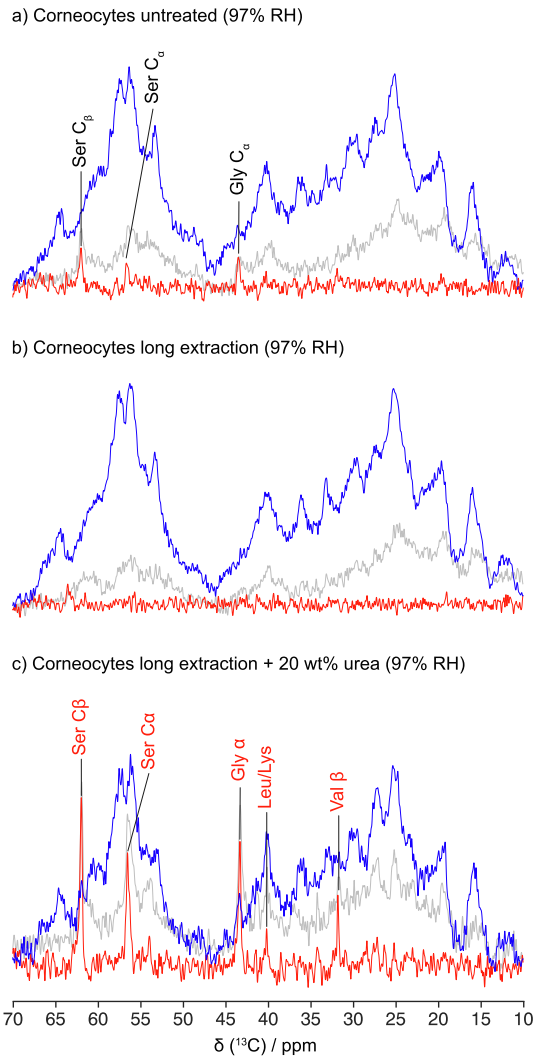

Fig. 6. ^{13}C MAS NMR spectra (DP; grey, CP; blue and INEPT; red) of a) corneocytes, b) corneocytes long extraction (24 h) and c) cornecytes long extraction (24 h) with addition of 20 wt% urea. After the extraction, all materials were dried before subsequent addition of chemical and hydration at 97% RH. All measurements were performed at 32°C. The red labelled peaks indicate the increase in mobility in comparison to the untreated corneocytes. The spectra are normalized with respect to the highest intensity in the CP spectrum. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

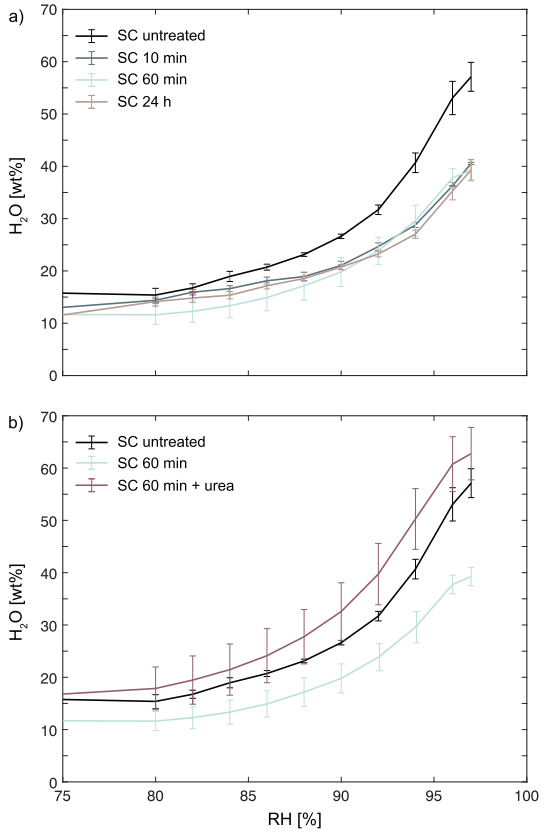

Fig. 7. Sorption measurements at 32°C for SC untreated, SC water-extracted for 10 min, SC water-extracted for 60 min, SC water-extracted for 24 h and SC water-extracted for 60 min + addition of urea (20 wt% based on the dry weight of SC extracted 60 min). The amount of absorbed H_2O (wt%) is based on the dry weight of the sample and expressed as a function of RH starting from 75%.

untreated SC. From this we can conclude that the water-extracted SC samples are less capable to swell in water as compared to the untreated SC reference sample. Interestingly, upon addition of urea the uptake of water was again increased to levels that are even higher than the untreated SC reference sample that contains its original NMF mixture.

4. Discussion

4.1. SC lipids and proteins are fluidized by osmolytes

In the present paper it is demonstrated that the properties of SC is strongly affected by osmolytes, either as the complex NMF mixture or as a single NMF component (Fig. 2). Here, the reference SC sample contains a mixture of small polar NMF molecules (Fig. 3), and the addition of water to this sample leads to induced mobility in both lipid and protein components (Fig. 4a). For the NMF-depleted SC, on the other hand, the molecular mobility is strongly reduced also at high humidities (97% RH), and the NMR spectra for this sample indeed resembles that of the completely dry SC (Figure S4 in SI). The reintroduction of a single polar compound (urea, PCA or potassium lactate) together with water (97% RH) to the NMF-depleted SC leads to regained molecular mobility in both the protein and lipid components. Similar effects is seen for close to fully hydrated SC (70 wt%, corresponding to close to 100 % RH). These data together infer that the response in SC molecular dynamics in relation to changes in hydration does not rely on the presence of a specific NMF composition, but indicates a more general mechanism of interactions.

The changes in SC molecular mobility when removing or adding NMF compounds correlate with the water uptake in SC at a given RH (Fig. 7), where the water uptake is higher in the more mobile sample. This is also in line with previous studies of water uptake in SC treated with osmolytes [65,19,66]. Similar relations have also been observed for hairless mice showing that a decrease of free amino acids in SC correlates with a decrease in skin surface conductance, as measured by a skin surface hydrometer, and a decrease in uptake of water in SC [63]. The increased fluidity in SC molecular components for the samples that contain NMF compounds will most likely aid the overall water uptake since fluid self-assembled systems can take up more water than the corresponding solid system. Such trends have also been demonstrated for e.g. lipid bilayer lamellar systems [67]. In addition, the polar compounds are generally hygroscopic and may therefore lead to uptake of water to some extent, depending on the humidity [68–70].

Previous studies of how osmolytes, such as urea and glycerol, influence lamellar phospholipid membranes in dry conditions have shown that these polar compounds with low vapor pressure can act to stabilize fluid lipid membranes at reduced water activities where solid membranes form in the absence of these compounds [71,72]. In this way, the osmolytes can in an unspecific way substitute for water [73]. In SC, the same osmolytes were shown to induce molecular mobility in both keratin and lipid components at a reduced level of hydration in a similar manner seen at a high level of hydration without any added compound [28], thereby replacing water in a close to ideal manner. It is here notable that the water content at a given (reduced) relative humidity was not significantly different from that in neat SC at the same humidity without added osmolyte [28,65]. The common terminology of "moisturizers" is thus a bit misleading, and the main function of these compounds in SC is likely not to increase the water content, but rather to replace water in dehydrated conditions and thereby retain fluidity in SC lipid and protein components. The present findings supports this proposed mechanisms in that the addition of polar compounds (water or NMF compounds) causes increased mobility in SC, and that the total amount of polar compounds (water + osmolyte) present in SC is more important for the overall behavior than the chemical identity of the added compounds.

Still, the precise response in SC molecular dynamics to the addition of water and NMF compounds will naturally depend on how the added compound distributes between the different regions in SC, and in particular on how it distributes between the extracellular lipids and the corneocytes. In our experiment with water-extracted corneocytes and addition of urea (Fig. 6), the effect on amino acid molecular mobility was shown to be greater in comparison to the water-extracted SC with addition of urea. This can be explained by that in the intact SC, urea is present in both lipid and corneocyte regions and likely distributes evenly in SC. However, other NMF compounds may not distribute in the same manner and may preferentially partition into one region on expens of the other. This could be the reason why the addition of potassium lactate shows stronger effect on the lipid acyl chains (TG) compared to urea and water (Fig. 5), which also is in line with the discussions in previous publications by others [31,64].

4.2. Molecular consequences from removal of polar and non-polar components in SC

The combined experimental data provide an overall picture of the response in SC lipid and protein components together with details at a molecular level. It is clear that the removal and addition of NMF compounds (NMF mixture or single NMF compounds) affect the molecular mobility in a small fraction of the lipid and protein components as inferred from the NMR experiments. On the other hand, there are hardly any detectable changes in the properties in the more ordered (solid) parts of the SC sample in the hydrated conditions, as inferred from the ATR-FTIR and SWAXD experiments. Together this illustrates the complementary use of the techniques, and leads to the overall conclusion that the majority of the SC remains in rigid and ordered structures for all conditions investigated, while the size of the small fraction of fluid regions are sensitive to the presence of NMF compounds and hydration. This also becomes evident when comparing our study with the one performed by Jokura et el. [29] who based a similar study on solely CPMAS NMR experiments (corresponding to the blue curves in Figs. 4–6 of the present study). Jokura et al. concluded that the mobility in the keratin filaments were lost upon removal of NMF but also that the mobility could not be regained by the addition of a small polar compound, which does not agree with our findings. The discrepancy between these studies is most likely due to the fact that the majority of the SC molecular components are solid at 32°C and the minor change in the fluid components induced by water or NMF compounds are difficult to detect in the CP spectra due to the relatively low sensitivity of the CP method to such changes.

It is notable that the removal of NMF components leads to increased mobility in both cholesterol and ceramide headgroups for the short and intermediate water extraction (10 and 60 min), where mainly small polar molecules are removed (Fig. 4b-c). However, the mobility in these segments completely disappear for the long water extraction (24 h). The analysis of the filtrates (Fig. 3) showed that the prolonged soaking in water also leads to extraction of certain lipids, presumably fatty acids. Important to have in mind regarding the long water extraction is the possibility of chemical degradation and, hence, the formation of degradation products, which is often the case in biological systems. It is here pointed out that NMF itself is the result of a degradation process and an induced degradation during the water extraction is therefore expected to just lead to an increased amount of NMF molecules. This is also consistent with the characterization of the different filtrates showing that the complexity of the filtrate did not increase with extraction time, but the concentration of the extracted compounds was however altered. The only main exception of this is the presence of fatty acids and small peptides in the filtrate from the long water extraction, albeit these are considered as degradation products. The fact that these larger molecules are only detected in the filtrate after the long extraction is plausibly due to that they have lower water solubility and slower diffusion as compared to the small water-soluble compounds.

4.3. Practical and biological implications

Exposure of the skin to a water-rich environment is a regular event in daily life, including events of washing, showering, bathing or usage of sanitary products like diapers. It is here shown that already after a relatively short period of soaking in water (here 10 min) there is a substantial removal of polar compounds from SC, which in turn clearly affects the molecular properties of SC components and the ability of SC to take up water. Alterations in SC molecular dynamics can in turn have direct impact on the SC mechanical and barrier function [74,75], which could be considered of either positive or negative nature depending on the conditions. More specifically, development of topical formulations could take advantage of an increased penetration rate, while in a diaper or other sanitary product one likely wants to avoid increased skin permeation. One practical implication of the present data concerns the design and interpretation of *in vitro experiments where pieces of SC are actually in direct contact with a solution or formulation, for example in diffusion cell studies. It is here again important to be aware of the fact that the extraction of the osmolytes from SC to a surrounding solution is relatively fast (minutes) and probably on the same time scale as the absorption of any active compound from the solution/formulation into the skin. Often such experiments are performed with a buffer solution, such as PBS, that may to certain extent counterbalance the osmotic imbalance between the solution and the SC sample. Still, extraction of NMF compounds due to concentration gradients will still occur, and indeed soaking of SC in PBS buffer for 24 h again leads to reduced mobility in SC lipids and proteins (Figure S5 in SI), similar to the sample that was extracted in water for the same time period (Fig. 4d).*

Several studies have shown a clear correlation between a decreased amount of NMF and severe skin dryness. Osmolytes like the ones present in NMF are also commonly found in skin care products. We have in this study shown that certain molecular properties in SC can be regained by replacing the complex NMF by a simplified single NMF component. However, this replacement is not necessarily enough for the reestablishment of all processes

and properties in the skin which are affected by removal of certain compounds. It is, for example, well known that both certain peptides and lipids have an important role of being antimicrobial and modulating inflammation in the epidermis [76] and their removal from SC during soaking in water could have a negative effect on the overall function of the skin. Questions regarding how the skin barrier properties and the desquamation process are affected by the removal of certain compounds are to be followed up based on the observations made in the present study.

5. Conclusions

In summary, this study shows that soaking of SC in water leads to removal of small polar compounds, i.e. the NMF, with the consequence of a decreased molecular mobility in both keratin filaments and lipid components in SC. However, through reintroduction of a single polar compound to the soaked SC the molecular mobility can be regained, both in the keratin filaments and lipid components. Interestingly, molecular mobility was also regained in the NMF-depleted SC through addition of a very high amount of water. Taken together, the here presented data suggests that the molecular mobility in SC is induced by a rather general mechanism that is mainly related to the amount of polar compounds present and less sensitive to the precise chemical identity of the polar compounds. In light of this, the terminology of a moisturizing agent in skin formulations appear misleading as it indicates that the added compound causes an increased uptake of water, while the mechanism rather seem to be that the added compound replaces the water [28]. With this insights one may deepen the understanding of the role of NMF in SC. In addition to this, practical issues regarding the experimental setups used in for example diffusion cell studies, where the skin is in full contact with a water solution for an extended period of time, have been raised.

CRediT authorship contribution statement

Maria Gunnarsson: Conceptualization, Investigation, Visualization, Writing - Original Draft, Writing - Review & Editing. **Enamul Haque Mojumdar:** Conceptualization, Writing - Review & Editing. **Daniel Topgaard:** Resources, Writing - Review & Editing, Supervision. **Emma Sparr:** Conceptualization, Writing - Original Draft, Writing - Review & Editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jcis.2021.07.012.

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