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Chemical penetration enhancers in stratum corneum — Relation between molecular effects and barrier function

Quoc Dat Pham [a], Sebastian Björklund [b,c], Johan Engblom [b,c], Daniel Topgaard [a], Emma Sparr [a,*]

[a] Division of Physical Chemistry, Chemistry Department, Lund University, P.O. Box 124, SE-221 00 Lund, Sweden
[b] Biomedical Science, Faculty of Health and Society, Malmö University, SE-205 06 Malmö, Sweden
[c] Biofilms — Research Center for Biointerfaces, Malmö University, SE-205 06 Malmö, Sweden

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ABSTRACT

Skin is attractive for drug therapy because it offers an easily accessible route without first-pass metabolism. Transdermal drug delivery is also associated with high patient compliance and through the site of application, the drug delivery can be locally directed. However, to succeed with transdermal drug delivery it is often required to overcome the low permeability of the upper layer of the skin, the stratum corneum (SC). One common strategy is to employ so-called penetration enhancers that supposedly act to increase the drug passage across SC. Still, there is a lack of understanding of the molecular effects of so-called penetration enhancers on the skin barrier membrane, the SC. In this study, we provide a molecular characterization of how different classes of compounds, suggested as penetration enhancers, influence lipid and protein components in SC. The compounds investigated include monoterpenes, fatty acids, osmolytes, surfactant, and Azone. We employ natural abundance ^{13}C polarization transfer solid-state nuclear magnetic resonance (NMR) on intact porcine SC. With this method it is possible to detect small changes in the mobility of the minor fluid lipid and protein SC components, and simultaneously obtain information on the major fraction of solid SC components. The balance between fluid and solid components in the SC is essential to determine macroscopic material properties of the SC, including barrier and mechanical properties. We study SC at different hydration levels corresponding to SC in ambient air and under occlusion. The NMR studies are complemented with diffusion cell experiments that provide quantitative data on skin permeability when treated with different compounds. By correlating the effects on SC molecular components and SC barrier function, we aim at deepened understanding of diffusional transport in SC, and how this can be controlled, which can be utilized for optimal design of transdermal drug delivery formulations.

1. Introduction

The human skin is a large interfacial film that separates regions with completely different properties. This fact implies several simultaneous transport processes occurring across the skin. Here, the skin barrier function is vital to prevent desiccation and to protect the body from uptake of foreign compounds. The skin also constitutes a mechanical protection in that it tolerates deformation from physical strain and stress. Taken together, the skin membrane has to fulfill several essentially different requirements, and these macroscopic material properties are determined by the organization and dynamics in the molecular components of the skin.

The barrier function of the skin is mainly assured by its outermost layer, the stratum corneum (SC). This is a thin (ca. 20 μm) [1] and dry layer that is composed of anucleated dead epidermal cells (corneocytes), which are filled with keratin filaments and embedded in a continuous multilamellar lipid matrix. The lipid composition is widely different compared to most other biological membranes in that it includes basically no phospholipids and the main components are long chain ceramides, fatty acids and cholesterol [2]. At ambient temperatures, the main part of both the lipid and the keratin components are solid. In hydrated conditions, minor fractions of the SC lipid and protein components become fluid (mobile disordered) [3–6]. The fluidity in these components can also be altered by the addition of small foreign compounds, for example compounds like urea or glycerol that are constituents of the so-called natural moisturizing factor [7]. The tiny fluid fraction is likely crucial for macroscopic material properties of SC, including barrier and mechanical properties. Increasing fluidity or fluid fraction is expected to lead to higher permeability to both polar and apolar compounds [8,9], and it may also account for the skin elasticity properties. One major challenge in the research field aiming at deepened understanding of the SC barrier functions is therefore to find

* Corresponding author.
E-mail address: emma.sparr@fkem1.lu.se (E. Sparr).

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methods that allow for characterizing this minor amount of disordered material in the highly ordered SC solid sample. Particularly relevant is to characterize how this disordered fraction changes in response to the external conditions or added compounds. In the present paper, we employ an NMR (Nuclear Magnetic Resonance) tool that provides completely novel information on how the fluid lipid and protein SC components are influenced by addition of different compounds.

The skin is highly attractive as a target for directed drug delivery. Still, there are only around 20 examples of drugs on the market that utilizes the transdermal route [10], and the low number is explained by the difficulties to overcome the skin barrier. One strategy for overcoming the barrier property entails the use of so-called chemical penetration enhancers, which can be combined with skin hydration ("occlusion") or increased temperature [11–13]. Physical forces such as electrical voltage (iontophoresis, electroporation), ultrasound (sonophoresis), microneedles, thermal ablation and microdermabrasion are also used to overcome the skin barrier [13]. Many different classes of compounds have been proposed as chemical penetration enhancers, including small hydrophobic and hydrophilic compounds, surfactants, lipids and solvents. These compound classes are very different with respect to their chemical and physical properties, and therefore expected to influence the SC molecular properties in different ways [14]. Most research on chemical penetration enhancers involves studies of these compounds added to a certain formulation, which can influence permeability to various active compounds or water (transepidermal water loss, TEWL) [15–17]. There are also examples of biophysical studies of chemical penetration enhancers in SC, including calorimetry and X-ray scattering studies of thermal transitions and lipid self-assembly in intact SC or SC model lipid mixtures exposed to different classes of chemical penetration enhancers [15,17,18]. From infrared spectroscopy studies, changes in the CH_2 stretching vibration in the lipid acyl chain upon addition of relevant compounds have also been investigated [19]. Still, the molecular characterization of how different compounds affect fluidity in SC lipid and protein components in intact SC is rather incomplete, in particular for the SC protein components, which indeed constitute ca 85 wt% in dry SC [2].

In the present study, we employ natural abundance ^{13}C solid-state (ss) NMR with $^{1}H \rightarrow {}^{13}C$ polarization transfer using CP (cross polarization) [20] and INEPT (insensitive nuclei enhanced by polarization transfer) [21] to obtain atomically resolved qualitative information on molecular dynamics in the intact SC [5]. We have previously introduced the acronym PT ssNMR (polarization transfer solid-state NMR) to denote this set of NMR measurements [22]. Despite the complexity of the NMR spectra from intact SC, we have been able to assign resonance lines from all major SC components, including amino acids of the keratin filaments, ceramides and cholesterol [5], and this is taken advantage of here. We investigate how different classes of compounds, that are relevant as potential penetration enhancers, influence the SC lipids, and we distinguish effects on the molecular mobility in acyl-chains (melting), ceramide headgroup and cholesterol. Furthermore, the influence on the SC proteins in the keratin filaments can be resolved, and we distinguish effects on the amino acids that are enriched in the terminal domains (rich in serine and glycine) or in the core (rich in leucine and lysine) within the keratin filaments (UniProt ID P04264 and P13645). The PT ssNMR measurements are sensitive to small changes in molecular mobility in the minor fluid fraction of SC lipids and proteins. From the combined INEPT and CP experiments, we obtain simultaneously information with molecular resolution on the dynamics in different segments of the lipid and protein molecular components in the presence of chemical penetration enhancers. From these experiments, it is possible to gain completely novel molecular insight into how various classes of penetration enhancers influence the balance between mobile and rigid components in the very same sample of intact SC. The added compounds are chosen to represent different classes of compounds relevant as chemical penetration enhancers [14,23]. We study intact SC together with polar compounds, surfactant, fatty acids and hydrophobic compounds. We also investigate how some representatives of these compounds influence SC permeability to a model drug, metronidazole (Mz), using flow-through diffusion cells. By correlating the molecular effects on SC to the macroscopic effects on SC barrier function, we aim at a deeper understanding of molecular transport across SC.

2. Materials and methods

2.1. Materials

Thymol, geraniol, carvacrol, oleic acid ($OA_{C18:1}$), dodecanoic acid ($DA_{C12:0}$), $DA_{C12:0}$-d23 (≥98 at.% D), Azone, urea, NaCl, $Na_2HPO_4 \cdot 2H_2O$, KH_2PO_4 were purchased from Sigma-Aldrich. Sodium dodecyl sulfate (SDS) was obtained from VWR, SDS-d25 (≥98 at.% D) was from ICON, glycerol was from Merck, stearic acid ($SA_{C18:0}$) was from BDH chemical and metronidazole was from Duchefa Biochemie. Milli-Q water was used to hydrate SC and to prepare phosphate buffer saline (PBS, 130.9 mM NaCl, 5.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4).

2.2. Preparation of dermal skin and stratum corneum

Porcine ears were obtained from a local abattoir and stored at −80 °C until use. Before use the ears were thawed and rinsed in cold running water. Hair was removed by a trimmer, and the skin tissue was dermatomed (TCM 3000 BL, Nouvag) from the inner ear to a thickness of approximately 500 μm. These dermatomed skin strips were used for flow-through diffusion-cell studies or further processed for preparation of separated SC.

In order to separate SC from tissue, these strips were placed on filter paper soaked in PBS solution with 0.2 wt% trypsin at 4 °C overnight. The sheets of trypsinated SC (hereafter simply referred to as SC) were removed from tissue by forceps, washed with PBS and further dried under vacuum. The dry SC was then pulverized to a fine powder with the use of a mortar and pestle to facilitate the mixing and equilibration. To reduce the contributions from biological variations between the different individuals, we prepared a batch consisting of pulverized SC from several individuals (ca. 15), and all internal comparisons are performed with samples from the very same batch. A second batch of SC was used for complementary NMR experiments on $DA_{C12:0}$-d23 and SDS-d25. Pulverized SC was dried again in vacuum and stored in a freezer until further use. In a previous study, we showed that there are no detectable changes in the PT ssNMR spectra between pulverized trypsinated SC and intact sheets of trypsinated SC [5], indicating that these samples do not differ on the molecular scale. However, the time needed for equilibration is clearly reduced for the pulverized SC.

For NMR experiments, approximately 30 mg of dry SC powder was mixed with 5 wt% of the compound of interest (based on the total weight of SC and compound) and then water was added (here 20 wt% or 40 wt% water, based on the total weight of SC and water). The samples were prepared in Milli-Q water and pH was not controlled. The samples were then transferred into tight ssNMR inserts (Bruker) and incubated at 32 °C for one day before NMR measurements. Two replicates were investigated for all samples containing 40 wt% water.

In order to get a good control of the experimental conditions, we mixed SC powder with known amounts of water and the added compound. We chose this approach in favor of trying to mimic some practical situations, for example by immersing the SC sheets into the formulations. With the present protocol, the compositions of all the samples are well controlled and known, and mixing is facilitated as we use pulverized SC.

We also prepared samples of SC sheets to directly mimic the conditions used in the diffusion-cell experiment. The SC sheets were immersed in 100 mL PBS solution saturated with the monoterpenes and comprising 0.75 wt% Mz (same condition as donor solution containing monoterpene in flow-through diffusion-cell studies) for 12 h at 32 °C. The SC sheets were then washed with 50 mL PBS buffer to remove the

excess monoterpenes from the surface of the SC sheets, wiped by tissue and transferred into tight ssNMR inserts.

2.3. Solid-state NMR experiments

NMR experiments were performed at ^{1}H and ^{13}C resonance frequencies of 500 and 125 MHz, respectively, on a Bruker Avance AVII - 500 NMR spectrometer equipped with a Bruker Efree 4 mm MAS (magic angle spinning) probe (for the first batch) or a 4 mm CP/MAS HX probe (for the second batch) and at a spinning frequency of 5 kHz. The CP [20] and INEPT [21] schemes for $^{1}H—^{13}C$ polarization transfer are commonly used to enhance the ^{13}C signal in NMR. Comparing the signal intensities acquired with DP (direct polarization)-CP-INEPT set of experiments in PT ssNMR, where DP is used as a reference, provides site-specific qualitative information about molecular mobility. In the PT ssNMR experiments, the following set-up was used: spectral width of 248.5 ppm, acquisition time of 0.05 s, 2048 scans per experiment, recycle delay of 5 s and ^{1}H and ^{13}C hard pulse at $\omega_1^{H/C} / 2\pi = 80.6$ kHz. The power of ^{1}H was ramped up from 72 to 88 kHz during the contact time of 1 ms in CP experiment. The INEPT experiments were performed with the delay times τ of 1.8 ms and τ' of 1.2 ms. All experiments were recorded under 68 kHz two-pulse phase modulation (TPPM) ^{1}H decoupling [24]. The ^{13}C spectra were externally referenced to the methylene signal of solid α-glycine at 43.7 ppm [25]. The temperature was calibrated by methanol [26]. The data were processed with a line broadening of 20 Hz, zero-filling from 1024 to 8192 time-domain points, Fourier transform, phase and baseline correction by in-house Matlab code partially from matNMR [27]. In previous studies [5], we were able to assign almost all peaks in the crowded NMR spectra of intact SC through comparisons with isolated SC components, model systems and databases. The transitions from rigid to mobile states in SC lipids and proteins were studied in relation to hydration and temperature, showing good agreement with previous published data when comparisons could be made. Importantly, we also confirmed reversibility in SC molecular mobility with temperature scans (32–60 °C).

2.4. Flow-through diffusion cell studies

Flow-through cell diffusion studies were performed in order to compare the effect of the different added compounds on the skin permeability of a model drug, Mz. Experiments were performed with four different compounds; thymol, geraniol, $DA_{C12:0}$ and $SA_{C18:0.}$ The experiments were designed so that the concentration of the model drug corresponds to 65% of its saturation concentration in the solvent of interest (here PBS + the added compounds) at 32 °C, which corresponds to 0.75 wt% Mz in PBS buffer matching the concentration used in commercial products. The donor solution was saturated with the monoterpenes and the fatty acids during the whole experiment by having the saturated solution in contact with excess solid (thymol, $DA_{C12:0}$ and $SA_{C18:0.}$) or liquid (geraniol).

The diffusion experiments were performed using flow-through cells which allows continuous flow (1.5 mL/h) through the receptor compartment [28]. The experiments were carried out at different occasions and with several replicates (2 to 7) each time. During the experiments, both the donor and receptor solutions were kept at 32 °C by a heater block (controlled by Techne TE-10A Thermoregulator) and stirred at 80 rpm by a magnetic stirrer (Multipoint 15 Magnetic Stirrer, Variomag). The area of the dermatomed skin separating the donor and receptor compartment was 0.64 cm^2. Before the experiment started, the skin was pre-equilibrated with the solutions of interest during 1 h (PBS + the added compounds (no Mz) in donor chamber and PBS buffer pumped through the acceptor compartment). The donor chamber was then emptied and filled with 2.5 mL PBS buffer saturated with the added compound of interest and with Mz (65% of saturation concentration). The experiments were then initiated, and the receptor solutions were collected in fractions every 2 h over 24 h. Collected fractions were analyzed by a Merck Hitachi high-performance liquid chromatography system equipped with L-6200A Intelligent Pump, L-4250 UV/visible detector, and Pharmacia LKB REC 1 recorder. Phenomenex SecurityGuard (Cartridges Gemini C18, 4 × 3.0 mm) and Phenomenex Gemini C18 5 μm column (100 Å, 100 × 4.6 mm) were used for separation. The mobile phase consists of methanol:phosphate buffer (10 mM KH_2PO_4) (20:80 v/v) and the flow rate of the mobile phase was 2.0 mL/min. The concentration of Mz was detected at the wavelength of 319 nm (the maximum between 200 and 450 nm) and calculated from the calibration curve of standard solutions prepared by dissolving known amounts of Mz in PBS buffer. Standard samples at low concentrations were obtained from dilution of stock solution.

2.5. Solubility of Mz in donor solutions

The solubility of Mz in the different donor solutions was determined to ensure that the thermodynamic activity (chemical potential) of the model drug is approximately the same for all donor solutions investigated. Donor solutions were prepared by shaking PBS buffer with excess amount of added compounds (thymol, geraniol, $DA_{C12:0}$ or $SA_{C18:0}$) and Mz in Eppendorf tubes at 32 °C for 3 days. The solutions were then filtrated through a 0.2 μm Anotop inorganic membrane filter (Whatman) to remove excess dissolved material, and then diluted in PBS to enable the analysis. The concentration of Mz was determined by UV/visible spectrophotometry (Cary WinUV, Varian) at the wavelength of 319 nm. The concentration was calculated from the calibration curve of the standard solutions. The solubility of Mz in the presence of the excess added compounds does not change significantly compared to the value in PBS (i.e. free from the added compounds) as shown in Table S1.

In the diffusion experiments, the donor solution is present together with an excess (separated solid or liquid) of the added compounds. In the case when the compound is a liquid (geraniol), we also had to consider the solubility of Mz in the excess geraniol solution, as this will affect the overall concentration in the donor solution. To investigate this influence, 1 mL PBS solution containing 0.75 wt% Mz was equilibrated with 0.5 mL of pure liquid geraniol. The samples were incubated and measured in the same way as in the solubility measurement. The results in Table S1 show that the amount of Mz in aqueous solution decreases significantly in the presence of liquid geraniol separated phase due to the high solubility of Mz in liquid geraniol (note log *P* for Mz is close to 0). To overcome this problem, the volume of excess geraniol solution (20 μL) in the diffusion experiments is very small compared to the volume of the saturated PBS solution (2.5 mL), and the amount of Mz that partitions into the excess geraniol is therefore considered negligible.

3. Results

In this study we use PT ssNMR to investigate how different classes of compounds, which are candidates for penetration enhancers, influence the molecular mobility in SC lipid and protein components at different hydration conditions. The molecular effects are then related to how the same compounds influence SC barrier properties through flow-through cell diffusion experiments. We investigate different groups of compounds, including monoterpenes (thymol, geraniol and carvacrol), saturated and unsaturated fatty acids ($DA_{C12:0}$, $SA_{C18:0}$ and $OA_{C18:1}$), one surfactant (SDS), Azone and small polar compounds (urea and glycerol). These compounds are utilized to study the effects of molecular size, hydrophobicity and functional groups. As the compounds are widely different, their molecular effects on the SC are also expected to differ. The water content in the SC samples was set to either 20 wt%, which corresponds to average water content in healthy skin in ambient air (around 75%–85% RH) (unpublished data), or 40 wt% water, which corresponds to hydrated/occluded conditions [29]. We chose to compare the samples with the same water content rather than the same relative humidity (RH) (water activity) due to uncertainties associated

with the equilibration of samples containing volatile compounds (i.e. monoterpenes) in vapor with controlled RH. The results are highly reproducible considering the complexity of the samples. With this study design, we are able to draw conclusion about the effects of different compounds on SC.

3.1. Molecular mobility in SC components as revealed from PT ssNMR

PT ssNMR was employed to investigate the effect of added compounds on the structure and dynamics of SC molecular segments. The major findings are summarized in Fig. 1 and example spectra are shown in Fig. 2. The ^{13}C MAS NMR spectra in PT ssNMR include data from three different experiments (DP, CP and INEPT) performed on the same sample. The DP spectrum generally shows resonances from all carbon segments in the sample. The signal for slow (with rotational correlation time $\tau_c > 0.1$ ms) and/or anisotropic segments is selectively enhanced in CP spectrum, while the INEPT technique only boosts the signal of mobile segments and yields no signal for rigid segments. The dependence of CP and INEPT intensities on the rate (τ_c) and anisotropy (order parameter S_{CH}) of the C—H bond reorientation is shown in Fig. S1 [30].

Essentially all of the resonances from lipid and protein components in the sample of SC were assigned [5] (Fig. 2B). The numbering of the cholesterol carbons and of the most relevant resonances from ceramides and fatty acids are included in Fig. 2C–D. For the acyl chains of fatty acids and ceramides, the resonances of the terminal methyl/methylene carbons (ωCH_3, $(\omega\text{-}1)CH_2$ and $(\omega\text{-}2)CH_2$) and of αCH_2 are located at 15, 23, 33 and 35 ppm, respectively. The majority of the lipid acyl chain $(CH_2)_n$ resonates within a range of 30 to 34 ppm, in which the all-trans conformation (AT) is visualized at 33 ppm and the liquid-like distribution of trans/gauche conformation (TG) is probed at 31 ppm [31]. A solid crystalline phase or gel phase have a high fraction of AT conformation (which is more enhanced in the CP spectrum), while the TG is a sign of disordered acyl chains in the liquid crystalline or isotropic liquid phases (which is more enhanced in the INEPT spectrum). The peaks of ceramide headgroup Cer C1 and Cer C2 are more readily observed in INEPT spectrum, at 55 and 61 ppm, respectively. Relevant markers of cholesterol include the molecular segments CHOL 4 (43 ppm), 9 (52 ppm), 10, 20, 22 (37 ppm) and 24 (40 ppm) (Fig. 2C). We do not specifically discuss the C═C segments which resonates at higher ppm values (from ca. 120 to 135 ppm) since they give relatively low signal intensity. The protein components of SC are dominated by the keratin filaments, of which the terminal domains are rich in glycine and serine [32] (UniProt ID P04264 and P13645) that can be probed at the Gly Cα, Ser Cα and Ser Cβ peaks (44, 57 and 62 ppm, respectively) [5]. The keratin filament core, on the other hand, is enriched in leucine and lysine (UniProt ID P04264 and P13645), which are examined at the resonances of Leu Cβ and Lys Cε (41 ppm).

Fig. 2A–B show PT ssNMR spectra for the reference samples (without added foreign compounds) at 20 and 40 wt% water. These data can be used to illustrate the information obtained from the present experiments. A first observation is that the majority of SC is rigid at both hydration conditions as implied from the dominance of the CP signal for most of the spectral range. The main contribution to the CP signals comes from the keratin filaments as dry SC consists of roughly 85 wt% protein [2]. Moreover, the rigid Cα resonances from all amino acid residues (except glycine Cα) can be observed as the broad and dominating CP peaks around 57 ppm. On the other hand, the rigid lipids with all-trans conformation are probed at the sharp and most prominent CP $(CH_2)_n$ AT peak at 33.4 ppm [31].

The increased hydration clearly causes increased mobility of the SC molecular components, as seen in the strongly enhanced INEPT signal of both lipid and protein segments (Fig. 2). At the lower water content (Fig. 2A), the keratin filaments are completely rigid as implied from the total absence of INEPT signal from proteins. Furthermore, a small fraction of mobile lipids co-exist with the solid lipids, as inferred from the low-amplitude INEPT peaks of the terminal methyl/methylene carbons (ωCH_3, $(\omega\text{-}1)CH_2$ and $(\omega\text{-}2)CH_2$), and from the presence of the INEPT $(CH_2)_n$ TG. When the water content is increased to 40 wt%, the major fraction of SC is still rigid, although there is an increasing fraction of mobile SC lipid and protein components, as implied from the increasing amplitude of INEPT signal from these segments (Fig. 2B). Increased SC lipid mobility is clearly observed for the lipid acyl chain and some CHOL segments. Increased mobility upon hydration is observed for all

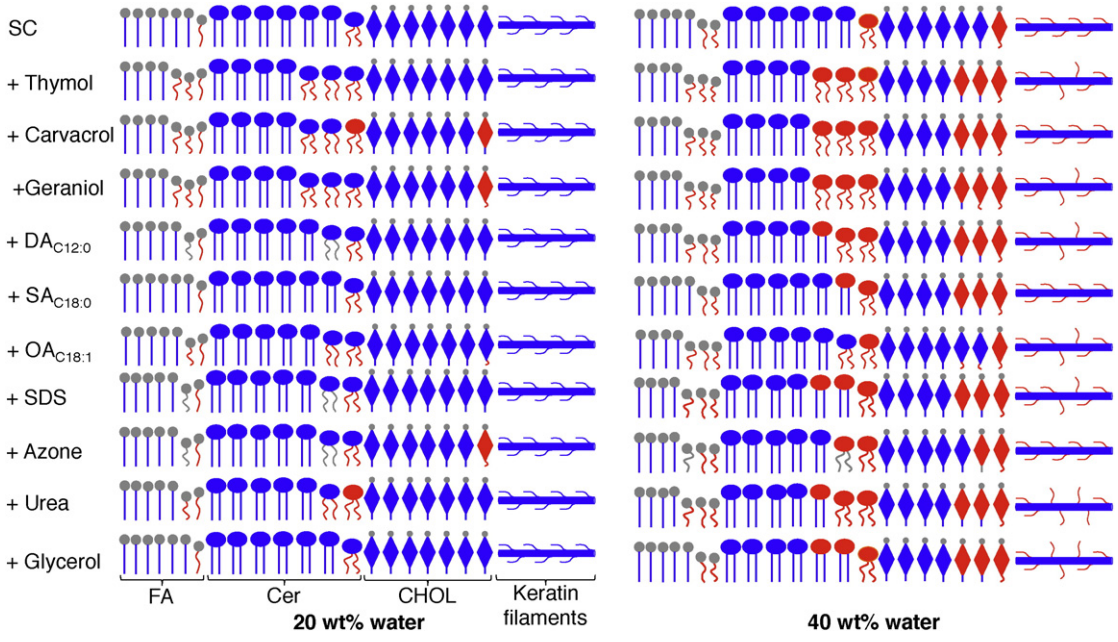


Fig. 1. Schematic interpretations of the effects of added compounds on the molecular mobility of the SC protein (keratin filaments) and lipid components (fatty acid (FA), Ceramide (Cer) and Cholesterol (CHOL)) at 20 and 40 wt% water. Blue represents rigid molecular segments (as seen from the (blue) CP spectra), while red represents mobile molecular segments (as seen from the (red) INEPT spectra). The mobility in the lipid acyl chains from SC lipids FA and Cer cannot be distinguished. Grey hydrocarbon chains indicate that the mobility of the SC lipid acyl chains cannot be distinguished due to overlap with the acyl chains of the added compounds. The experiments do not provide information on the mobility in the headgroups of FA and CHOL, which therefore are also marked in grey.

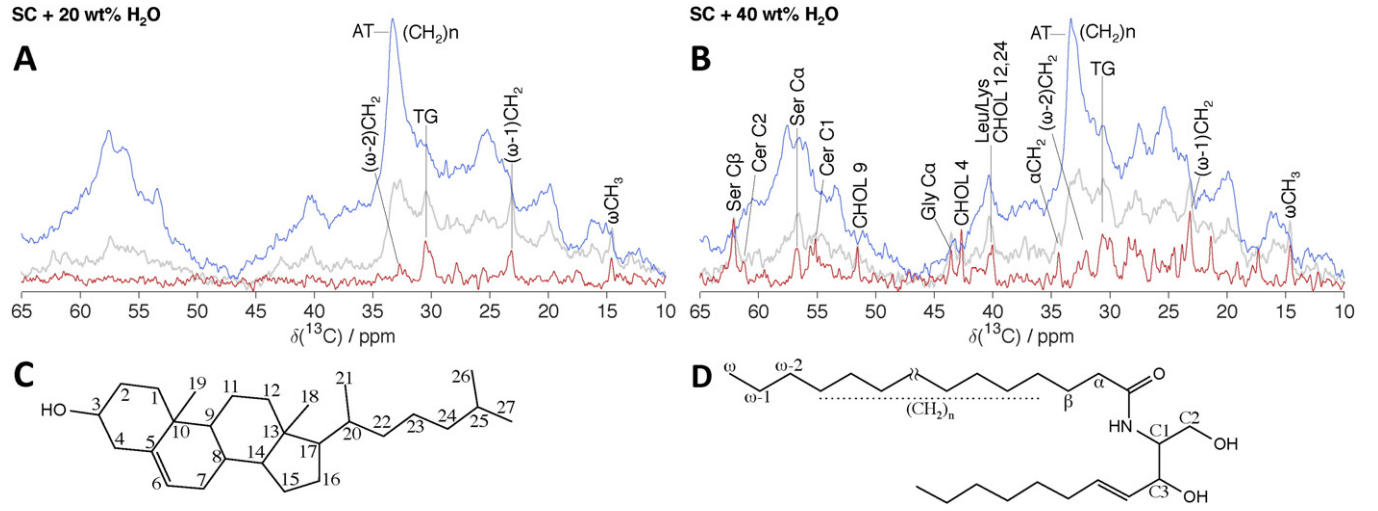


Fig. 2. ^{13}C MAS NMR spectra (DP: grey, CP: blue, INEPT: red) from the SC at 32 °C and at 20 wt% (A) and 40 wt% water (B). Chemical structures with numbered segments of relevant SC lipids (illustrated here with cholesterol (C) and a ceramide NS (non-hydroxy sphingosine) (D)). Different ceramide headgroups resonate at almost the same chemical shifts in the shown spectral region and cannot be distinguished [5].

the segments of the lipid acyl chain (ωCH_3, $(\omega\text{-}1)CH_2$, $(\omega\text{-}2)CH_2$, αCH_2 and $(CH_2)_n$ TG), and for some CHOL segments (C4, C9 and C12/C24). A minor increase in the INEPT signal is also seen for the ceramide headgroups (C1 and C2). There is a prominent effect for Gly (Cα) and Ser (Cα and Cβ), which are abundant in the terminal domains of the individual proteins assembled into keratin filaments. Increasing hydration also gives rise to the INEPT signal at the resonance characteristics of Leu (Cβ) and Lys (Cε), which are enriched in the keratin core. However, these resonances cannot be resolved from CHOL 12, 24 segments, and no solid conclusions can be drawn for these amino acids. These findings are consistent with previous studies on SC hydration [5].

3.2. Molecular dynamics in SC components upon the addition of foreign compounds

The PT ssNMR techniques together with the SC peak assignment provide a sensitive and unique tool to reveal how different added compounds influence SC at a molecular level [5]. We focus on representative molecular segments from the lipids and the keratin cores and terminals (examples in Fig. 2B) [5] and the main results are summarized in Fig. 1 and Table 2. In the analysis made in Table 2, we compare the INEPT intensities of the selected resonances of SC lipid and protein molecular segments in the presence and absence of the added compounds at the very same water content. The DP signal of the same resonance and the broad CP signal of the protein Cα centered around 57 ppm are chosen as internal references to compare the INEPT intensities in different samples. They are the most appropriate references in these systems since their intensities change the least. The carbon segments of amino acids in the corneocyte resonate in the whole the spectral range of interest, constituting the broad background of the DP and CP spectra. In most of the cases investigated, the mobility of proteins does not change significantly with the added compounds, resulting in the negligible changes in DP background and the protein CP Cα region. Still, the DP intensity of one segment is influenced by the overlapping resonances and also change with dynamics [30]. In NMR, the carbon with slow motion and random orientation gives broad signal while the resonance of the mobile segment is sharp. The DP sequence gives signals from all segments including mobile and solid carbons while the INEPT is only observable for the mobile carbons. It is pointed out that this analysis is qualitative and we only draw conclusions if there is an increase in the mobility upon adding compounds and if this increase is pronounced. Examples are shown in Fig. S2. For the lipid acyl chains and protein segments, the change is concluded if it is observed in more than one segment. However, the influences on ceramide headgroup and cholesterol may vary between segments. The mobility of Cer C2 on the branch is expected to be more sensitive than Cer C1 and the bulky steroid and the acyl tail of cholesterol may have different mobilities.

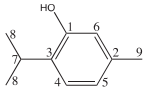
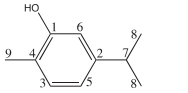
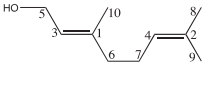
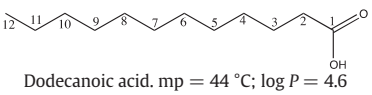
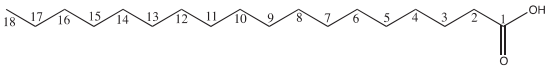
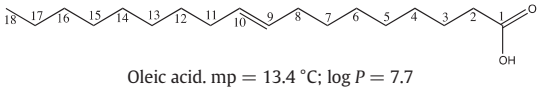
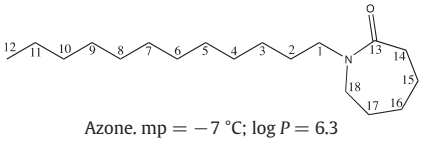
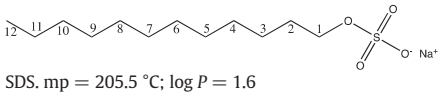
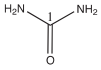
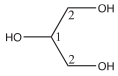
Experiments were performed for two different hydration conditions, and we investigate the effect of added compounds on SC samples with varied properties, as implied from the differences in molecular mobility. By comparing samples with 20 wt% and 40 wt% water, we are able to explore the effects on both solid and fluid SC components. At low water content when the SC is very rigid, it is straightforward to recognize the effect of added compounds from the increase in INEPT signal. A general observation is that the effects vary between the classes of compounds, between compounds in the same class and between hydration conditions. In other words, more SC components are affected at high hydration which is relevant to occlusion conditions that will be described further in more detail below.

3.2.1. Monoterpenes

Monoterpenes are well-known as natural penetration enhancers and also possess many interesting biological effects, for example antimicrobial and antioxidant activities [33]. These hydrophobic compounds have two isoprene units and three representatives are studied here, including thymol, carvacrol and geraniol (Table 1). Thymol and carvacrol are two structural isomeric cyclic monoterpenes with the hydroxyl group at different positions on the benzyl ring, resulting in different melting temperatures: carvacrol is in liquid form while thymol is solid at 32 °C. Geraniol has linear structure and the same number of carbon as the thymol and carvacrol.

Fig. 3 shows PT ssNMR data for intact SC in the presence of different monoterpenes at 20 and 40 wt% water. There is a clear enhancement of INEPT signals in all spectra, which reveals that all monoterpenes investigated strongly affect the molecular mobility in SC. In particular, the addition of monoterpenes leads to an increased mobility in the lipid acyl chain segments. This effect is most prominent at the lower water content, as compared to the reference sample at the same water content. In the more hydrated conditions (Fig. 3D–F), there is already some mobility in SC lipids in the neat SC sample, and the addition of the monoterpenes leads to further increase in lipid mobility in several segments (acyl chain, ceramide headgroups and cholesterol). Another striking observation is that there are no or very minor changes in the molecular mobility in the protein components (Table 2, Fig. 3). The analysis of the samples with thymol is complicated by the fact that the INEPT peak from the $(\omega\text{-}1)CH_2$ in the lipid chains overlaps with the peak

Table 1
Chemical structures with numbered segments of compounds used in this study. Melting point (mp) and logarithm of octanol/water partition coefficient (log *P*) of the added compounds are also shown. Log *P* is used as a facile reference value. It does not directly correlate with the lipid/corneocyte partitioning as it does not account for amphiphilicity and for differences in solubility due to variations in the fluid and solid structures.

Monoterpenes	 Thymol mp = 52 °C; log *P* = 3.3	 Carvacrol mp = 1 °C; log *P* = 3.1	 Geraniol mp = −15 °C; log *P* = 2.5
Free fatty acids	 Dodecanoic acid. mp = 44 °C; log *P* = 4.6		
	 Stearic acid. mp = 69.3 °C; log *P* = 8.2		
	 Oleic acid. mp = 13.4 °C; log *P* = 7.7		
Azone	 Azone. mp = −7 °C; log *P* = 6.3		
Surfactant	 SDS. mp = 205.5 °C; log *P* = 1.6		
Osmolyte	 Urea. mp = 132.7 °C; log *P* = −2.1		 Glycerol. mp = 18.1 °C; log *P* = −1.8

originating from the thymol C8 carbon at 23 ppm. However, by comparing the intensities of the overlapping peaks with those originating from the same thymol molecule (THY C7 and C9) and from other parts of the lipid acyl chain, we can conclude that there is indeed an INEPT enhancement of lipid (ω-1)CH_2 segment.

To summarize the results in Fig. 3, we conclude that monoterpenes investigated all fluidize the SC lipid acyl chain, and that this effect is stronger at lower hydration (compare Figs. 2 and 3). It is also noted that the thymol itself gives rise to INEPT signal (Fig. 3A, D) which is not the case for neat (solid) thymol at the same temperature 32 °C [34]. From this we conclude that thymol is solubilized in fluid regions of the SC, as previously also reported for thymol in lipid model systems [34]. A closer inspection of the spectra in Fig. 3 reveals some differences between the different monoterpenes investigated. At 20 wt% water, thymol has no detectable effect on cholesterol or ceramide segments, while the addition of carvacrol leads to a small increase in the mobility of the ceramide headgroup and cholesterol steroid ring. The strongest effect on the cholesterol mobility is seen for geraniol, where INEPT signal for several carbons in the terminal acyl tail and the steroid ring are resolved (Table 2). Moreover, geraniol has the strongest effect on the mobility in the $(CH_2)_n$ TG segments in the lipid chain at the lower water content. At higher water content, there is no significant difference between the effects of the different monoterpenes on SC lipid and protein components.

3.2.2. Fatty acids

Fatty acids are used as penetration enhancers to increase the permeation of both hydrophilic and hydrophobic compounds. These compounds have shown no or limited irritation and toxicity when applied on the SC [14]. Fatty acids with long saturated acyl chains are also naturally present as main components of SC lipids [2]. Here, we investigated two saturated fatty acids, with different hydrocarbon lengths (dodecanoic acid, $DA_{C12:0}$ and stearic acid, $SA_{C18:0}$), and one unsaturated fatty acid, oleic acid ($OA_{C18:1}$) of the same chain length as $SA_{C18:0}$ (Table 1).

The effects of the fatty acids on SC molecular components clearly vary with the fatty acid acyl-chain composition and the hydration conditions (Table 2 and Fig. 1, Fig. S3). In general, the SC components are more affected at high water content (Fig. S3D–F), and only the fatty acid with lowest melting temperature ($OA_{C18:1}$) showed some significant effects at the lower water content (Fig. S3A–C). Most of the resonances of the added fatty acid chains overlap with the peaks of SC lipid chains (ωCH_3, (ω-1)CH_2, (ω-2)CH_2, $(CH_2)_n$ and αCH_2), which makes it difficult to distinguish their effect on SC lipid chain mobility. However, in some cases, the addition of fatty acids also leads to increased mobility of ceramide headgroups and cholesterol. This effect is strongest for $DA_{C12:0}$ at high water contents. The addition of $DA_{C12:0}$ and $OA_{C18:1.}$ also cause increased mobility in SC protein components at high water contents.

From the present data, we can draw some conclusions on how the added fatty acids behave when present in SC, and this information can be used as a hint to reveal their location in SC domains. The long saturated fatty acid, $SA_{C18:0}$, does not cause any significant changes in the mobility of the SC lipid acyl-chains at any hydration condition (Fig. S3B, E). Still, the resonances from different segments of the $SA_{C18:0}$ molecules itself can be observed in the spectra as an increase in DP and CP intensity. No mobility of $SA_{C18:0}$ molecules can be detected in INEPT spectrum, although it causes a slight increase in mobility in the ceramide headgroups and in some carbons in cholesterol at the highest water content (Table 2, Fig. S3E). The SC protein components appear unaffected by the addition of $SA_{C18:0}$ at all conditions investigated. These results imply that at least a fraction of the added $SA_{C18:}$ is present in the SC structures, presumably in the SC solid lipid domains.

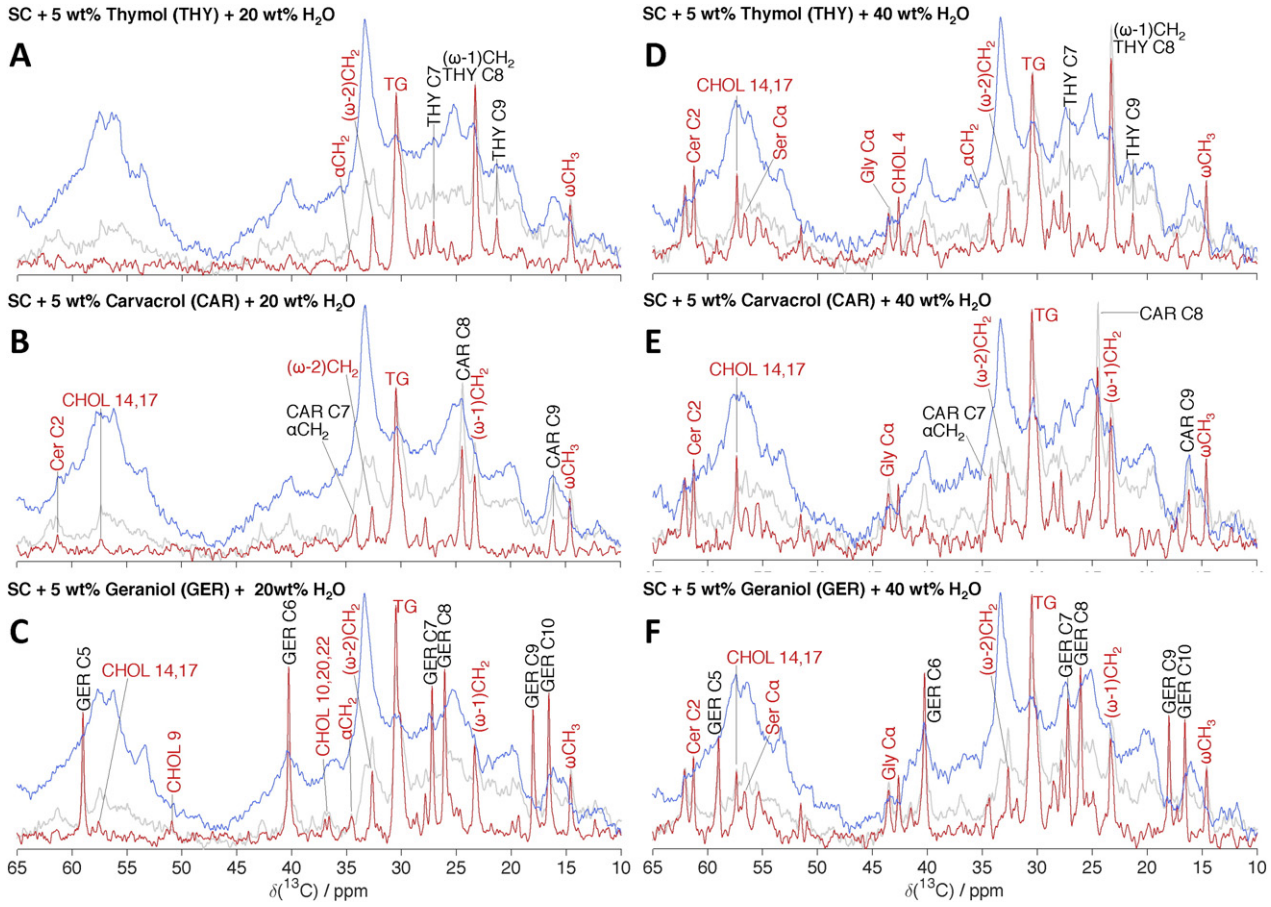


Fig. 3. ^{13}C MAS NMR spectra (DP: grey, CP: blue, INEPT: red) of the SC at 32 °C and at 20 wt% (left) and 40 wt% water (right) with 5 wt% thymol, carvacrol and geraniol. The red labeled peaks are the enhanced INEPT signal compared to the reference SC sample without added compounds at the same water content.

Table 2
The relative changes in the INEPT intensity of selected resonances of lipid and protein molecular segments in the SC with added compounds compared to the reference SC (without added compounds at same water content). The DP signal is used as the reference. The changes were evaluated at both water contents i.e. 20 wt% and 40 wt% water. Approximate increase in the INEPT: ↑ and ↑↑ correspond an increase and a strong increase; 0 signifies no detectable change; * signifies that the INEPT signal appears with added compounds and is not visible in the reference sample; N.A. means non applicable as the effects cannot be resolved due to the overlap of the resonances from the SC lipids and the added compound. # indicates an exception with the addition of $OA_{C18:1}$, where the SC acyl chain resonances are not resolved from the added compounds, still the induced mobility in the SC acyl chain is inferred from the decrease of CP $(CH_2)_n$ AT peak. ^ indicates the INEPT enhancement although the peaks overlap. For systems where duplicate samples were investigated (all samples with 40 wt% water, Figs. 2B, 3D–F, S3D–F, S4B, S5B, S6C–D, S7), only changes observed for both replicates are indicated.

Added compounds		Water content (wt%)	Acyl chain					Lipid								Protein	
												CHOL	Cer head			Ser	Gly
			ωCH3 (14.6 ppm)	(ω-1) CH2 (23.3 ppm)	(ω-2) CH2 (32.7 ppm)	(CH2)nTG (30.5 ppm)	α CH2 (34.2 ppm)	4 (42.7 ppm)	9 (51.5 ppm)	14, 17 (57.4 ppm)	10, 20, 22 (ca. 36.7 ppm)	24 (40.0 ppm)	C1 (55.4 ppm)	C2 (61.3 ppm)	Cα (56.6 ppm)	Cβ (62.1 ppm)	Cα (43.5 ppm)
Monoterpene	Thymol	40	↑↑	^	↑↑	↑↑	↑	↑	0	↑↑	0	0	0	↑↑	↑	0	↑
		20	↑↑	^	↑↑	↑↑	*	0	0	0	0	0	0	0	0	0	0
	Carvacrol	40	↑↑	↑	↑↑	↑↑	N.A.	↑	0	↑↑	0	0	0	↑↑	0	0	0
		20	↑↑	↑↑	↑↑	↑↑	N.A.	0	0	*	0	0	0	*	0	0	0
	Geraniol	40	↑↑	↑	↑↑	↑↑	0	0	0	↑↑	0	N.A.	0	↑↑	↑	0	↑
		20	↑↑	↑↑	↑↑	↑↑	*	0	*	*	*	N.A.	0	0	0	0	0
Fatty acid	$DA_{C12:0}$	40	^	^	^	^	0	↑	↑	↑↑	0	0	↑	↑↑	↑	0	↑
		20	N.A.	N.A.	N.A.	N.A.	0	0	0	0	0	0	0	0	0	0	0
	$SA_{C18:0}$	40	0	0	0	0	0	↑	↑	↑	0	0	↑	↑	0	0	0
		20	0	N.A.	0	0	0	0	0	0	0	0	0	0	0	0	0
	$OA_{C18:1}$	40	N.A.	N.A.	N.A.	#	0	0	0	0	↑	↑	0	0	↑	0	↑
		20	N.A.	N.A.	N.A.	#	0	0	0	0	*	0	0	0	0	0	0
Surfactant	SDS	40	^	^	0	^	0	↑	↑	↑↑	0	0	↑	↑↑	↑	↑	0
		20	N.A.	N.A.	N.A.	0	0	0	0	0	0	0	0	0	0	0	0
Azone	Azone	40	N.A.	N.A.	N.A.	N.A.	0	0	0	↑↑	N.A.	0	0	↑	0	0	0
		20	N.A.	N.A.	N.A.	N.A.	*	*	0	0	N.A.	*	0	0	0	0	0
Osmolyte	Urea	40	0	0	0	↑	↑	↑	↑	↑↑	0	0	↑	↑↑	↑↑	↑	↑
		20	↑	↑	0	↑	0	0	0	0	0	0	0	*	0	0	0
	Glycerol	40	0	0	0	0	0	↑	↑	↑↑	0	0	↑	↑↑	↑	↑	↑
		20	0	0	0	0	0	0	0	0	0	0	0	*	0	0	0

For the $OA_{C18:1}$ and $DA_{C12:0}$, we observe very strong INEPT signals in the acyl chain regions (Fig. S3A, C, D, F). Although the INEPT signals of the SC lipid acyl chains overlap with the prominent INEPT peaks from the fatty acids, the AT peak in the CP spectra decreases after the addition of $OA_{C18:1}$. This indicates melting of a fraction of SC lipids. Moreover, the addition of $OA_{C18:1}$ leads to an increased mobility in cholesterol chain segments at both water contents, while $DA_{C12:0}$ influences both Cer headgroup and CHOL at the highest water content (Table 2). To distinguish the effects of $DA_{C12:0}$ on the SC acyl chains, additional NMR experiments were performed on SC with ^{2}H-labeled $DA_{C12:0}$, which does not contribute to ^{13}C spectra acquired with $^1H \rightarrow {}^{13}C$ polarization transfer. By comparing the spectra of SC with ^{2}H-labeled $DA_{C12:0}$ and reference SC sample from the same batch at the same water content (40 wt% water) (Fig. S8A–B), we confirm the fluidizing effect of $DA_{C12:0}$ on the SC acyl chains.

3.2.3. Surfactant

Surfactants are major ingredients in skin detergents, soaps and cleansers. It has been shown that SDS, one commonly used surfactant, can alter the transepidermal water loss, and that it also influences the transition temperature of SC lipids and disturb the SC SAXS diffraction patterns [35]. At 20 wt% water, the addition of SDS leads to a slight enhancement of INEPT signals at some of these overlapped resonances arising from the lipid acyl chains (Fig. S4A, Table 2). The presence of SDS is also observed as an increase of CP AT peak. Apart from the overlapping effect in the lipid acyl-chains, there are no changes in the mobility of SC components upon the addition SDS compared at the lower water content. At higher water content, the addition of SDS leads to increased mobility in cholesterol and ceramide headgroups as well as a slight increased mobility in the terminal segments in keratin filament (Fig. S4B, Table 2). Additional experiment on SC with deuterated SDS confirms an increase in mobility in the lipid acyl chains (Fig. S8A, C).

3.2.4. Azone

Azone is a hydrophobic compound that was specifically designed as a skin penetration enhancer. It has been shown to increase the skin permeability for hydrophilic and hydrophobic drugs [14,36]. Azone is a liquid at room temperature, and it gives rise to very strong INEPT signals, many of them overlap with the signals from the SC lipid acyl chain segments (Fig. S5). At the low water content investigated, the addition of Azone causes increased mobility of αCH_2 and cholesterol (Table 2). At the higher water content, apart from the effect on cholesterol, we also observed increased mobility in the ceramide headgroups upon the addition of Azone. At all conditions investigated, Azone has no or negligible effect on the molecular mobility in the protein components.

3.2.5. Osmolytes

Osmolytes are small polar compounds that can protect membrane systems against osmotic stress. Osmolytes like urea and glycerol are naturally present in SC as parts of the so-called natural moisturizing factor (NMF) [37,38]. These compounds are also commonly used in commercial skin care lotions and creams, then called humectants [39]. Here we studied two different small polar compounds in SC, urea and glycerol. At 20 wt% water, the addition of glycerol does not lead to any significant changes in the mobility of SC lipid chain, while the addition of urea leads to a slight increase of the INEPT signal from some of the carbons in the lipid chain (Table 2 and Fig. S6A, B). Moreover, both urea and glycerol cause increased mobility in the ceramide headgroups. At the higher water content, the addition of urea or glycerol does not cause any significant additional mobility in the SC lipid chain as compared to the hydrated SC (Table 2, Fig. S6C, D), and it is clear that urea and glycerol have less effect on the SC acyl chain compared to e.g. monoterpenes in all conditions investigated. On the other hand, urea and glycerol have stronger effect on the mobility in the terminal segments of keratin filaments compared to the other compounds used here (Table 2).

3.3. Skin permeability

We investigated the effect of the more hydrophobic compounds and fatty acids on the dermatomed skin permeability of a model drug, Mz, using flow-through diffusion cells. We selected compounds that either showed strong effect (thymol, geraniol and $DA_{C12:0}$) or no effect ($SA_{C18:0}$) on the SC lipid mobility. Commercial skin formulations generally contain many components, including active compounds and excipients. It is clear that several of these ingredients might influence the mobility in SC molecular components. In recent studies from our laboratory we have studied the effect of different polar and apolar solvents on SC (unpublished data), and we have not been able to identify any solvent that does not influence the mobility in SC lipids. For SC samples soaked in solvent, there are additional complications with extraction of, for example, fluid SC lipids. We therefore designed the diffusion experiments in a slightly unconventional way to enable studies of different compounds with no interference from other formulation components. In order to enable quantitative comparisons of the measured permeabilities and fluxes, the thermodynamic activities (chemical potentials) of the added compounds and Mz are adjusted to be the same for all systems investigated. In this way, the driving force of the diffusional transport of Mz across the dermatomed skin is considered to be the same in all cases, and the observed differences are due to the presence of the added "penetration enhancing" compounds in the skin. The experimental data can also be compared to previous studies of how the hydrophilic compounds urea or glycerol influence the diffusional flux of Mz [40]. The hydrophobic compounds investigated all have very low solubility in water and are expected to readily partition into SC. We therefore maintain saturation concentration of these compounds in the donor solution by keeping this solution in direct contact with a reservoir of the pure substance. The conditions are well defined in that the thermodynamic activity of added compounds is close to unity in the donor solution over the whole experiment. On the other hand, the concentrations of the added compounds in the dermatomed skin may vary due to differences in the skin/water partition coefficient. Furthermore, the concentration of the added compound in the SC is not expected to be the same as in the NMR studies described above. For the discussion of the permeability data, additional NMR experiments were performed for sheets of SC exposed to the same conditions as the donor solution containing monoterpenes (Fig. S9). The addition of thymol has significantly stronger effects in decreasing the CP AT signals (Fig. S9 top) and increasing the INEPT signals from SC lipids compared to geraniol. As an example, the INEPT TG/CP AT ratio of $(CH_2)_n$ segments is 19 for thymol and 4 for geraniol. The very prominent INEPT peaks from the monoterpenes in the SC sample further show that these molecules are present at very high concentration when SC is equilibrated with saturated aqueous solutions at concentrations that by far exceed those used in the NMR experiments in Fig. 3.

Table 3
Steady-state flux of metronidazole (Mz) J_{ss} (μg cm^{-2} h^{-1}, mean ± SD) across skin, the permeability P (μm h^{-1}, mean ± SD) of skin and the permeability enhancement ratio ER ($P_{compound}/P_{PBS}$) for different donor formulations. n: number of replicates after removing outliers. N.A. means not applicable due to the lack of steady-state condition.

	Neat PBS ($n = 3$)[a]	Geraniol ($n = 18$)[b]	Thymol ($n = 7$)[c]	Stearic acid ($n = 7$)[a]	Dodecanoic acid ($n = 16$)[b]
J_{ss}	10 ± 1	52 ± 5	N.A.	12 ± 1	125 ± 21
P	13 ± 1	68 ± 7	>1000	16 ± 1	165 ± 28
ER	1	5	>100	1	13

[a] The linear region for PBS and $SA_{C18:0}$ was 18–24 h, and the concentration of Mz in the donor cell changed with <1% after 24 h.
[b] For geraniol and $DA_{C12:0}$, the linear region was chosen from 12 to 16 h, and the concentration of Mz in the donor cell changed with <3% (geraniol) and 9% ($DA_{C12:0}$) after 16 h.
[c] For thymol, the concentration of Mz in the donor solution varies over the experiment and steady-state is not reached. The permeability is then obtained from the slope of the curve and the actual (changing) boundary concentrations using to Eq. (1).

The results obtained for the steady-state flux and the permeability for the different donor formulations are shown in Table 3. Thymol, geraniol and $DA_{C12:0}$ significantly enhance the flux and the SC permeability of Mz, while $SA_{C18:0}$ has negligible effect. The data were analyzed from curves of cumulative permeated mass per membrane area, as a function of time. The outliers were detected by using the generalized extreme Studentized deviate test with significance level of 0.05 [41] and then removed. In order to maintain steady-state conditions, the boundary conditions should be kept constant, meaning constant composition of the donor and the receptor solutions during the measurement. If this is fulfilled, the steady state flux of Mz (J_{ss}) is obtained from the slope of the linear region of the curve, and the SC permeability to Mz (P_{SC}) is calculated from

$$J_{ss} = P_{SC}\Delta c = P_{SC}\left(c_{donor} - c_{receptor}\right) = P_{SC}c_{donor} \quad (1)$$

where c_{donor} is the concentration of Mz in the donor solution, which is assumed to be constant, and $c_{receptor} = 0\ \mu g\ mL^{-1}$ is the concentration of Mz in the receptor solution (sink conditions). Steady-state conditions were considered fulfilled for the neat PBS solution as well as the solutions with geraniol, $SA_{C18:0}$ and $DA_{C12:0}$ (Fig. S10A–D). In case of thymol, steady-state conditions are not fulfilled, as the boundary conditions in Mz concentration are not maintained during the measurement time due to high SC permeability (Fig. S10E). For this case, the permeability is instead obtained from the slope of the fluxes at different actual boundary concentrations in Mz using Eq. (1) for each 2-h time interval (Fig. S10F).

4. Discussion

4.1. Hydrophobic and amphiphilic compounds mainly affect SC lipids, while more hydrophilic compounds affect both SC lipids and proteins components

SC is a composite, where the major fraction is composed of corneocytes (85 wt% in dry SC) [2] filled with keratin filaments, which protruding terminal chains are rich in hydrophilic amino acids. The corneocytes are therefore seen as relatively polar regions of SC that can take substantial amounts of water [42]. The extracellular lipid matrix, on the hand, is mainly hydrophobic and contains long lipids with very low water content [43]. When foreign compounds, such as the penetration enhancers, are added to SC, they partition between the different SC regions on basis of their solubility in the different media. If the compounds alter the balance between fluid and solid lipids, the overall partitioning between SC lipid and protein regions will be affected due to the changes in solubility in the lipid domains. The results presented here show how different types of compounds influence the mobility (fluidity) in SC lipid and protein components (Table 2 and Fig. 1). It is clear that the hydrophobic and amphiphilic compounds, including monoterpenes, fatty acids, Azone and surfactant mainly influence the SC lipids, while hydrophilic compounds, including osmolytes and water, influence both the SC lipid and protein components. From the present experiments, we obtain detailed information about the molecular consequences of adding different compounds to SC, while we cannot draw conclusion about direct contacts between the added compound and, for example, certain lipid species. It is here pointed out that we cannot confirm complete "dissolution" of the added compounds in SC from the present data. However, as we in all cases observe effects on specific SC components, it is inferred that at least a fraction of the added compounds are present in the SC matrix to interact with its lipid and/or protein components.

Hydrophobic and amphiphilic compounds partition more readily into extracellular lipid regions in favor of water-rich regions inside corneocytes. This is consistent with the present observations that these compounds show stronger tendency to fluidize SC lipids, including lipid acyl-chains, cholesterol and ceramide headgroups. These results can be compared to the general phenomena of melting point depression caused by the addition of small amounts of hydrophobic compounds or impurities to solid lipid bilayer systems [34,44]. Previous studies of SC treated with the same hydrophobic or amphiphilic compounds have also shown suppression of SC lipid transitions [15,17,18,35,45–47], as well as alterations in lipid acyl-chain packing [18,35,48] and conformation [45,49]. Here we add to the understanding of these systems, providing detailed molecular information on the balance between mobile and rigid SC lipids and protein components. It has been suggested that monoterpenes, $OA_{C18:1}$ and Azone form segregated fluid domains within SC that may dissolve small amounts of SC lipids [15,45,46,48–50]. From the present NMR data, there are clear indications that at least a fraction of these compounds is incorporated in SC lipid matrix, as implied from the changes in molecular mobility in headgroups and chains of SC lipids. We further conclude that the addition of hydrophobic compounds, fatty acids and surfactant have very minor or no effects on the SC proteins. The results are also consistent with previous studies using Fourier transformed infrared (FT-IR) spectroscopy and calorimetry showing that monoterpenes and $OA_{C18:1}$ do not to alter the SC protein conformation [17,51].

Hydrophilic compounds are expected to partition in the polar domains in the SC. We conclude strong effects of urea and glycerol on the mobility of serine and glycine, which are enriched in the terminal segments of the protein filaments inside the corneocytes. The presence of urea and glycerol in the narrow aqueous layers within the extracellular lipid matrix also influences the lipid self-assembly. The larger fraction of polar components in the extracellular SC lipid regions leads to increased mobility of ceramide headgroups in the bilayer interface, as well as increased fluidity in the hydrophobic regions of the bilayer (Fig. S6). These results are consistent with previous studies of these compounds in model lipid systems and intact SC [7,52,53].

4.2. Molecular effects of added compounds on SC depend on SC hydration

The molecular consequences of the added compounds strongly depend on the degree of SC hydration (Table 2, Figs. 3 and S3–S7). As shown in Fig. 1, the addition of water leads to increased molecular mobility of both proteins and lipid SC components [5], which can be compared to the hydration-induced solid-fluid transition in model lipid bilayers at constant temperature [54,55]. A general observation from this study is that a larger number of SC molecular segments are affected by the addition of the different compounds at the higher water content. In particular, the effects on the molecular mobility in the SC protein components are only seen at 40 wt% water. At this water content, mobile lipid and protein regions are already present in neat SC [5], and the added compounds can more easily dissolve in the more fluid system to further shift the balance between fluid and solid phases.

The samples were prepared in pure water and pH was not controlled. Although there are some uncertainties on the ionization of fatty acids, the majority of the SC and added fatty acids are assumed to be in the deprotonated form (the intrinsic pK_a of the free fatty acid is ca 4.8) [56]. It is noted that self-assembly as well as variation in water content might influence on the degree of fatty acid protonation. The change in water activity can alter the local electrostatic interactions and the dissociation equilibrium of the charged components, which can lead to variations in the pK_a [57]. This is also relevant to the skin as the several studies show that the measured pH at the skin surface depends on skin occlusion [58].

4.3. Specific effects on ceramides and cholesterol in SC

Most of the compounds investigated have the effect to increase the fraction of mobile (fluid) lipid acyl-chains. Beside this, we are also able to distinguish between changes in specific SC lipid components, including ceramide and cholesterol segments. Almost all of the added compounds were shown to affect the mobility in both cholesterol and the ceramide headgroup at high water contents (Table 2). The observed

mobility of the ceramide headgroup segments, especially Cer C2, infers that these lipids are present in a fluid phase, which may also contain fatty acids and cholesterol. Moreover, the changes in the headgroup mobility imply the influences at the lamellae interfacial layers of the added compounds. When comparing the different added compounds, we notice that $OA_{C18:1}$ has negligible effect on the mobility of ceramide headgroup, while other compounds, for example monoterpenes, influence these segments. The observed effect of monoterpenes can be compared to previous FT-IR studies, suggesting that these molecules can break the strong hydrogen bonds between ceramide headgroups [59]. In contrast to monoterpenes, Schaffer et al. reported phase segregation in monolayers composed of ceramide and $OA_{18:1}$ [60] and other studies have shown that only small amounts of $OA_{18:1}$ are miscible in ceramide-enriched domains in model SC lipid system [61].

It is well known that cholesterol (ca. >25 mol%) leads to the formation of a liquid-ordered lamellar phase in mixtures with, e.g., fatty acids and phospholipids [62–65]. This phase is stable at temperatures and water activities where a solid lamellar phase forms in the corresponding neat lipid systems. On the other hand, ceramide has been shown to displace cholesterol from liquid-ordered lipid domains in model lipid system composed of phospholipid-cholesterol-ceramide [66,67]. Our NMR data show no signature of the liquid-ordered lamellar phase, which would give rise to CP signal for the lipid acyl-chain carbon resonances. In fact, we observed the simultaneous increase in mobility of ceramide and cholesterol in most cases (Table 2). It has also been shown that cholesterol preferentially mixes with lipids that have acyl-chain length ranging from 14 to 18 carbons, and that cholesterol promotes segregation in mixtures with longer or shorter lipids [68–70]. In mixtures with several lipid components, the cholesterol miscibility typically increases [71]. In SC, the lengths of the lipid chains range between C14-C32, with C20-C24 being most common [2,72]. As the fraction of mobile lipid increases upon hydration and/or addition of other compounds, the acyl-chain composition in the fluid lipid domains is expected to change, which might in turn influence CHOL miscibility. However, we are not able to distinguish any such effect from the present data.

4.4. Comparison between linear and cyclic monoterpenes

We conclude that among the investigated compounds, the monoterpenes are the most efficient compounds to melt SC lipids. The effect of monoterpenes is more pronounced at lower hydration (Fig. 3). Similar behavior was also observed for monoterpenes in model lipid system [34]. At 20 wt% water, geraniol shows the strongest effect on enhancing the mobility of carbons in the TG acyl chains and cholesterol (Fig. 3). This implies that the addition of the linear monoterpene causes melting of a larger fraction of long-chain fatty acids and ceramides compared to the other monoterpenes. Melting of long-chain lipids would give additional weight to the INEPT signal of TG resonance, resulting in the pronounced change in the INEPT signal of TG acyl chains compared to the negligible differences in the INEPT signals of other acyl chain segments, e.g. ωCH_3. Out of the two cyclic isomeric monoterpenes, carvacrol has the strongest effect on cholesterol mobility. As the hydroxyl group is expected to locate close to the polar interfacial region, the change in the position of this functional group on the benzyl ring may influence its orientations in the lamellae, and thereby its effect on the surrounding lipid molecules.

4.5. Comparison between linear compounds with different acyl chains

The effects caused by adding fatty acids to SC strongly depend on the hydrocarbon chain composition. While it is not possible to draw definite conclusions on the effects of these compounds on the mobility in the SC lipid chains due to overlapping signals, it is clear that the addition of fatty acids leads to increased mobility in cholesterol and ceramide headgroup segments. This effect is less pronounced for the long saturated $SA_{C18:0}$ compared to the short saturated $DA_{C12:0}$. Based on these observations, we propose that the added fatty acids are present in different regions of the SC extracellular lipids, where $DA_{C12:0}$ is enriched in the fluid SC lipid domain, and $SA_{C18:0}$ is present in solid domains. It is also possible that the solid domain is partly segregated $SA_{C18:0}$ co-existing with solid SC lipids. Furthermore, the addition of $DA_{C12:0}$ causes a small increase in the mobility of protein at high water content, which was not observed for the longer saturated fatty acid. This can be related to the differences in hydrophobicity between the fatty acids.

Even though the resonances from the acyl chain of the SC lipids cannot be resolved from the peaks originating from the hydrocarbon chains in the added fatty acids, we conclude increased fluidity in SC lipids upon addition of the long unsaturated $OA_{C18:1}$ based on the decrease of CP AT signal (Fig. S3). Furthermore, the addition of $OA_{C18:1}$ causes increased mobility in the acyl tail of cholesterol (Table 2). Due to its high potential as SC penetration enhancer, the effects of adding $OA_{C18:1}$ to SC has previously been investigated by different experimental approaches, including FT-IR, differential thermal analysis and molecular dynamics simulations [15,45,49,73]. Based on simulations, Hoopes et al. proposed that the incorporation of $OA_{C18:1}$ in model SC lipid bilayers causes an increase in the lateral diffusion of cholesterol in bilayer, and no effects due to interactions with the ceramide headgroups [73]. This is consistent with the molecular changes in these SC lipids as observed here for intact SC (Table 2). It is here noted that the addition of $OA_{C18:1}$ at the high water content results in an increased protein mobility (Table 2), which is similar to $DA_{C12:0}$.

Hydrophobic mismatch has been shown to induce fluidity in lipid system [74], which is consistent with the present observation that $DA_{C12:0}$ is more efficient in fluidizing SC lipids compared to $SA_{C18:0}$. SDS has the same hydrocarbon chain as $DA_{C12:0}$, and it also gives rise to very similar response in the mobility of cholesterol and ceramide headgroup. Still, their differences can be seen in the INEPT signals of the unresolved acyl chains. On the other hand, the more hydrophobic compound with C12:0 chain, Azone, shows a different behavior in that it causes higher mobility at lower hydration, and less effect on cholesterol and ceramide headgroups at high hydration.

4.6. Comparison between different small polar compounds

Urea and glycerol are polar compounds that are not expected to partition into the hydrophobic regions of the lipids, but will rather locate in the aqueous regions between the lipid lamellas as well as inside the corneocytes. It is noted that urea and glycerol has the strongest fluidizing effect on the terminal segments (that are abundant in glycine and serine residues) in the protein filaments among all compounds investigated (Table 2, Figs. 2 & S6). In particular, it is interesting that we do not observe major changes in the protein mobility of the filament cores. Urea is known to weaken the hydrophobic interactions and it is commonly used for protein denaturation [75]. This weakening is apparently not sufficient to solubilize or disturb the solid core of the protein keratin filaments, which are still solid. In addition, the polar compounds also influence the lipids. The effects on the lipids can be explained by the increase in the polar components (water + urea/glycerol) in the SC, which was previously shown to cause melting of lipid and protein components [7,53]. Urea shows slightly stronger effect on lipid chain segments compared to glycerol. This observation is also consistent with previous studies [7,53].

The present study was designed to keep the concentration of added compounds the same in all samples (based on wt% relative to the dry SC), and comparisons are made to reference SC samples at the same water content (20 or 40 wt% water). It should be noted that even though the amount of water in the SC sample is the same, the water activity (or, equally, chemical potential of water, directly related to the vapor pressure or the RH) is most likely not the same. In particular, the addition to the water-soluble compounds leads to clear reduction in the water activity, which has previously been shown to cause reduced mobility in both lipid and protein components [5]. For those cases, it is therefore

impossible to maintain both water content and water activity constant through the comparison. In a previous study where urea and glycerol were added to intact SC equilibrated at the same RH = 80%, but different water content (>20 wt% for all samples investigated), a clear effect on both protein and lipid mobility was seen [7]. In the present study, which is performed at lower water contents (20 wt%), the effect of the same compounds on SC lipid and protein components is very small (Fig. S6 A,B). When comparing the present and previous studies, we need to keep in mind that when we add urea or glycerol to the sample, we also reduce the water activity. We cannot distinguish between the effects of the added compound and the effect of dehydration, and we only see the combined effect. This complication is of less importance when comparing studies with hydrophobic compounds, as these compounds have very low water solubility and thus very minor influence on water activity.

We finally note that at high water content (40 wt%), the fluid lipids are already present in SC (Fig. 2B), and the addition of the polar compounds has only minor influence on the mobility of the SC lipid acyl chains (Table 2 and Fig. S6C, D). This is in contrast to the more hydrophobic compounds and fatty acids (e.g. monoterpenes and $OA_{C18:1}$) which cause significant enhancement of the mobility in the lipid carbon chain segments for all hydration conditions investigated.

4.7. Link between SC molecular mobility and SC barrier function

From the diffusion cell experiments with selected compounds (Table 3), we confirmed increased permeability of the dermatomed skin to the model drug Mz (log $P = 0$) for the same compounds that cause increased mobility in SC lipid and protein components. No changes in skin permeability were shown for the $SA_{C18:0}$ which was also shown to have very minor effects on the SC molecular dynamics. Previous studies on formulations with more complex compositions in terms of solvents and excipients (water activity not specified) have also shown that monoterpenes and $OA_{C18:1}$ increase the permeability to hydrophilic and hydrophobic drugs [15–17,76,77], and it has been reviewed that terpenes, including monoterpenes, are effective enhancers for drugs with different hydrophobicities [14]. For saturated fatty acids, the highest permeation enhancement was seen with hydrocarbon chains with 10–12 carbons [15,78]. In case of polar substances like urea and glycerol, we have previously shown that penetration can be enhanced at slightly reduced hydration conditions in the presence of these substances [40]. Although quantitative comparisons with the present NMR results cannot be done as these experiments were performed in very different conditions, the qualitative trends are reproduced with respect to which compounds that cause increased SC permeability and different hydration conditions.

In the flow-through diffusion cell studies, the thermodynamic activities of the monoterpenes and fatty acids in the donor solution are all close to unity during the whole experiment. The concentrations of these compounds in the skin are likely not the same and they are also different from the conditions used in the NMR studies (compare signal intensities in Figs. 3 and S9). This difference in concentration can explain the observed differences in penetration enhancement between thymol and the other compounds investigated. Indeed, thymol has higher octanol/water partition coefficient than geraniol [79], therefore it is expected to have higher concentration in the skin. In the NMR experiments for SC sheets treated with the donor solutions used in the diffusion cell studies, it was also shown that thymol have stronger effect than geraniol on SC lipid mobility (Fig. S9). The permeability in solid bilayers is much lower compared to fluid bilayers [8,9], and this difference can be attributed to higher solubility and diffusivity of the diffusing compounds in the fluid structures. In previous studies, we found that SC hydration causes increased mobility in SC lipid components [5], which can be related to many observations of increased SC permeability in similar hydrated conditions (occlusion) [11,80]. Another potentially important aspect is the arrangement of the segregated fluid and solid domains in SC with the added compound. In lipid bilayer system, anomalous high permeability behavior has been observed in segregated bilayer membranes and attributed to the domain interfaces [8]. Experiments in model lipid systems have indeed also shown that thymol is clearly more effective in inducing phase segregation compared to geraniol [34], which can in turn affect the diffusional pathway and the overall permeability.

4.8. Implications to formulations

One essential aspect in the development of (trans)dermal drug delivery and cosmetics formulations is to understand the mechanisms behind the penetration enhancing activity in order to optimize the use of excipients. As shown in this study, PT ssNMR is a useful tool to distinguish the effects caused by different types of compounds on the SC molecular constituents. In addition, this method can help to understand the roles of the individual components and the types of interactions in complex formulations, for example non-interactive, additive, synergistic and antagonistic interactions. The concentration dependence of different compounds in formulations can also be revealed by this method [7].

There are many compounds that have been considered as chemical penetration enhancers on basis of their hydrophobicity and partition coefficients [81]. We argue that another key aspect to understand the penetration enhancement efficiency is to consider the phase changes promoted by the added compounds when incorporated into the SC in a certain condition. This will depend on the molecular structure as well as other properties of the formulation, for example, water activity. The hydration dependence is also highly relevant to evaluate the use of the non-occluding or occluding conditions when applying cosmetic or pharmaceutical formulations on the skin [29].

The hydrophobicity of the added compound is crucial in determining their distribution between lipid and protein regions in SC. This is highly relevant in relation to targeting effect on certain SC components. It can also have implications to formulations aiming at treating, for example, diseased or very dry and scaly skin. Furthermore, transport of drugs with different hydrophobicity can take different routes in the skin. If the mobility of a specific region of the composite SC increases, the solubility of most drug molecules also increases, and this also have implication for the drug permeability. For hydrophilic drugs, the hydrophobic regions in the extracellular continuous matrix constitute the main barrier. For hydrophobic drugs, on the other hand, the solubility in the aqueous regions inside the corneocytes is expected to be low, and the extracellular lipid regions are considered to constitute the dominant transport route. The general implication of this is that the addition of compounds that induce fluidity in SC lipids (including hydrophilic, hydrophobic and amphiphilic compounds, see Fig. 1) - are expected to increase SC permeability to both hydrophilic and hydrophobic (small) active compounds. For hydrophilic drugs, additional penetration enhancement might be achieved by adding compounds that also induce mobility in the protein components (include hydrophilic and amphiphilic compounds, Fig. 1). One example that illustrates that melting of lipids indeed affect diffusional transport of polar compounds is found in increase in water vapor permeability of porcine SC at elevated temperatures and constant water activity [82] where proteins are rigid and lipids are mobile [5]. Another example is the increased permeation of polar molecules zidovudine and 5-fluorouracil in the presence of monoterpenes [16,76], which were here shown to only affect SC lipids.

5. Conclusions

In this work we investigate the effects of different compounds relevant as chemical penetration enhancers on the molecular mobility of SC components and on the permeability of the skin in well-defined and controlled systems. The obtained data clearly demonstrated that

(i) PT ssNMR is a good method to distinguish the influence of added compounds on different SC molecular constituents.

(ii) hydrophobic compounds, fatty acids and surfactant mainly affect SC lipids while polar compounds affect both SC lipid and protein components.

(iii) molecular effects of added compounds on SC depend on SC hydration which should be taken into account when preparing and applying formulations.

(iv) the molecular mobility can be linked to the macroscopic permeability of the skin barrier membrane.

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Appendix A. Supplementary data

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