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Stratum corneum molecular mobility in the presence of natural moisturizers

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The outermost layer of skin, stratum corneum (SC), is a lipid-protein membrane that experiences considerable osmotic stress from dry and cold climate. The natural moisturizing factor (NMF) comprises small and polar substances, which like osmolytes can protect living systems from osmotic stress. NMF is commonly claimed to increase the water content in SC and thereby protect the skin from dryness. In this work we challenge this proposed mechanism, and explore the influence of NMF on the lipid and protein components in SC. We employ natural-abundance ^{13}C solid-state NMR methods to investigate how the SC molecular components are influenced by urea, glycerol, pyrrolidone carboxylic acid (PCA), and urocanic acid (UCA), all of which are naturally present in the SC as NMF. Experiments are performed with intact SC, isolated corneocytes and model lipids. The combination of NMR experiments provides molecularly resolved qualitative information on the dynamics of different SC lipid and protein components. We obtain completely novel molecular information on the interaction of these NMF compounds with the SC lipids and proteins. We show that urea and glycerol, which are also common ingredients in skin care products, increase the molecular mobility of both SC lipids and proteins at moderate relative humidity where the SC components are considerably more rigid in the absence of these compounds. This effect cannot be attributed to increased SC water content. PCA has no detectable effect on SC molecular mobility under the conditions investigated. It is finally shown that the more apolar compound, UCA, specifically influence the mobility of the SC lipid regions. The present results show that the NMF components act to retain the fluidity of the SC molecular components at dehydrating conditions in such a way that the SC properties remain largely unchanged as compared to more hydrated SC. These findings bring new molecular insight on how small polar molecules in NMF and skin care products act to protect human skin from drying.

A. Introduction

Most organisms in nature are in one way or another affected by osmotic stress. Marine life is exposed to high salinity in ocean water, while terrestrial life has to deal with desiccating conditions in the form of low relative humidity (RH) and cold climate. These situations may cause an imbalance between the internal and external osmotic pressure. Most organisms that experience osmotic stress have in common the presence of small water-soluble substances in relatively high concentrations, which in contrast to elevated concentrations of electrolytes do not lead to malfunctioning of the living system.[1] The functional role of these molecules is then to prevent osmotic stress by balancing the osmotic pressure of the external environment, and to preserve the conditions required for biological functions. This type of substances can be referred to as osmolytes and comprise molecules such as carbohydrates, polyols, amino acids, methylamines, and urea.[1] For humans, the outermost layer of skin (called the stratum corneum, SC) is particularly exposed to osmotic stress from dry and cold climate of the external environment. Therefore, it is perhaps not surprising that osmolytes are naturally present in SC. In this case, the osmolytes are referred to as the natural moisturizing factor (NMF) and comprise a mixture of free amino acids, amino acid derivatives (such as pyrrolidone carboxylic acid, PCA, and urocanic acid, UCA), lactic acid, urea and glycerol.[2-5] The manifestation of NMF components in SC is well characterized and their presence is considered crucial to maintain SC hydration in conditions of osmotic stress from desiccation.[2-5] In addition, defective skin conditions and certain skin diseases, for example, winter xerosis and atopic dermatitis, are associated with decreased NMF levels in SC.[6, 7]

The SC is organized in stacks of terminally differentiated cells (called corneocytes), which are flat, hexagonal-like shaped, and enclosed by the cornified cell envelope.[8] The cell envelope comprise a macromolecular assembly of cross-linked proteins and a covalently bound lipid layer facing the extracellular matrix.[9, 10] The intracellular space of the corneocytes is filled with keratin filaments together with other proteins, such as the

filament-associated filaggrin.[11] The extracellular material is comprised of lipids, which surrounds the corneocytes and thus form a continuous lipid matrix.[8] The main lipid species in SC are long-chained ceramides and free fatty acids, with high degree of saturation, and cholesterol, which together are arranged as multi-lamellar structures.[12, 13] The SC represents an excellent barrier for molecular diffusion, which is a prerequisite for water homeostasis, and it also provides protection from harmful substances entering the body.[14]

The water content in SC is determined by its water-holding capacity and by the water activity in the regions that it separates, which are the viable epidermis and the ambient air. The SC hydration is vital to maintain cohesion and flexibility of the outermost skin barrier.[15] It has been shown that the enzymatic degradation of the protein filaggrin into NMF components is dependent on SC hydration[2] and that there is an inverse correlation between the water gradient in SC and the gradient of several NMF components.[4] As previously suggested[2], it seems that SC has, to a certain degree, a self-adjusting capability to introduce natural moisturizers in the SC regions that are most exposed to osmotic stress. Supposedly, this occurs when the corneocytes migrate from the hydrated lower regions toward the dry outermost surface of SC where enzymatic driven production of NMF components is initiated.[2] The NMF components are derived from different sources, such as filaggrin-degradation into amino acids and their derivatives (e.g. UCA and PCA), sweat constituents (e.g. urea), the epidermis via circulation, or from triglyceride turnover in sebaceous glands (e.g. glycerol).[4, 11] Depending on the origin of the NMF components they may be present in different regions of SC, but it is most likely that the polar regions in SC have relatively higher concentrations of NMF as compared to the apolar regions.

In this paper we ask the question of how natural moisturizers protect SC against osmotic stress. We have previously shown that both urea and glycerol can stabilize fluid lipid bilayers at reduced water activities where solid bilayers form in the absence of these compounds.[16, 17] The implication of this is that small polar compounds strongly influence the properties of the apolar hydrocarbon regions in the lipid layer. We investigate whether the same mechanism applies also to NMF components in the SC, where these molecules may influence the properties of both the lipid and the protein components at reduced hydration. It is evident that alterations of SC mechanical and barrier properties, related to dry or hydrated skin, cannot only be understood in terms of changes of SC water content.[18-21] More important, the barrier properties depend on the organization and molecular dynamics of the non-aqueous SC lipid and protein components.[19-24] We hypothesize that naturally occurring osmolytes may influence the molecular dynamics of the SC constituents in a similar manner as water does.[21, 22] To investigate this, we employ natural-abundance ^{13}C solid-state NMR methods that provide molecular resolution in the chemical shift scale in combination with selective screening of mobile or rigid segments of the SC lipid and protein components.[21, 25] We study intact SC as well as isolated corneocytes and a model lipid bilayer system. The NMF components considered here are glycerol, urea, PCA, and UCA, which are all important components of the NMF. Glycerol and urea are also common substances in commercial skin care lotions and creams and have been demonstrated to have a beneficial effect on dry skin conditions.[26]

B. Materials and methods

Materials

Glycerol, urea, imidazole-4-acrylic acid (*trans*-urocanic acid, UCA), (-)-2-pyrrolidone-5-carboxylic acid (PCA), trypsin, chloroform, and methanol were obtained from Sigma-Aldrich. NaCl, $Na_2HPO_4•2H_2O$, KH_2PO_4, KNO_3, K_2SO_4, and KCl were purchased from Merck. 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC) was obtained from Avanti Polar Lipids Inc. Phosphate buffered saline (PBS) was prepared from Milli-Q water (130.9 mM NaCl, 5.1 mM $Na_2HPO_4•2H_2O$, 1.5 mM KH_2PO_4, pH 7.4).

Stratum corneum preparation

Pig ears were given to us from a local slaughterhouse (Dalsjöfors Slakteri, Sweden) and stored at -80°C until use. Skin strips were cut out from the inside of the outer ear using a dermatome (TCM 3000 BL, Nouvag). Dermatomed skin strips were placed on filter paper soaked in 0.2% trypsin in PBS solution (or Milli-Q water for skin used to prepare corneocyte samples) for 12 h at 4 °C. SC sheets were removed with forceps and washed thoroughly in PBS solution and dried in vacuum. Dry SC was ground to a fine powder using a pestle and mortar and then stored in a freezer until use. In a previous study on SC, employing the same natural-abundance ^{13}C solid-state NMR methods as here, we concluded that a comparison of NMR spectra from SC in the powder form with the corresponding spectra from intact SC sheets showed no differences.[21] This implies that the pulverization of SC occurs on a macroscopic scale, which results in unaffected SC properties on the molecular scale.

Preparation of lipid extracted stratum corneum (corneocytes)

Dry SC sheets were prepared according the procedure described above, except that Milli-Q water was used as solvent instead of PBS solution. SC sheets were placed in 60 ml chloroform:methanol (2 h in each of the following compositions 2:1, 1:1, 1:2) and collected each time by filtration. This sequence was repeated one more time with 30 min as extraction time. Next, an extraction in methanol was performed overnight. Finally the isolated corneocyte material was rinsed in Milli-Q water and dried, before pulverized and stored in a freezer until use.

Sample preparation

Approx. 30 mg of dry SC or corneocyte powder was mixed with urea, glycerol, PCA, or UCA in known proportions (5 or 20wt% based on SC dry weight). To assure proper mixing, the NMF was solubilized in a small amount of water that was added to the SC or corneocyte sample, followed by rigorous mixing. Excess water was then allowed to evaporate in a desiccator under vacuum before the sample was placed in a chamber of controlled RH at 32 °C. Aqueous solutions with dissolved NaCl at 5.12 and 1.19 mol kg^{-1} were used to regulate the RH at 80% and 96% RH, respectively.[27] The samples were weighed before (dry samples) and after 48 h inside the RH chamber. When handling biological material, like the SC, we need to optimize the sample preparation to reach equilibrium conditions in reasonably short time, which is

Table 1 Theoretical PT ssNMR signal intensity relations for a CH_2 segment in dynamic regimes with different correlation times (τ_c) and order parameters (S_{CH}) at 11.72 T magnetic field and 5 kHz MAS.[25] The theoretical values are calculated with input parameters equal to the experimental settings.

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

why we used pulverized SC. We confirmed that the water uptake reached a stable value after 48 hours, and no signs of degradation or bacterial growth during this time period were observed, as previously commented.[21] Throughout the article, we describe the samples with respect to the RH at which it was equilibrated. The measured water content in each sample is given in Table 2 and defined as m_w/m_{tot}, where m_w is the amount of water and m_{tot} is the total mass of the hydrated SC (or corneocyte) sample (including the NMF component). We also give the content of water and the NMF, which is defined as $(m_w+m_{NMF})/m_{tot}$. The DMPC samples were prepared according to the procedure previously described[17] and equilibrated at 27 °C in closed chambers with relative humidity of 84% and 96% RH, controlled with saturated solutions of KCl and K_2SO_4, respectively.

Solid-state NMR – Experimental settings

For NMR measurements, the samples were packed in watertight inserts (15 µl) and placed in 4 mm rotors (Bruker). All measurements were carried out on a Bruker Avance-II 500 spectrometer, equipped with a 4 mm cross polarization (CP)/magic-angle spinning (MAS) double resonance probe at a magnetic field of 11.74 T with ^{13}C and ^{1}H resonance frequencies of 125 and 500 MHz, respectively. The ^{13}C spectra were acquired under MAS frequency of 5 kHz and 68 kHz TPPM ^{1}H decoupling.[28] 2048 scans were obtained for SC and corneocyte samples, while 64 scans were collected for DMPC samples, with 5 s recycle delay in both cases, resulting in an experimental time of around 170 min and 5 min, respectively. Solid α-glycine (43.7 ppm) was used as external reference.[29] The acquisition time was 50 ms and the ^{1}H and ^{13}C hard pulses were applied at $\omega_1^{H/C}/2\pi = 80$ kHz. For CP experiments, the contact time (τ_{CP}) was 1 ms, using $\omega_1^{C}/2\pi = 80$ kHz and $\omega_1^{H}/2\pi$ linearly ramped from 72 to 88 kHz. In INEPT, the delay time τ was 1.8 ms, while τ' was 1.2 ms. Time-domain data were processed with line broadening of 30 Hz for SC and corneocyte samples and 10 Hz for DMPC samples. Zero-filling from 1597 to 8192 time-domain points was used. Fourier transform, automatic phase correction,[30] and baseline correction was performed in Matlab using in-house code partially derived from matNMR.[31] The temperature was controlled at 32 °C (SC and corneocytes) or 27 °C (DMPC) with a BVT-2000 controller and a BCU-05 air cooler.

Using PT ssNMR for studying molecular dynamics

In the present study we investigate the effect of glycerol, urea, PCA, and UCA on the molecular dynamics of SC components using PT ssNMR (polarization transfer solid-state NMR).[25, 32] PT ssNMR combines three different pulse sequences: DP (direct polarization), ramped CP[33] (cross polarization), and refocused INEPT[34] (insensitive nuclei enhanced by polarization transfer). The DP spectra show resonances from all ^{13}C present in the sample, while the CP and the INEPT spectra display either rigid ^{13}C or mobile ^{13}C, respectively. PT ssNMR provides segmental resolution of ^{13}C in different molecular segments in the chemical shift scale and qualitative information on the molecular dynamics of each resolved ^{13}C segment, as previously shown for a number of different colloidal systems with low water contents.[17, 32, 35] In CP, polarization transfer from ^{1}H to ^{13}C occurs via (through space) dipolar couplings, which are effective polarization transfer facilitators for segments with anisotropic ^{1}H-^{13}C bond vector reorientation and/or segments with slow motion. In contrast to CP, the INEPT sequence transfers polarization from ^{1}H to ^{13}C via (through bond) *J*-couplings, which are effective polarization transfer mediators for segments undergoing fast and isotropic reorientation. The intensity of the DP, CP, and INEPT signals is dependent on the rate and anisotropy of the ^{1}H-^{13}C bond reorientation. The ^{1}H-^{13}C bond dynamics can be quantified by the rotational correlation time τ_c and order parameter S_{CH}, which describes the motional rate and reorientation anisotropy, respectively.[25] The relative intensities of the DP, CP, and INEPT signals in different dynamic regimes is summarized in Table 1.[25]

Isothermal sorption microcalorimetry

A double twin isothermal microcalorimeter was used to study the water vapor sorption of the pure substances. A detailed description of the instrument is presented elsewhere.[36] In brief, the instrument involves a two-chamber calorimetric cell, with the sample chamber on the top and water chamber on the bottom, which are connected by a tube (9 mm in diameter) to allow for water diffusion between the chambers. The cell is kept at isothermal conditions, together with the reference cell. From this method one can simultaneously monitor the water activity (a_w) and the partial molar enthalpy of mixing of water (H_w). The experiments were performed at 32 °C.

C. Results

The goal of this study is to characterize how naturally occurring moisturizers influence the molecular dynamics of protein and lipid components in SC. We explore the following four substances that are naturally present in SC as NMF: glycerol, urea, PCA, or UCA. The compounds were added and mixed with intact SC, and then the samples were equilibrated at 32 °C and 80% RH, and finally characterized by ^{13}C PT ssNMR. The compositions of these samples, expressed as wt%, are summarized in Table 2. To enable deeper molecular insight, we complemented the studies on SC by investigating also isolated corneocytes and model lipids at similar conditions. We note that the corneocyte samples contain a minor fraction of lipids that are covalently bound to the cornified cell envelope.[10] However, from

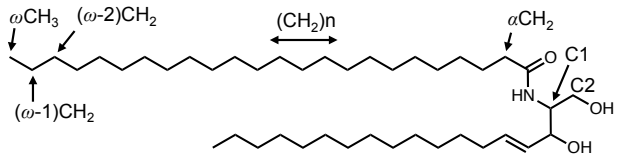

Fig. 1 Labels of relevant lipid carbon resonances (here illustrated with Cer NS).

here on we simply refer to those samples as "corneocytes". The model lipid is DMPC, which is not a typical skin lipid. This lipid system is chosen because it undergoes a phase transition between solid and liquid crystalline lamellar phases upon hydration,[37, 38] which is similar to the observed changes in phase properties for SC lipids at increasing hydration.[21]

From the PT ssNMR measurements we obtain information on the mobility in different molecular segments in SC, corneocytes or model lipids by comparing the intensities of the INEPT, CP, and DP signals. We have recently employed PT ssNMR on SC and given a detailed peak assignment of most ^{13}C resonances, which then allowed us to explore the effects of hydration and temperature on the molecular dynamics of different SC lipid and protein components.[21] For this study we will, in particular, compare the signal intensities from representative molecular segments from either proteins or lipids, all of which resonate at different chemical shifts. For the protein components (mainly keratin filaments) we foremost refer to the peaks located around 44, 57, and 62 ppm from Gly C_α, Ser C_α, and Ser C_β, respectively, see Fig 2 for example. The resonances around 15, 23, and 30 ppm from ωCH_3, $(\omega\text{-}1)CH_2$, and $(CH_2)_n$, respectively, are used as markers for lipids in the multilamellar matrix, see Fig. 2 for example.[21] The molecular segments of the most relevant lipid carbon resonances from ceramides (or fatty acids) are shown in Fig. 1, while the standard numbering of the cholesterol carbons is given in Fig. S1 in the Electronic Supplementary Material. We focus on the spectral range (5-75 ppm) where all these resonances are located. We do not discuss the carbonyl region due to the fact that the INEPT polarization transfer requires protonated carbons, which, in practice is also true for CP under the present experimental settings. Also the alkene region is left out because the olefin resonances in SC give relatively low signal intensity.[21] The expected ^{13}C resonances from the carbons in the different NMF compounds are given in Table S1 and also marked with asterisks in all figures.

Sorption data for the pure compounds are shown in Fig. S2. The substances studied here are all small molecules with low vapor pressure. Glycerol is a hygroscopic liquid and its sorption isotherm shows continuous water uptake upon increasing RH (Fig. S2). The other compounds are solid in the pure form at 32 °C. The RH (directly related to the vapor pressure or the water activity), at this temperature, is around 72% and 92% for urea and PCA, respectively. The sorption isotherm for UCA is associated with non-equilibrium effects at high RHs (above 90% RH), which can be related to amorphous recrystallization.[39] UCA also differs from the other compounds in that it is less polar and readily partitions into lipid bilayers.[40]

Table 2 Water content (defined as m_w/m_{tot}) of SC and corneocyte samples and the total content of water and NMF (defined as $(m_w+m_{NMF})/m_{tot}$). All concentrations are given as wt%.

Sample	RH	Water	Water and NMF
SC*	80%	24	-
SC*	96%	50	-
SC 5wt% Glycerol	80%	25	29
SC 5wt% Urea	80%	21	25
SC 5wt% PCA	80%	18	22
SC 5wt% UCA	80%	12	16
SC 20wt% Glycerol	80%	23	38
SC 20wt% Urea	80%	26	41
SC 20wt% PCA	80%	14	28
SC 20wt% UCA	80%	23	36
Corneocytes*	80%	11	-
Corneocytes*	96%	64	-
Corneocytes 20wt% Glycerol	80%	31	43
Corneocytes 20wt% Urea	80%	59	66
Corneocytes 20wt% PCA	80%	13	28
Corneocytes 20wt% UCA	80%	39	50

*Sample contains no added NMF

Urea induces mobility in stratum corneum components in a similar manner as water

We first investigate how urea influences the molecular mobility in SC lipid and protein components at ambient conditions and we compare this to the effects of SC hydration. The results are shown in Fig. 2. Experiments were performed for SC and isolated corneocytes at 80% and 96% RH, and with varying amount of urea at 80% RH. A first observation is that different conditions clearly influences the signal from the CP, INEPT and DP sequences, thereby demonstrating changes in the dynamics of the molecular components in SC.

For the pure SC sample at 80% RH (Fig. 2) the CP signal from rigid molecular segments is dominating, with the $(CH_2)_n$ resonance at 33 ppm being the most prominent peak. The acyl chains in solid crystalline or gel phases have a higher fraction of all-trans (AT) conformers compared to the conformationally disordered chains of solid amorphous or liquid crystalline phases, which is expected to result in different chemical shifts.[41] This effect can be seen in the DP spectrum, which displays one peak centered around 33 ppm for rigid acyl chains with all-trans conformation and one peak centered around 31 ppm for more disordered acyl chains with a distribution of trans and gauche (TG) conformers. Further, it is seen that the peak around 33 ppm is selectively more enhanced in the CP spectrum, while the peak around 31 is more enhanced in the INEPT spectrum, showing that a small fraction of methylene carbons undergo fast trans-gauche isomerization. Additional signs of lipid mobility are the INEPT resonances from ωCH_3, $(\omega\text{-}1)CH_2$, $(\omega\text{-}2)CH_2$, and αCH_2 around 15, 23, 33, and 35 ppm, respectively,[21] see Fig. 1 for carbon labels.

The broad and dominating CP peaks centered around 57 ppm in the spectra obtained from both SC and corneocytes (cf. all spectra in Fig. 2 and 3) are mainly ascribed to C_α resonances of the peptide bond from all amino acid residues (except Gly C_α). The prominent CP enhancement in the C_α region, together with the total absence of INEPT peaks from proteins, show that the keratin filaments are completely rigid at 80% RH.[21] Glycine and serine are the most abundant residues of the keratin filaments, and also highly abundant in the cornified cell envelope.[9] In the

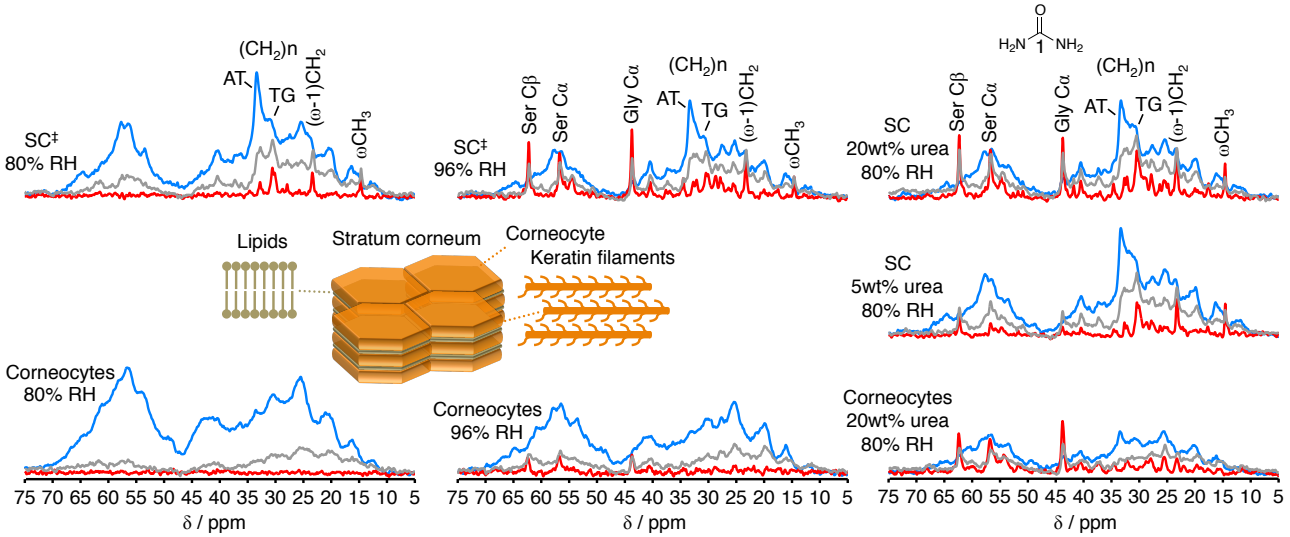

Fig. 2 PT ssNMR ^{13}C spectra (DP in grey, CP in blue, INEPT in red) from SC and corneocytes at 32 °C and 80% RH (left, no NMF), 96% RH (middle, no NMF), and at 80% with 5wt% and 20wt% urea (right). Signal intensity is given on the same scale for all samples. AT stands for all-trans and TG for trans/gauche. ‡Data from Björklund et al.[21]

keratin filaments, these residues are predominantly located in the flexible N- and C-terminal domains of the individual protein chains, making up the filaments, and it may therefore be expected that the dynamics of these residues are most prone to respond to changes in the water-rich regions in the heterogeneous SC.

Fig. 2 shows that the addition of urea to the SC at the same RH and temperature (80% RH, 32 °C) has a strong effect on the molecular dynamics, even though the water content of this sample is similar to the pure SC sample at 80% RH (Table 2). The addition of 20wt% urea (defined with respect to SC dry weight) results in a striking enhancement of the INEPT signal from Gly C_α, Ser C_α, and Ser C_β resonances, as compared to the spectra of pure SC at the same RH (Fig. 2). This is consistent with increased mobility of the more flexible N- and C-terminal domains of the individual protein chains in the keratin filaments. The Gly C_α, Ser C_α, and Ser C_β segments do not provide any CP resonances with similar line shape as the DP or INEPT peaks, implying that these molecular segments are in the fast dynamic regime with isotropic reorientations (cf. Table 1). It is still noted that the major fraction of the amino acids are rigid also in the presence of urea, as evident from the elevated CP signal in the spectral range around 57 ppm from the C_α resonances. Finally, the INEPT signal from the ωCH_3, $(\omega\text{-}1)CH_2$, and $(CH_2)_n$ peaks are also higher as compared to the pure SC at 80% RH, and this implies higher lipid mobility due to shorter τ_c and/or lower anisotropy of these segments. The same trends in protein and lipid dynamics are also detected at lower urea contents (5wt% with respect to SC dry weight), although the effects are less pronounced (Fig. 2). Again, there is a notable increase of the INEPT signal from Gly C_α, Ser C_α, and Ser C_β resonances and also a slight increase of the ωCH_3, $(\omega\text{-}1)CH_2$, and $(CH_2)_n$ INEPT peaks, as compared to the pure SC samples at the same RH. One important conclusion from the comparisons in Fig. 2 is that addition of urea has very similar influence on the mobility of the SC components as elevated water content at increased RH. In the spectra corresponding to SC at 96% RH (Fig. 2) a prominent enhancement of the INEPT signal from Gly C_α, Ser C_α, and Ser C_β resonances is observed, together with an increase of the ωCH_3, $(\omega\text{-}1)CH_2$, and $(CH_2)_n$ INEPT peaks. This demonstrates the hydration effect on the mobility of the SC components and the outcome is comparable to the spectra obtained for SC with 20wt% urea at 80% RH (Fig. 2).

The effect of hydration on SC lipids and proteins has previously been analyzed in detail for intact SC,[21] and here we also provide new data showing the influence of hydration on isolated corneocytes. The notable signal reduction from resonances assigned to lipids in the INEPT, DP, and CP spectra shows that the extraction procedure successfully removes the extracellular lipid lamellae and allows us to focus on the effects on the protein components. However, it should be noted that the covalently bound lipids in the corneocyte samples can be observed from PT ssNMR at elevated temperatures.[21] The spectra obtained for the corneocyte samples show that the addition of urea leads to increased INEPT signals for Gly C_α, Ser C_α, and Ser C_β resonances, implying that the keratin filament terminals are mobile. Still, the CP signal dominates most of the spectral range, suggesting that the main fraction of protein components is rigid. The hydration effect on the molecular mobility of the protein components of the corneocyte sample is similar at 96% RH as compared to the situation when urea was added to the corneocyte sample at 80% RH, but less pronounced as judged from the lower INEPT signal from the Gly C_α, Ser C_α, and Ser C_β resonances.

A comparison between different natural moisturizers

We continue to make a comparison between different compounds that are all relevant as NMF components, and we study their influence on the mobility of SC molecular components. Fig. 3 shows PT ssNMR data for intact SC and isolated corneocytes in the presence of glycerol, PCA or UCA (5wt% and 20wt%) at 80% RH and 32 °C. A first inspection reveals that all compounds, except PCA, strongly influence the molecular mobility of the SC components, although the molecular details vary between the compounds.

For SC or corneocytes with 20wt% glycerol at 80% RH (Fig. 3) the results are, indeed, similar to those obtained for pure SC or corneocytes at 96% RH (Fig. 2), and those obtained for pure SC or corneocytes at 80% RH in the presence of 20wt% urea (Fig 2).

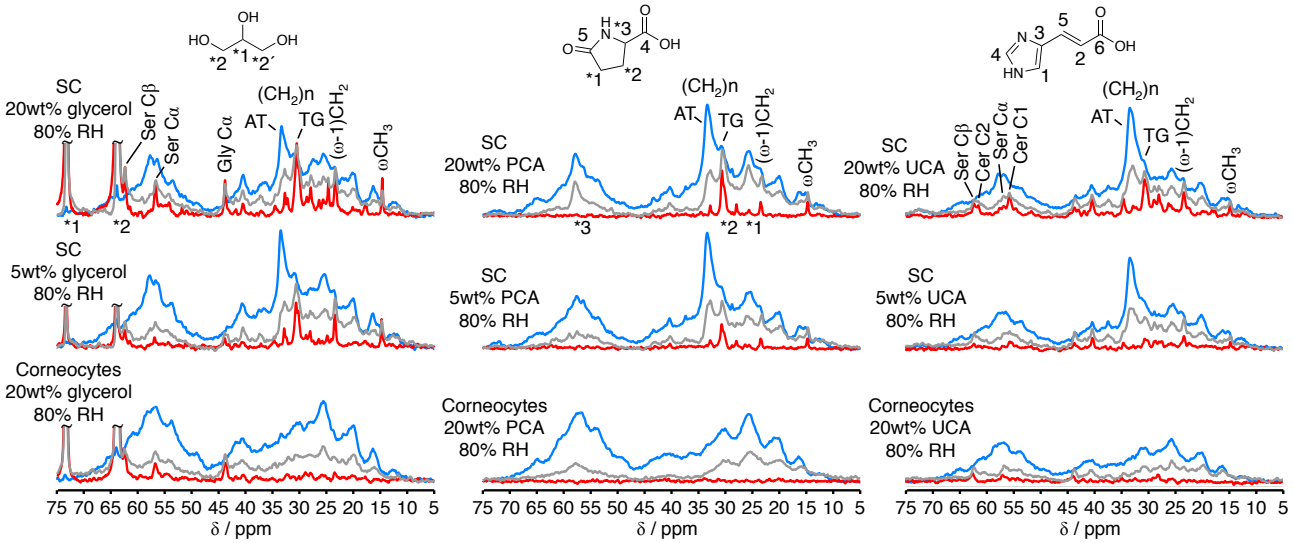

Fig. 3 PT ssNMR ^{13}C spectra (DP in grey, CP in blue, INEPT in red) of SC and corneocytes at 32 °C and 80% RH with glycerol (left), PCA (middle), and UCA (right). Resonances from glycerol and PCA carbons are marked with asterisks. All resonances from UCA are located outside the 5-75 ppm spectral window shown here. Signal intensity is given on the same scale for all samples. AT stands for all-trans and TG for trans/gauche.

Compared to pure SC at 80% RH (Fig. 2), the addition of glycerol leads to a significant enhancement of the INEPT signal from the Gly C_α, Ser C_α, and Ser C_β resonances, as well as the ωCH_3, $(\omega\text{-}1)CH_2$, and $(CH_2)_n$ segments. These effects are also observed in the spectra of SC with 5wt% glycerol, but to a lower degree. We also note the appearance of an INEPT peak at 24.6 ppm in the spectra from SC with added glycerol. This peak is most pronounced at the higher glycerol concentrations, although it is not from glycerol itself. As it is not present in any spectra from corneocyte samples, the most likely interpretation is that this peak originates from some type of lipid. Finally, the glycerol carbons give rise to prominent INEPT peaks in all spectra (marked with asterisks in Fig. 3), as well as small CP signals. This shows that glycerol is in the fast dynamic regime with low, but finite, anisotropy, probably located in aqueous regions surrounded by anisotropically organized objects like keratin filaments or lipid lamellar structures. A similar comparison with urea is not possible due to that its non-protonated carbonyl carbon results in total absence of INEPT and CP signal.

The addition of PCA to SC or corneocytes at 80% RH (Fig. 3) does not lead to any significant change in the spectra, in comparison to the pure SC or corneocytes at the same RH (Fig. 2). The asterisks around 25, 30, and 57 ppm in Fig. 3 indicate the chemical shifts for the expected peaks from the carbons in PCA. Because there is no INEPT peak at 57 ppm from PCA, it can be assumed that the INEPT peaks around 25 and 30 ppm, from SC lipid segments, are not influenced to any significant degree by contribution from the PCA resonances. This is supported by the total absence of INEPT peaks at these lipid-dominating resonances observed in the corneocyte sample, which does not contain the lipid lamellae matrix. The DP and CP signals are, however, increased at these resonances, which makes the comparison between DP and INEPT or CP biased for these particular resonances. The INEPT signal from the ωCH_3 and $(\omega\text{-}1)CH_2$ resonances, which are not coinciding with the chemical shifts of PCA, is reduced both for 20wt% and 5wt% PCA, as compared to the case of pure SC at 80% RH. Taken together, the results indicate that the presence of PCA at 80% RH has no influence on the protein mobility, and that it potentially leads to reduced mobility of the SC lipids. These effects can be related to that pure PCA is solid at 80% RH (Fig. S2). It is likely that this is the case also when PCA is present in SC at the same RH, which is consistent with the inefficient INEPT signal enhancement from the carbons in PCA (cf. corneocyte sample with PCA in Fig. 3).

The most intriguing observation in the spectra from SC with UCA (5wt% and 20wt%) in Fig. 3 is the INEPT peaks at 55.7 and 61.4 ppm, next to the Ser C_α and Ser C_β resonances. These INEPT peaks are most intense for the highest UCA concentration, and they are completely absent in the spectrum from the corneocyte sample. Together, this suggests that these peaks are attributed to mobile lipid species. More specifically, the headgroup carbons of the ceramides (Cer C1 and Cer C2) are expected to resonate at these shifts,[21] see Fig. 1 for carbon labels. The relatively strong INEPT peak around 35 ppm can be assigned to αCH_2 of ceramides or fatty acids and, therefore, be an additional sign of headgroup mobility in the presence of 20wt% UCA in SC. Also, the prominent INEPT peak around 41 ppm, which can be assigned to cholesterol C12/C24 may be related to increased lipid mobility. These INEPT peaks may also be associated to mobility of protein components, but as these peaks are virtually absent in the spectra from the corneocyte sample this is it not likely in this case. This demonstrates that UCA influences the molecular dynamics of the extracellular SC lipids. Further inspection of the spectra in Fig. 3, from the SC sample with 20wt% UCA, show that the INEPT peak from $(CH_2)_n$ is also relatively intense and similar to the case when 20wt% glycerol or urea are added to SC (Fig 2 and Fig. 3, respectively), while the intensity of the ωCH_3 and $(\omega\text{-}1)CH_2$ INEPT peaks is lower and fall in between the case of SC at 80% RH and 96% RH in Fig. 3. Finally, the INEPT spectra show peaks at the Gly C_α, Ser C_α, and Ser C_β resonances with higher intensity as compared to the case of pure SC at 80% RH, but lower as compared to SC at 96% RH (Fig. 2). The effect on these amino acid residues is clearly smaller as compared to the effect of urea or glycerol on the same

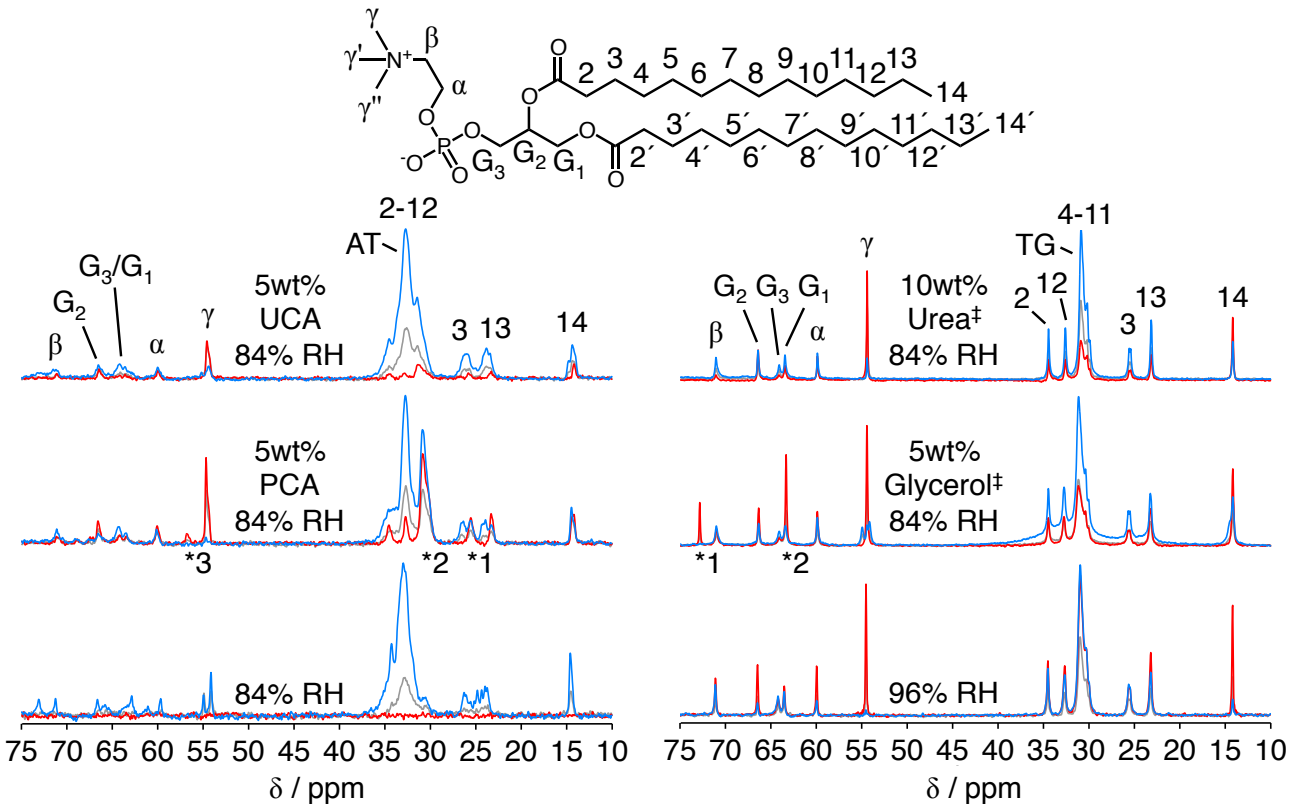

Fig. 4 PT ssNMR ^{13}C spectra (DP in grey, CP in blue, INEPT in red) of DMPC at 27 °C and 84% or 96% RH for pure DMPC, and DMPC with 5wt% UCA, 5wt% PCA, 5wt% glycerol, and 10wt% urea. Intensities are normalized to give equal CP signal for the most intense peak located between 30 and 35 ppm. AT stands for all-trans and TG for trans/gauche. ‡Data from Nowacka et al.[17]

molecular segments. For the SC sample with lower UCA concentration (5wt%, Fig. 3), the same effects are observed, but less pronounced. Note that all of the carbons of UCA resonate at higher chemical shifts than the range shown in Fig. 3 (see Table S1).

Natural moisturizers in model lipid bilayers

To further characterize the effects of the different moisturizers on fluid and solid lipid bilayers, we studied defined ternary lipid model systems composed of DMPC, water and one of the NMF components. The binary DMPC-water system is well characterized, and previous studies have shown that hydration induces a phase transition between a solid lamellar phase and liquid crystalline lamellar phases.[38] At 27 °C, this transition takes place at 93% RH.[37] This temperature was also chosen here to enable direct comparisons with previous studies.[16, 17, 37]

Fig. 4 summarizes the PT ssNMR data obtained for DMPC with added PCA or UCA at 84% RH. To enable comparisons we included data on the binary DMPC-water systems at 84% RH (gel lamellar phase) and at 96% RH (liquid crystalline lamellar phase, L_α), as well as previous reported data[17] for ternary DMPC-water-urea and DMPC-water-glycerol systems at 84% RH. The complete lack of INEPT signal in the spectra for DMPC without NMF at 84% RH implies a solid bilayer phase. One signature that can be utilized to establish the presence of the solid phase is the double CP peak at 55 ppm, which originates from different molecular conformations of the methyl groups in the DMPC headgroup.[17] If the headgroup is mobile, as in the liquid crystalline L_α phase, the molecular motions average the resonance frequencies of these methyl carbons, and only a single CP peak is observed, together with elevated INEPT signal. This feature can be very useful to distinguish phase co-existence, as previously described.[17, 32] Similar, at 84% RH the major peak in the CP spectra at 33 ppm is assigned to non-resolved resonances from C_2-C_{12}, and it signifies an all-trans (AT) conformation of the acyl-chains.[41, 42] At 96% RH, the main peak originating from disordered acyl-chains (TG) of DMPC is visible at 31 ppm and the appearance of INEPT signal from all resonances indicates lipid mobility. The presence of INEPT and CP signals with the same chemical shifts and line shapes is indicative of a liquid crystalline lamellar phase.[17, 32] At 96% RH the C_2 and C_{12} segments are also resolved from the main fraction of methylene carbons due to increased mobility.

All four NMF compounds cause increased mobility of the DMPC molecules at 84% RH, as implied from the appearance of INEPT signal in Fig. 4. The spectra obtained for DMPC in the presence of PCA indicate phase co-existence between a solid phase and a liquid crystalline lamellar phase. The CP signal at 33 ppm implies a large fraction with all-trans conformation in the acyl-chains, while the INEPT signal at 31 ppm indicates that a fraction of acyl-chains are disordered. Note that the peak (DP, CP, and INEPT) at 31 ppm is coinciding with the resonance of one the PCA carbons (marked by asterisk). However, the resonances from the other PCA carbons show relatively low intensity from the INEPT signal, which suggests that the INEPT peak at 31 ppm is not dominated by the PCA carbon resonance. The slight shift between the CP and INEPT signals at 25 ppm and 15 ppm suggests phase co-existence. This can be compared to the

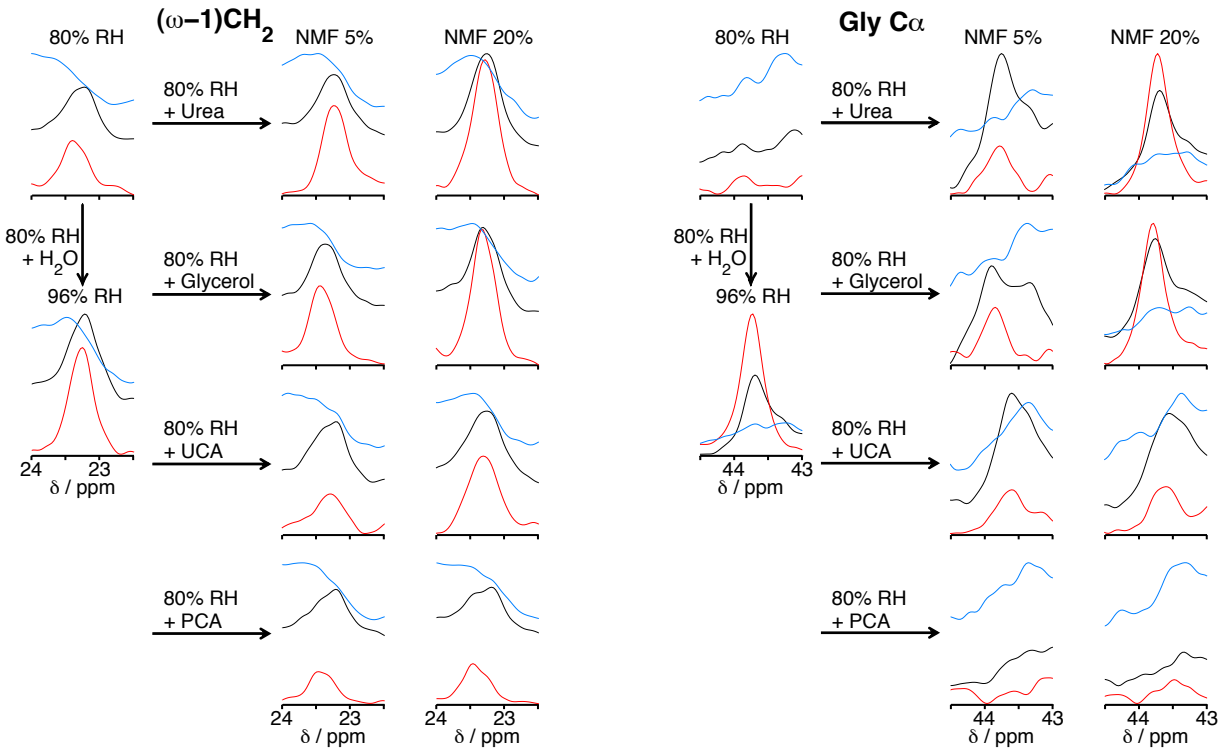

Fig. 5 PT ssNMR ^{13}C spectra in close-up of $(\omega\text{-}1)CH_2$ and Gly C_α resonances in the presence or absence of glycerol, urea, PCA, or UCA. DP in grey, CP in blue, INEPT in red.

situation when urea or glycerol is added to DMPC at the same RH (Fig 4), where the spectra imply even higher fraction of the fluid phase.[17] When UCA is added to DMPC at 84% RH, there is slight increase in the mobility of the lipid headgroup and acyl-chain carbons (Fig. 4). The spectra show only small INEPT signals, suggesting that a major fraction of the lipids is solid. Phase co-existence is implied from the weak double CP peak and the corresponding single INEPT peak around 55 ppm, and from the slight shift between the CP and INEPT signals at 25 ppm and 15 ppm.

D. Discussion and conclusions

Natural moisturizers influence stratum corneum molecular mobility

Water act as a plasticizer for skin.[15] Similarly, the NMF and osmolytes in skin care products can serve to increase skin smoothness and elasticity, and this has been explained by that the addition of the hygroscopic compounds leads to increased water content in SC.[4, 26, 43] The main message from the present study is that the addition of NMF compounds to SC at reduced RH does only marginally influence the water content, but more importantly, it alters the dynamical properties of the SC lipid and protein components. This can indeed be compared to SC hydration because addition of water influences these SC components in a similar manner.[21, 22] In the present study we present data from PT ssNMR measurements, where the combination of the INEPT, CP, and DP experiments provide detailed information on the SC molecular mobility and reveal how the different natural moisturizers affect the dynamical properties of SC components. In particular, an enhancement of the INEPT signal reflects an increase in the molecular mobility. A closer inspection of the spectra reveals that the presence of NMF components has a significant effect on the intensity of the INEPT signal, both from lipid (ωCH_3, $(\omega\text{-}1)CH_2$, and $(CH_2)_n$) and protein (Gly C_α, Ser C_α, and Ser C_β) molecular segments. This increase in INEPT signal intensity (i.e. molecular mobility) is clearly illustrated in Fig. 5, which provides the spectral region, in close-up, where $(\omega\text{-}1)CH_2$ or Gly C_α resonances dominate the signal. The corresponding case for ωCH_3 and $(CH_2)_n$ resonances is given in Fig. S3, while Fig. S4 shows the peaks from Ser C_α, and Ser C_β molecular segments in close-up. These figures highlight the most important findings of this work and form a basis of this discussion.

Urea and glycerol influence apolar stratum corneum components

Glycerol and urea are polar compounds that are mainly distributed in the aqueous regions, and are expected to be so also in the heterogeneous SC material. Still, their presence clearly affects the properties of the apolar lipid components in the membrane. Here, we show that the addition of these compounds leads to increased mobility (melting) of the lipid acyl-chains in intact SC, as seen from the enhanced INEPT signal from relevant lipid segments (Fig. 5 and Fig. S3). This effect is similar to what has previously been shown in model lipid systems upon addition of glycerol and urea[16, 17] (cf. Fig. 4), and is also very similar to the case of increasing hydration of SC (Fig. 5 and Fig. S3). The fact that the DP scheme gives signal from all of the ^{13}C in the SC (rigid and mobile carbons from all molecular segments at a particular chemical shift), while the INEPT sequence only enhances the signal from the mobile ^{13}C fraction, makes it difficult to quantify the increase in mobility. However, by fitting

Table 3 Comparison of I_{INEPT}/I_{DP} ratios for selected resonances of lipid and protein molecular segments in SC at 96% RH without NMF and SC with urea or glycerol at 80% RH.

Sample	RH / %	ωCH_3	$(\omega\text{-}1)CH_2$	Gly C_α	Ser C_β
SC*	96	1.4	1.1	1.9	1.7
SC 5wt% Urea	80	1.7	1.2	0.4	0.8
SC 20wt% Urea	80	2.3	1.3	1.9	1.6
SC 5wt% Glycerol	80	1.4	1.0	0.8	0.8
SC 20wt% Glycerol	80	2.1	1.2	1.8	1.0

*Sample contains no added NMF

the INEPT peak according to a Lorentzian line shape and using this fit (chemical shift and peak width kept constant) in combination with additional Lorentzians one can fit the DP signal and derive the corresponding DP peak. Using this deconvolution approach we estimated the signal intensity ratio of INEPT and DP (I_{INEPT}/I_{DP}) for some specific molecular segments that showed the most prominent INEPT signal. The result from this analysis is summarized in Table 3. The I_{INEPT}/I_{DP} ratio can in theory vary between 0 for rigid and approx. 4 for mobile segments, depending on τ_c and S_{CH} of the particular molecular segment.[25] The data in Table 3 show that addition of 5wt% urea or glycerol at 80% RH has about the same effect on the mobility of the lipid ωCH_3 and $(\omega\text{-}1)CH_2$ segments as compared to hydrating the SC at 96% RH. At higher amounts of urea or glycerol (20wt%) the mobility of the lipid ωCH_3 and $(\omega\text{-}1)CH_2$ segments increases even more, and these segments are thus more mobile as compared the hydrated SC at 96% RH. From Table 3, we further note that the addition of urea or glycerol has less effect on the SC protein components compared to the SC lipids. The addition of 5wt% urea or glycerol at 80% RH has only marginal effect on the mobility in the protein Gly C_α and Ser C_β segments. At higher osmolyte contents, the I_{INEPT}/I_{DP} ratio for these protein segments is similar to that of hydrated SC.

The observed effects can be explained by that the polar compounds have low vapor pressure and therefore remain in the SC membrane system also at reduced RH when water evaporates. In this way, the osmolytes can substitute for the water under dehydration in such a way that the properties of the system remain largely unchanged as compared to a more hydrated state. This is likely an important role of NMF in SC, and it can be related to the effects of osmolytes in other biological systems under osmotic stress.[1] In principle, the same qualitative behavior is also expected for other small polar compounds with low vapor pressure, including carbohydrates, polyols, amino acids, methyl amines, and methyl sulfonium solutes, etc., provided that the compound is dissolved in the aqueous phase at reduced RH.

Urea and glycerol are commonly used in formulations for treatment or prevention of defective dry skin conditions.[26] These compounds are classified as humectants with role to moisturize the skin. We find this terminology to be somewhat misleading in that urea or glycerol has the effect of increasing the mobility of the lipid and protein components at dry conditions (Fig. 4, Fig. S3, Fig. S4), while the water content is almost unchanged. Table 2 summarizes the water content in the different SC systems investigated. The table also gives the total amount of added polar components (here meaning water + NMF substance). A first observation is that the increase in RH leads to an increased water content in SC and in the isolated corneocytes, which is consistent with previous sorption studies.[15, 24] It is notable that the addition of urea or glycerol to SC at 80% RH does not lead to an increased water uptake, although the total amount of polar components increases towards a value similar to the hydrated SC (96% RH). The present observed changes in SC molecular mobility upon addition of these compounds can therefore not be simply related to the water content, but should be considered in relation to the total amount of polar molecules. Finally, it is noted that the water content in the corneocyte samples increases with the addition of urea and glycerol. This can possibly be related to changes in the protein conformation and filament assembly during the extraction process. It is also noted that the macroscopic measure of mass does not distinguish between water absorbed in the corneocytes, and water that wets the surface between adjacent isolated cells, which can occur by capillary condensation.[44]

Natural moisturizers have different effects on stratum corneum components

In the previous section, we discussed the general effects of adding osmolytes (glycerol or urea) to SC at reduced RH. We will now turn the focus to the differences between the substances investigated. Such comparison is possible as we have a method that is sensitive to changes in molecular mobility of different SC components. Urea is known to weaken the hydrophobic interactions and it is commonly used for protein denaturation.[45] This weakening is apparently not sufficient to solubilize or disturb lipid bilayer structures in the SC or the DMPC model systems.[17] The addition of urea has a clear effect on the SC protein components, leading to significant increase in the mobility of the Gly C_α, Ser C_α, and Ser C_β segments, comparable to the case of pure SC at 96% RH (cf. Fig. 5, Fig. S4, and Table 3). This effect is most prominent for the corneocyte sample (Fig. 2). We have previously observed that SC pretreated in a solution with 20wt% urea for 24 h at 32 °C is associated with weaker Bragg diffraction from the protein components, which potentially can be associated with a decrease in the fraction of solid protein structures.[23]

UCA is less polar as compared to the other small compounds used in this study, and it has been shown to partition into lipid bilayers in favor of water.[40] It is therefore expected to be present in the extracellular SC lipid structures. The addition of 20wt% UCA increases the mobility in the hydrocarbon chains to some extent (Fig. 5 and Fig. S3), which is consistent with melting point depression when an apolar compound is dissolved in the hydrocarbon layer. More importantly, the addition of UCA to SC has a clear effect on the ceramide headgroup carbons C1 and C2 and likely also on the αCH_2 segments of ceramide and fatty acid headgroups (Fig. 3). In addition, UCA seems to influence the cholesterol mobility. These observations from the SC samples are also consistent with the influence of UCA on the molecular mobility of the DMPC bilayer system (Fig. 4).

In case of PCA, Fig S2 shows that it is only solubilized in water at RH>93%, and at lower RH it is present in its solid form. The solubility limit can be shifted towards lower RH if dissolution occurs in the lipid-containing phase, as previously demonstrated for urea.[16] This can explain the observed effect of PCA on the molecular mobility of the DMPC bilayer system at 84% RH (Fig. 4), while the dissolution limit of PCA in the solid composite SC membrane is apparently not reached at 80% RH resulting in unaffected SC molecular mobility (Fig 3). The fact

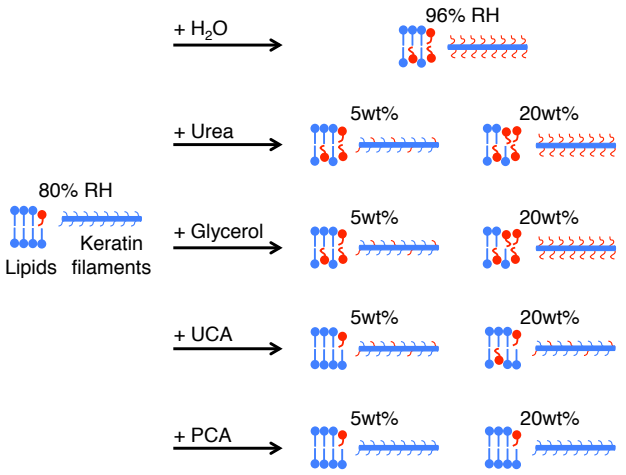

Fig. 6 Schematic interpretation of the effect of urea, glycerol, PCA, and UCA on the molecular mobility of the SC protein (keratin filaments) and lipid components at 80% RH with respect to the effect of hydration (96% RH). Blue represents rigid molecular segments (as seen from the CP scheme), while red represents mobile molecular segments (as observed from the INEPT pulse sequence).

that PCA remains solid (undissolved) in the SC sample at 80% RH explains why the water content in this SC samples is lower compared to the pure SC sample at the same RH. In other words, PCA only dilutes the SC sample without absorbing any water resulting in lower water content. This is in contrast to the sodium salt of PCA that absorbs water at lower RH.[3]

Figure 6 provides a schematic interpretation of the present results to summarize how urea, glycerol, UCA, and PCA affect the mobility of the SC protein and lipid components.

Moisturizers in stratum corneum – Implications to biological functions and barrier properties

Glycerol, urea, PCA and UCA are all naturally present in skin as part of NMF, with the role of protecting the SC from severe drying.[4] Glycerol and urea are also commonly used in topical treatment of both healthy and dry skin and have been shown to influence SC properties, including hydration, smoothness, and recovery of barrier function after perturbation by tape stripping SC with adhesive tape or by treatment with sodium lauryl sulphate.[26, 43, 46] Our present findings provide new insight on the molecular action of these compounds in SC at reduced RH. The clear effect on the mobility of the lipid and protein SC components in the presence of glycerol or urea (Fig. 5, Fig. S3, Fig. S4, and Table 3) can be related to increasing pliability and softness of SC treated with these compounds under dry conditions and to the beneficial properties of glycerol or urea in skin treatment.

The change in the content of fluid lipid and protein components of SC strongly relate to its barrier function.[20, 47] We have previously performed *in vitro* studies on the permeability of skin membranes showing that the transport of a model drug remains high and similar to that of a fully hydrated skin membrane when glycerol or urea are added to aqueous formulations with reduced water activity.[23] In the absence of these molecules, the SC permeability is reduced ~ 5-fold, when comparing flux values at similar reduced water activities.[20] This effect was related to S(W)AXD data of dehydrated SC samples with glycerol or urea, treated in the same manner as in the permeability study, showing similar diffraction profiles from the extracellular lipid lamellae as in the fully hydrated SC in the absence of urea or glycerol.[23] This is consistent with the present data, demonstrating that the molecular mobility of the lipid components in SC samples containing glycerol or urea is similar, or even enhanced, compared to the SC sample at 96% RH (Fig. 5, Fig. S3, and Table 3).

Concepts involving more intricate biochemical processes are emerging, where osmolytes are suggested to play important roles for SC homeostasis.[46, 48] For example, proper desquamation, where the outermost corneocytes are discarded requires degradation of the corneodesmosomes, which link corneocytes together and provide SC cohesion.[49] These structures are degraded by various enzymes, of which some are found in the extracellular lipid matrix.[49] It has been shown that the enzyme activity depends on the external RH and is also influenced by application of glycerol.[48] From these observations it has been hypothesized that maintaining fluidity of the SC extracellular lipids under desiccating conditions can play an important role for enzyme activity.[48] The results from this work demonstrate that both glycerol, urea (Fig. 5, Fig. S3, and Table 3), and also UCA to some extent (Fig. 5, Fig. S3), indeed increase the fluidity of the SC lipids at low RH, which thus may have a beneficial outcome in respect to important enzyme regulated processes in SC, such as desquamation and recovery of the SC barrier after perturbation.

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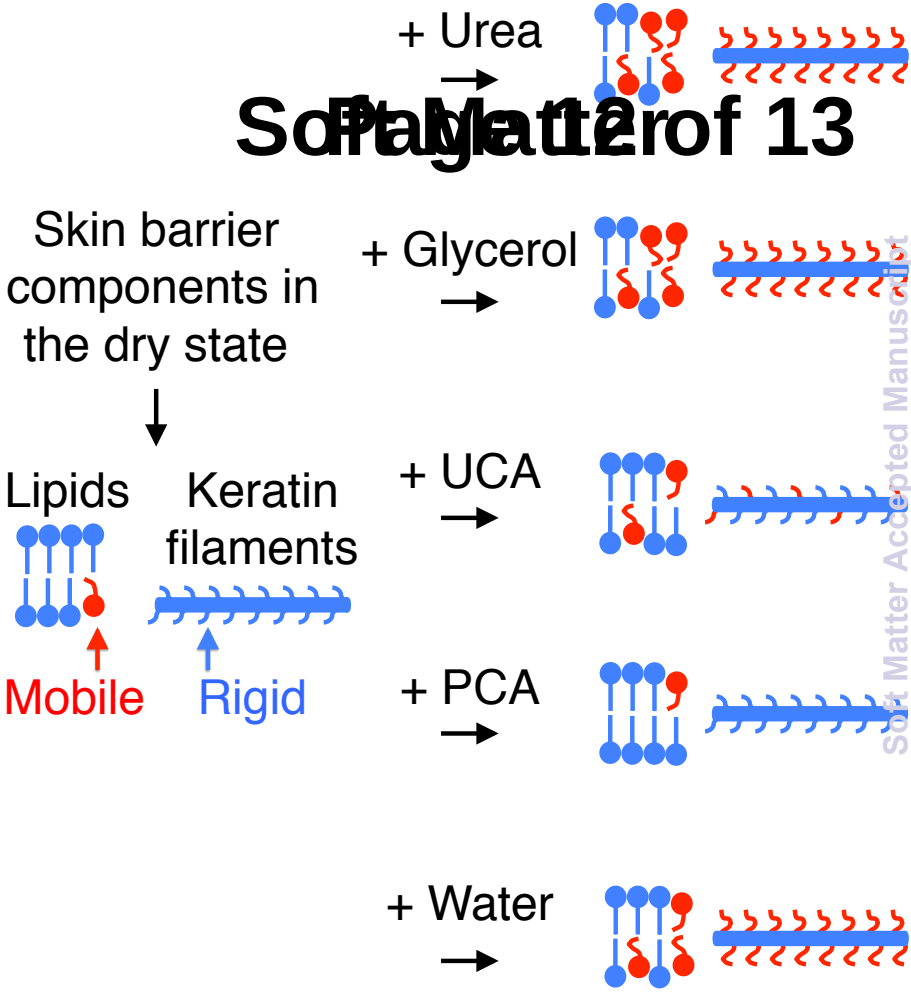

Natural moisturizers act to retain the fluidity of the skin barrier molecular components at dehydrating conditions – a situation similar to skin hydration.