Tracking solvents in the skin through atomically resolved measurements of molecular mobility in intact stratum corneum

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Solvents are commonly used in pharmaceutical and cosmetic formulations and sanitary products and cleansers. The uptake of solvent into the skin may change the molecular organization of skin lipids and proteins, which may in turn alter the protective skin barrier function. We herein examine the molecular effects of 10 different solvents on the outermost layer of skin, the stratum corneum (SC), using polarization transfer solid-state NMR on natural abundance $^{13}$C in intact SC. With this approach it is possible to characterize the molecular dynamics of solvent molecules when present inside intact SC and to simultaneously monitor the effects caused by the added solvent on SC lipids and protein components. All solvents investigated cause an increased fluidity of SC lipids, with the most prominent effects shown for the apolar hydrocarbon solvents and 2-propanol. However, no solvent other than water shows the ability to fluidize amino acids in the keratin filaments. The solvent molecules themselves show reduced molecular mobility when incorporated in the SC matrix. Changes in the molecular properties of the SC, and in particular alternation in the balance between solid and fluid SC components, may have significant influences on the macroscopic SC barrier properties as well as mechanical properties of the skin. Deepened understanding of molecular effects of foreign compounds in SC fluidity can therefore have strong impact on the development of skin products in pharmaceutical, cosmetic, and sanitary applications.

keratin filaments | solid-state NMR | extracellular lipids | corneocytes | phase behavior

The human skin makes up a large interfacial barrier film that protects the body from desiccation and uptake of hazardous chemicals. In many situations in everyday life, the skin is, consciously or unconsciously, exposed to solvents and other chemicals. The uptake of solvents in the skin may lead to changes in the molecular organization of skin lipids and proteins, which in turn may alter macroscopic properties of the skin, including skin flexibility, softness, and permeability (1–6). The exposure to solvents may also cause irritation, corrosion, or irritant contact dermatitis (7). When the skin is exposed to solutions or creams with complex composition—such as skin lotions, cleansers, washing agents, or pharmaceutical products—several molecular mechanisms operate simultaneously due to interactions between molecular components in the skin and molecules in the complex solution, in which a solvent is often the main component.

The barrier function of the skin is assured by its outermost layer, the stratum corneum (SC), which consists of dead keratin-filled cells, corneocytes, embedded in a multilamellar lipid matrix (Fig. 1) (8, 9). SC differs from most other biological membranes in that the main fraction of both lipids and proteins is solid at ambient conditions (10, 11). Still, a minor fraction of lipids and protein components is mobile at physiological temperatures in conditions of hydrated SC, and after the addition of so-called moisturizers or penetration enhancers (6, 11–15). The macroscopic material properties of the SC barrier membrane naturally depend on the molecular properties of its building blocks. In particular, transport properties as well as mechanical properties and water-holding capacity can be altered by changing the balance between solid and fluid structures in the complex SC material (13, 14, 16–19). Increasing fluidity is expected to lead to higher solubility and a higher diffusion coefficient for most added compounds, and thus increased effective SC permeability (14, 18, 19). A clear correlation between SC fluidity/molecular mobility and permeability of small molecules in SC has been demonstrated in previous studies (11–14, 16, 17, 20). The presence of fluid domains in the SC matrix is likely also important for other biological functions, for example the enzyme activity in the SC intercellular space (21). There is a rather extensive literature describing how solvents and excipients influence the extracellular solid SC lipids based on studies using scattering, diffraction, and infrared spectroscopy techniques (2, 3, 5, 22, 23). Calorimetric studies have further shown that solvents may alter the thermal solid–fluid transitions in SC (2, 3, 5), which typically occur at nonphysiological elevated temperatures. However, these studies provide very limited information on how solvents influence the minor fractions of SC lipid and protein components that are fluid at ambient temperatures.

In this study, we use polarization transfer solid-state NMR (PT ssNMR) (24) on natural abundance $^{13}$C to obtain atomically resolved information on molecular dynamics in intact SC (11). We are able to track the solvent molecules when incorporated into the SC complex matter and study their interactions with SC matrix via changes in their molecular dynamics. Simultaneously, we obtain information on the molecular effects caused by the added solvent on SC lipid and protein components in the very same sample. The present NMR method is sufficiently sensitive to characterize the solvent molecules when they have been incorporated in SC, thus allowing for an atomically resolved mapping of solvent molecules in SC.

**Significance**

Our skin is regularly exposed to solvents in cosmetics, washing and sanitary agents, and drug formulations. The uptake of solvents into the skin may change essential properties of the skin, for example, its protective barrier function, as well as its flexibility and softness. Herein different solvents relevant to skin formulations and sanitary products were added to samples of intact stratum corneum (SC), which is the outer layer of the skin. The solvent molecules can be tracked inside SC, showing reduced mobility. Furthermore, the solvents induce fluidity in SC components. These changes depend on solvent identity and concentration and on SC hydration conditions. Changes in SC components can be related to changes in macroscopic properties of SC, including skin barrier function.

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sensitive to detect changes in the minor fraction of mobile components, and it also gives information on the major fraction of rigid components (11, 24), thereby providing information on SC molecular dynamics with a molecular detail that has not been reachable before. In addition, it is possible to distinguish several coexisting fractions of the same type of molecular component with different molecular dynamics.

We report the molecular effects of 10 different solvents representing different molecular classes with respect to structure and hydrophobicity (Table 1). We use samples of intact porcine SC treated with solvents and, in the very same experiments, we study the molecular dynamics (“fluidity”) in solvent, as well as the SC lipid and protein components. When added to the complex structure of SC the solvent molecules will partition between the relatively hydrophilic domains in the keratin-filled cornocytes cells and hydrophobic extracellular lipid regions (Fig. 1). It has previously been shown that substantial amounts of water can be taken up into the cornocytes (25), which has a strong effect on the molecular mobility in the hydrophilic amino acids that are enriched in the terminal chains of keratin filaments (11). The extracellular matrix contains hydrophobic lipids with long acyl chains and very low water content (26, 27). Still, tiny amounts of water and other polar solvents can be present in the vicinity of the polar headgroups of the lipids (3, 11, 12). Apolar compounds, on the other hand, likely partition into the hydrocarbon layer in the extracellular lipid matrix. In many cases there is an effect of the treatment with the external chemicals on the fluidity in lipids and keratin filaments (2, 4, 12, 13, 28), which in turn may alter the solvent partitioning balance.

Most of the solvents investigated here are found in pharmaceutical, cosmetic, and sanitary applications. Methanol and ethanol are common laboratory solvents. Ethanol is also frequently used in washing and sanitizing as well as in (trans)dermal patches with long skin exposure times. Some of the polar and apolar solvents [e.g., octamethyltrisiloxane (OMTS) and 1-decanol] are typical (co)solvents in personal care, cosmetic, and topical pharmaceutical formulations, where propylene glycol (PG) is also used as a penetration enhancer (6). Glycerol is naturally present in SC as a component of the so-called natural moisturizing factor (29) and it is commonly used in skin care products as humectant and/or penetration enhancer (13, 15, 20). DMSO is used in industry and topical applications as a solvent for unsaturated and aromatic hydrocarbons. DMSO has also been shown to enhance percutaneous absorption of certain chemical agents (30) and it possesses some biological functions including antiinflammatory activity (31).

We aim at deepened understanding of interactions between various solvents and SC lipid and protein components in well-defined controlled conditions in terms of hydration and solvent concentration. We investigate samples of intact SC using PT ssNMR experiments, with the following objectives:

- Characterize molecular dynamics and partitioning of the varying classes of solvent molecules between different regions within the complex structure of SC.
- Elucidate how the different solvents influence the molecular mobility in different segments of SC lipids and proteins.
- Distinguish general trends of how different classes of solvents influence SC molecular components and relate this to the properties of solvents.
- Investigate the consequences of washing SC in excess solvent.

**Results**

**PT ssNMR to Monitor Molecular Conformation and Dynamics in Molecular Matter with Complex Composition.** We aim at molecular characterization of the interaction between solvent molecules and lipids and proteins within intact SC. From the PT ssNMR measurements we observe changes in molecular dynamics of the solvent molecules inside intact SC. Simultaneously, we detect changes in the molecular mobility in SC lipid and protein components. We start by shortly introducing the PT ssNMR method to study intact SC (Fig. 1) and a summary of the main findings (Fig. 2 and Table 1). This is followed by a more detailed presentation of the results for each individual case.

In a $^{13}$C NMR spectrum the molecular moieties of the SC components and solvents can be differentiated based on their chemical shifts, and the mobility of each segment is further obtained by using polarization transfer. The PT ssNMR experiments are sensitive to the minor fraction of mobile components in intact SC, and they simultaneously provide information on the rigid/obstructed components in the very same sample. We can distinguish the same molecular segment that resonates at different chemical shifts depending on molecular conformation and dynamics. Even at the same chemical shift, chemically identical segments with different molecular mobility can be discerned based on the line shapes and intensities of the peaks. Furthermore, for each molecular segment, it is possible to characterize the timescale and anisotropy of C–H bond reorientation.
and SI Appendix, Fig. S1) by comparing the signal intensities obtained with the direct polarization (DP), cross-polarization (CP) (32), and insensitive nuclei enhanced by polarization transfer (INEPT) (33) acquisition schemes. Example spectra for hydrated intact SC are shown in Fig. 1. The DP spectrum generally shows resonances from all carbons in the sample and is here used as reference. Under the present experimental conditions, the DP signal is quantitative when the rotational correlation time $\tau_c < 10$ ns and gradually disappears at longer $\tau_c$ (24). CP is efficient in boosting the signal of segments with slow ($\tau_c > 0.1$ ms) and/or anisotropic motions. The CP signal is not observable for nearly isotropic reorientation (order parameter $J_{SCCH} < 0.01$) with fast dynamics ($\tau_c < 10$ ns). However, the signal from mobile segments ($\tau_c < 10$ ns) is selectively enhanced in the INEPT spectra, whereas the INEPT signal is not visible for slower motion ($\tau_c > 0.1$ ms) and/or highly anisotropic reorientation ($J_{SCCH} > 0.5$) (24). The dependence of CP and INEPT intensities on $\tau_c$ and $J_{SCCH}$ of the C–H bond reorientation is shown in SI Appendix, Fig. S1. In addition, the lineshape of the CP resonances also provides information on the molecular dynamics in different segments. Under the experimental conditions used here, maximum line broadening due to motion-decoupling interference is expected at $\tau_c$ around 0.1 $\mu$s (24, 34), and this effect gradually disappears at both faster and slower motions. Furthermore, a segment with molecular reorientation on the timescale of magic-angle spinning (MAS) ($\sim 0.1$ ms) would give rise to a broad CP signal due to spinning-motion interference, and this broadening effect also dissipates at both faster and slower motions (35). In fact, a segment with a range of molecular conformations and microenvironments with different chemical shifts, as observed in the present system, would give rise to a broad signal when $\tau_c > 0.1$ ms because the motion is not fast enough to average the resonance frequencies (36). Taken together, the line narrowing effect due to faster motion is expected to be observed at $\tau_c \sim 0.1$ $\mu$s.

To facilitate the presentation of the data in this paper, we define “mobile” and “rigid” segments for SC components according to the rate and anisotropy of C–H bond reorientation (SI Appendix, Fig. S1). The INEPT signal indicates a “mobile” segment, which can be “fast isotropic” or “fast anisotropic.” In the latter case, the INEPT signal is accompanied by a sharp CP signal. The term “rigid” is defined by the conditions when only CP signal is detected. The solvent molecules are characterized as isotropic, fast anisotropic, or “obstructed,” where the latter signifies solvent

<table>
<thead>
<tr>
<th>Solvent</th>
<th>$\log P$</th>
<th>$M_w$</th>
<th>Information</th>
<th>Solvent concentration, wt %</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5</td>
</tr>
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<td>18</td>
<td>N.D.</td>
<td>N.D.</td>
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<td>92</td>
<td>obs</td>
<td>obs &amp; iso</td>
</tr>
<tr>
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<td>78</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>PG</td>
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<td>76</td>
<td>obs</td>
<td>obs &amp; iso</td>
</tr>
<tr>
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<td>32</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Ethanol</td>
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<td>46</td>
<td>obs</td>
<td>obs &amp; iso</td>
</tr>
<tr>
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<td>obs</td>
<td>obs</td>
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<tr>
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<td>158</td>
<td>fast ani &amp; iso</td>
<td>fast ani &amp; iso</td>
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<tr>
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<td>226</td>
<td>fast ani &amp; iso</td>
<td>fast ani &amp; iso</td>
</tr>
<tr>
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<td>237</td>
<td>obs</td>
<td>obs, fast ani &amp; iso</td>
</tr>
<tr>
<td>Trisiloxane</td>
<td></td>
<td></td>
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Here, the solvent was added at varying concentrations to dry SC with no additional water. The solvent concentration was varied to identify a concentration regime where an obstructed/fast anisotropic fraction of solvent coexists with an isotropic fraction of solvent. The logarithm of octanol/water partition coefficient ($\log P$) and the molecular weight ($M_w$, g/mol) of the solvents are also listed. $\log P$ is used as a facile reference value for the hydrophobicity of the solvents. It does not account for differences in solubility due to variations in the fluid and solid structures. The colors indicate the types of solvents: polar solvents (blue), apolar solvents (yellow), and the solvent with $\log P \approx 0$ (green). In the case of water, the molecular dynamics of the solvent is not studied, because water does not contain any carbons. The effects of the solvents on SC lipid chain mobility are compared with the effects of water at the same concentration. The symbols should be read as weaker (<), stronger (>), and much stronger (>>) fluidizing effect compared with water. Abbreviations: fast ani, fast anisotropic; iso, isotropic; N.A., not applicable; N.D., not detected; and obs, obstructed. All experiments were performed at 32 °C.
segments that are detected in the CP and DP spectra but not in the INEPT spectra and that have slower motion (τ_c < 0.1 µs) compared with fast isotropic reorientation (Fig. 2A). For the present system with solvents incorporated in SC we can rule out the interpretation of sharp CP signal of the solvents as a sign of solid crystals or the fast (τ_c < 10 ns) and highly anisotropic (|S_C1H| ≥ 0.5) motion that is expected for rotator- and liquid-ordered phases (37).

**Characterizing Solvents Within the SC.** We investigate interactions between SC and 10 different solvents (Table 1) that are widely used in skin products. NMR spectra obtained for SC with added solvents are shown in Figs. 3 and 4 and SI Appendix, Figs. S2–S4. As reference, we compare with dry SC (Fig. 1C). In cases where the solvent molecules contain one or more C–H bonds, the PT ssNMR experiments provide detailed information on molecular dynamics of the solvent molecules. In neat solutions, the molecular motion of the solvents is isotropic and fast. When the solvent molecules are present within the SC they may interact with lipid and protein components, which can alter the molecular dynamics of the solvent molecules.

The comparison between results obtained for the different systems (Table 1) reveals that all polar solvents are strongly affected by being incorporated into SC. At low concentrations, one or several segments of the solvent molecules are obstructed with τ_c < 0.1 µs, as inferred from the broad CP signals accompanied by DP signals and no INEPT intensity at their resonances (Figs. 3A and 4A and SI Appendix, Fig. S2A, C, and E). From these data, it is shown that the correlation time for the solvents in SC is several orders of magnitude lower compared with solvents in neat solutions [for comparison, τ_c ~ 0.5 ps, 4 ps, and 0.6 ns in neat solutions of methanol, DMSO, and glycerol, respectively, at 25 °C (38–40)]. The reduction in molecular mobility is a strong indication of interaction between the polar solvent molecules and SC molecular components. When the amount of solvent in SC is increased (Fig. 3B and C and SI Appendix, Fig. S2B, D, and F), the motion of the polar solvents becomes faster, as implied from the sharper CP signals. At the highest solvent concentrations investigated we also observe a coexisting fraction of solvent in the sample that has the characteristic of being fast isotropic (Fig. 3B and C and SI Appendix, Fig. S2B, D, and F). Similar behavior as shown for the polar solvents is also seen for the slightly more apolar 2-propanol (Fig. 4B and SI Appendix, Fig. S3) in SC.

The apolar solvents are less affected than the polar ones by being incorporated into SC. As summarized in Table 1, at least a fraction of the apolar solvents show the characteristics of being fast isotropic also at the lowest concentrations investigated...
Concentration dependence of different solvents in SC. Different solvents in SC at the same concentration of 20 wt % solvent. (Table 1). For the hydrocarbon solvents (Figs. 3 D and E and 4C and SI Appendix, Fig. S4A) we detect two coexisting populations of solvent molecules at all solvent concentrations. One population of the solvent exhibits fast isotropic motion, and the other population shows fast anisotropic motion (Fig. 2C and Table 1). Finally, the apolar siloxane solvent molecules (OMTS) only show fast isotropic motion when added to SC (SI Appendix, Fig. S4B), similar to a neat isotropic solution of OMTS. From the present analysis, we are not able to pinpoint the location of fast isotropic solvent molecules, and we cannot distinguish between cases when the solvent is present in isotropic fluid domains within SC (11, 41–44) and when it is present in an excess solution. However, we combine the studies of the molecular dynamics of the solvent molecules with an analysis of how the added solvent influences the molecular dynamics in SC lipid and protein components. Based on the combination of information on molecular dynamics in the solvent as well as in SC lipid and protein components we are able to draw conclusions on the uptake of solvents into the SC.

PT ssNMR to Study Molecular Effects on SC Molecular Components. In previous studies, we managed to assign the chemical shifts of all major peaks in the crowded 13C spectra to carbons in lipids and amino acids in intact SC (11). The assignment was based on samples of intact SC together with reference systems of isolated SC lipids and corneocytes as well as simplified model systems. In the analysis of the spectra, we here focus on representative SC lipid and protein molecular segments (SI Appendix, Table S1), as illustrated in Fig. 1E. The Gly Cα, Ser Cα, and Ser Cβ peaks are used as signature peaks for the terminal domains of the keratin filaments that are rich in glycine and serine (45) (UniProt ID codes P04264 and P13645). The core of the keratin filament is enriched in leucine and lysine (UniProt ID codes P04264 and P13645), which are examined at the resonances of Leu Cα and Lys Cα. For the SC lipids, the acyl chains of fatty acids and ceramides can be probed at the resonances of the terminal methyl/methylene carbons [ωCHn, (ω-1)CH2, and (ω-2)CH2] and of αCH2. In addition, the majority of the lipid acyl chain (CH2)n resonates within a range of 30–34 ppm, in which the all-trans conformation (AT) predominating in solid phases is visualized at 33.4 ppm in the CP spectrum, whereas the liquid-like distribution of trans/gauche conformations (TG) are probed at 31 ppm in the INEPT spectrum (46). Different segments of cholesterol (CHOL 4, 9, 10, 20, 22, and 24) and ceramide headgroup (Cer C1 and C2) can also be distinguished. The strategy when analyzing the crowded spectra from SC is to look for consistency in the observed changes. In other words, we draw conclusions about changes in molecular mobility for a certain molecule if we observe changes in more than one segment in the same molecule (if applicable).

A general conclusion from the combination of data presented here is that all spectra obtained for SC with added solvents at 32 °C (Figs. 1 C–E and 3–5 and SI Appendix, Figs. S2–S7) are dominated by the CP signal for most of the spectral range. This implies that the majority of SC lipid and protein components are

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**Fig. 3.** Concentration dependence of different solvents in SC. 13C MAS NMR spectra (DP, gray; CP, blue; and INEPT, red) of the SC with 10 (A), 20 (B), and 40 (C) wt % glycerol, 5 (D) and 20 (E) wt % hexadecane, and 20 wt % (F) deuterated hexadecane at 32 °C. Resonances from solvent carbons are marked with asterisks.

**Fig. 4.** Different solvents in SC at the same concentration of 20 wt % solvent. 13C MAS NMR spectra (DP, gray; CP, blue; and INEPT, red) of the SC at 20 wt % PG (A), 2-propanol (B), and 1-decanol (C) at 32 °C. Resonances from solvent carbons are marked with asterisks.
Changes in SC Molecular Dynamics in the Presence of Solvents. We investigated how various solvents influence the molecular dynamics in different segments of SC lipid and protein components, and the findings are summarized in Fig. 2 B and C and Table 1. In dry SC, the keratin filaments are completely rigid, as apparent from the total absence of INEPT signal from protein carbons (Fig. 1C). For the lipid components, however, there is a minute fraction that is mobile, which gives rise to the low-amplitude INEPT peaks of the terminal methyl carbons (CH₃) and the trans/gauche conformation (CH₂) TG (Fig. 1C). Upon hydration, the fraction of mobile SC lipid increases (Fig. 1 D and E). The protein components remain solid up to water content of at least 24 wt % (Fig. 1D) (11), whereas at higher water contents (40 wt %, Fig. 1E) the amino acids in the terminal segments of the keratin filaments become mobile.

We then replace water with another solvent. For all systems investigated, it is shown that the addition of solvent leads to increased mobility of the SC lipid acyl chains. The magnitude of this fluidizing effect varies with the identity and the concentration of the solvents. A comparison between different solvents at the same concentration shows the strongest effect for the apolar solvents (1-decanol, hexadecane, and siloxane), as well as for 2-propanol (Table 1). Among the polar solvents investigated, only water shows detectable effects on cholesterol and ceramide headgroups at very high concentration (40 wt % in SC, Fig. 1E), whereas the apolar solvent 1-decanol influences the molecular segments of cholesterol already at lower concentration (20 wt % in SC, Fig. 4C). It is notable that the protein components remain rigid for all solvents investigated at concentrations up to 40 wt % in SC, with water as the only exception (Fig. 1E).

Molecular Mobility of Polar Solvents Within Intact SC. One striking observation from the present data is that the polar solvent molecules themselves seem obstructed when present inside SC at low concentrations (Table 1, Figs. 3A and 4A, and SI Appendix, Fig. S2A, C, and E). Further examination of the lineshape of the peaks reveals more detailed information on the molecular dynamics of these solvent molecules. The CP resonances of carbons bound to hydroxyl group of glycerol (Fig. 3A), PG (Fig. 4A), methanol and ethanol (SI Appendix, Fig. S2 A and C), and the carbon of the –CH₃ group of DMSO (SI Appendix, Fig. S2E) are broad and accompanied by DP resonances, indicating that these carbons are obstructed with τₑ ~ 0.1 µs (Fig. 2A). However, the –CH₃ groups of PG and ethanol have faster motion as seen by their sharp CP peaks. Taken together, the obstructed character of the solvents strongly implies interactions between the solvent molecules and SC components, likely involving the hydroxyl groups in the solvent molecules. At higher solvent concentrations, the average motion of the obstructed solvent molecules becomes faster, and another coexisting fraction of solvent molecules with isotropic motion can be detected. Among the solvents investigated, the lowest concentration limit where the coexisting isotropic fraction appears is shown for glycerol (Fig. 3 A–C).

All polar solvents investigated induce mobility in the SC lipid acyl chains as concluded from the increased INEPT intensity and the concomitant decrease in the CP intensity of (CH₃), AT segments, although the magnitude of this effect varies between the different solvents. A minor increase in lipid mobility is observed when adding PG, methanol, ethanol, and glycerol to SC. The fluidizing effect of all these solvents is smaller compared with water at the same concentration of 20 wt %. However, the addition of DMSO leads to a slightly more pronounced effect on lipid mobility compared with water. When the concentration of solvents increases from 20 wt % to 40 wt %, the mobility of SC acyl chains further increases (Figs. 3 A–C and 4A and SI Appendix, Fig. S2 A–D and F).

To further investigate the interaction between DMSO and protein filaments in the corneocytes, we performed experiments where DMSO was added to isolated corneocytes at different concentrations (20 and 40 wt %) (SI Appendix, Fig. S5). Again, we confirm that the addition of DMSO has no effect on the mobility in any amino acid segments of the keratin filament. Still, there is a dramatic effect on the DMSO mobility by being incorporated into the corneocyte sample. Similar to the observation when DMSO was added to intact SC, it is observed that the solvent molecules show the characteristics of being obstructed when present in the isolated corneocytes at low concentrations. At higher DMSO concentrations, the DMSO molecule fraction gains faster motion and coexists with a fraction of isotropic fluid solvent in the corneocyte sample.

Molecular Mobility of Apolar Solvents Within Intact SC. Among the 10 solvents investigated, the apolar ones are clearly more mobile compared with the polar ones when present in SC (Table 1). The difference is most pronounced at the lowest concentrations and the apolar hydrocarbon solvents give rise to both sharp CP and strong INEPT signals (Figs. 3 D and E and 4C and SI Appendix, Fig. S4A) even when present at 5 wt % in SC (Fig. 3D and SI Appendix, Fig. S4A). One major complication in the analysis of the NMR data for the hydrocarbon solvents is that most of the resonances from the solvent hydrocarbon chains overlap with the peaks from the SC lipid acyl chains. For 1-decanol, the carbon atom bound to hydroxyl group (C1) can be uniquely identified at 62.5 ppm, and based on the intensities of this peak (Fig. 4C) we conclude coexistence of two populations of 1-decanol that differ in their molecular mobility: one fast isotropic fraction and one slower/anisotropic fraction. To enable detailed characterization...
of hexadecane in SC, we performed additional experiments using the deuterated version of the same solvent. The uniformly $^2$H-labeled hexadecane (Fig. 3F) does not contribute to $^{13}$C spectra acquired with $^1$H→$^{13}$C polarization transfer. A comparison of the spectra from SC with hydrogenated and deuterated hexadecane (Fig. 3E and F) shows that the sharp CP signals at the overlapping resonances (Fig. 3E) indeed originate from the hexadecane molecules. Based on these data, we conclude that hexadecane behaves similar to 1-decanol when added to intact SC, and that it is present as two populations.

A comparison between all solvents investigated (Table 1) shows that the apolar solvents and 2-propanol are more efficient in melting of SC lipids compared with the polar solvents, including water (Figs. 3E and F and 4B and C and SI Appendix, Fig. S4B) at the same concentration. For the hydrocarbon solvents, the effects on the lipids are indicated by the decrease of the AT peak in the CP spectra, compared with the dry SC sample (Fig. 1C). The effect is most clear for 1-decanol, which also leads to increased mobility of cholesterol in the SC lipid matrix. The experiment in which we use deuterated hexadecane (Fig. 3F) makes it possible to resolve the effects of the solvent on SC lipid chain mobility, and it was clearly shown that this solvent has strong fluidizing effect on the SC lipids. The apolar siloxane solvent OMTS also fluidizes SC lipids, as is obvious from the INEPT spectra, although the effect is weaker compared with 2-propanol and the apolar hydrocarbon solvents.

**Comparison Between PG and Glycerol in Hydrated Skin.** Glycerol and PG are small polar molecules with similar structure motifs that are commonly used in skin formulations. As described above, both solvents induce mobility in SC lipids but have no effect on SC proteins in the absence of water (Table 1). To get a more detailed understanding of how these polar compounds influence SC in varying hydration conditions, they were also added to SC as cosolvents (5 wt %) together with water (40 wt %) (Fig. 5). Both PG and glycerol show the characteristics of being fast and isotropic when added to hydrated SC, because their resonances are only seen in the DP and INEPT spectra. This observation is in clear contrast to the situation when the same solvents were added to dry SC (Figs. 3A→C and 4A and SI Appendix, Fig. S2F). The most prominent effect of PG and glycerol in hydrated skin is the fast anisotropic mobility of the solvent molecules when present within SC. This is revealed by a close inspection of the spectral region of, for example, Ser and Gly (Fig. 5C). In particular, PG has a strong fluidizing effect in the terminal segments in the protein filaments (Fig. 5C). With respect to the SC lipid fraction in the hydrated SC, glycerol and PG show no or minor effects on the lipid acyl chain mobility, although they both cause increased mobility of cholesterol and ceramide headgroups. In summary, the comparisons between experiments in Fig. 5 and Figs. 3A→C and 4A and SI Appendix, Fig. S2F show that the effects of glycerol and PG are clearly different when added to dry and hydrated SC.

**Molecular Mobility of SC Lipid and Protein Components After Washing in Excess Solvents.** Washing of skin in excess solvent likely leads to incorporation of solvent molecules into SC. It may also cause extraction of SC components to an excess solvent solution. To relate to practical situations where skin is exposed to solvents, we investigated changes in SC molecular mobility in samples that had been washed in excess solutions of methanol, ethanol, or hexane (Fig. 2E and SI Appendix, Fig. S6). After the washing step, the solvent was evaporated, and thereafter the SC samples were equilibrated at 84% relative humidity (RH). As a reference sample, we use SC equilibrated at the same RH without prior washing in solvent (SI Appendix, Fig. S6A).

The main conclusion from these experiments is that washing SC in solvent strongly influences the SC INEPT spectra, demonstrating decreased SC molecular dynamics in all cases (Fig. 2E). For the reference SC sample at 84% RH, there is a minor fraction of SC lipid acyl chains that are fluid, whereas for the SC samples that had been washed in excess solvent and equilibrated at the same RH no fluid components were detected (SI Appendix, Fig. S6A→D). In addition, washing in excess ethanol or methanol also leads to a reduction in the signal from the rigid SC lipid acyl chains (SI Appendix, Fig. S6B and C), implying that also solid lipids are lost in the washing step. For the sample that was washed in hexane (SI Appendix, Fig. S6D) we observe an increased intensity in the CP spectra at resonances corresponding to the AT lipid acyl chains, indicating reduced mobility in this fraction. No effects on SC protein components are detected after washing in any of these solvents.

**Discussion**

**Tracking Solvents in a Complex SC Matrix.** We use PT ssNMR to investigate the molecular mobility of solvent molecules within the complex biological material of SC. Simultaneously, we study changes in molecular dynamics of the SC lipids and proteins caused by the presence of the solvent. This combination of information enables deepened understanding of molecular interactions between external chemicals and the outer layer of the skin. The PT ssNMR method is highly sensitive for observing small changes in the minor fluid fraction (τ < 10 ns) of SC components, and it simultaneously detects solid components. Thanks to the high sensitivity to variations in molecular dynamics with close to atomic resolution, the method is a powerful tool to study fluidity in intact SC and to complement the extensive literature that is mainly focused on characterization of solid SC components (2, 3, 5). The method is not quantitative in the sense that it does not provide a value on the fraction of mobile to rigid $^{13}$C for a certain segment, mainly due to the nonlinear response in INEPT and CP signals to changes in molecular dynamics and anisotropy (SI Appendix, Fig. S1), but also because of the difficulties of resolving overlapping peaks in the crowded spectra from intact SC.

A variety of solvent molecules differing in size, hydrophobicity, and chemical structure (Table 1) were used, and it is concluded that all of them are partly or completely incorporated in SC. The experiments reveal interactions between solvent molecules and SC components, as demonstrated by the reduced molecular mobility of the solvent molecules when present within SC. The most prominent effects are shown for polar solvents, which appear obstructed when incorporated into SC even when present in rather high amounts (Table 1). From the present data it is not possible to draw any conclusions about the molecular mobility in water inside SC because it is not visible in the $^{13}$C spectra. Polar molecules such as water, ethanol, methanol, PG, DMSO, and glycerol as well as 2-propanol can be present in narrow interlamellar layers [measured to ca. 2 Å at 60 wt % water (10)] in the extracellular lipid matrix, as well as in between the solid keratin rods inside the corneocytes. The polar solvents are then expected to interact with the ceramide lipid headgroups or polar amino acid side chains, for example, serine. Confinement and interactions with polar groups can explain the obstructed character of these small polar molecules in SC. The apolar hydrocarbon solvents also show reduced mobility compared with isotropic solutions. The most likely explanation for this is that the apolar hydrocarbon solvents partition into the hydrophobic layers of the SC lipid lamellae and fluidize the lipid hydrocarbon chains, which is also consistent with previous studies on 1-decanol in model systems and human skin (4, 47). Similar behavior has previously been suggested for, for example, isopropyl myristate in SC (3). The apolar hydrocarbon solvent molecules in the lipid layer show fast anisotropic reorientation rather than obstructed motion.

At higher solvent concentrations a coexisting fraction of isopropyl myristate is detected together with the obstructed/fast anisotropic polar and apolar solvent molecules (Table 1). The concentration...
where the coexisting isotropic fraction occurs varies between the different solvents, and it is generally lower for the apolar solvents compared with the polar ones. Adding more polar solvents to SC gradually leads to swelling of the corneocytes, and the solvent dynamics approaches that of the neat liquid. The addition of apolar solvents leads to an increased amount of fluid SC lipids, which also shows the characteristics of an isotropic fluid (11, 41–43). At high concentrations SC will be saturated with the solvent. From the present data we cannot determine the location of the isotropic solvent. However, it is notable that increasing the amount of isotropic solvent in most cases is also associated with an increased fluidity in SC components (Table 1), clearly indicating the presence of solvents within the SC matrix. We note that the molecular dynamics of the polar solvents and 2-propanol in SC increases with increasing concentration and increased fluidity in SC lipids. This indicates not only a higher fraction of fluid SC lipids but also a faster dynamics of these fluid domains where the solvents are located. It is finally noted that the solvent of intermediate polarity, 2-propanol, shows characteristics in between the polar and apolar solvents in terms of its molecular mobility when incorporated into SC and the concentration where the coexisting isotropic fraction of solvent is detected.

Water is Critical for SC Protein Mobility. Water is the only solvent among those investigated that shows any detectable effect on the SC protein components when added to dry SC (Fig. 1 C and E). The other polar solvents do not affect SC protein mobility unless they are added together with water. Still, we expect the polar solvents to be present between the solid keratin rods inside the corneocytes. It was confirmed that relatively high amounts of DMSO are taken up in isolated corneocytes and intact SC (SI Appendix, Figs. S2E and S5). The DMSO molecules then have characteristics of being obstructed, which can be explained by confinement in polar microdomains in the solid keratin filament structures. Previous infrared microspectroscopic imaging studies have also demonstrated colocalization of DMSO and PG with proteins in intact porcine skin (48). Similar behavior is also seen for the polar solvents ethanol, methanol, PG, and glycerol when incorporated into SC.

The outstanding effect of water on the terminal segments in the keratin filaments can be related to the fact that water is a better solvent for polar amino acids than any of the other studied solvents. Previous studies have shown that the interactions between, for example, DMSO and the polar uncharged amino acid glycine is highly unfavorable, which correlates with precipitation of proteins by DMSO (49). Methyl, ethyl, and propyl alcohols are also often used to precipitate water-soluble proteins (50), which can be explained by reduced solubility due to the lower dielectric constant of the medium. In hydrated conditions the keratin filaments can be seen as solid rods covered by mobile terminal segments enriched in hydrophilic uncharged amino acids (UniProt ID codes P04264 and P13645) (11). The increased mobility in the terminal segments gives rise to long-range steric entropic repulsive forces between the filaments, which may explain the strong swelling capacity of corneocytes at high water contents (41). This behavior is analogous to polymer brushes that can expand or collapse depending on interactions with the solvent (51). Because the swelling capacity of the corneocytes can be related to SC flexibility and strength (17), one can also expect these mechanical properties of the skin to change when water is replaced by another solvent. It has indeed been shown that the ability of corneocytes to take up water decreases with increasing concentration of ethanol, and that the uptake of neat ethanol is negligible compared with that of neat water (52).

PG and Glycerol Show Different Effects on SC Lipids and Proteins When Added As Cosolvents Together with Water. PG and glycerol are small polar molecules with two or three hydroxyl groups, respectively. They are widely used in skin-care products as solvents, penetration enhancers, and humectants (6). The present data imply that the interactions of these compounds with SC components clearly depend on the water content in SC (Fig. 5), which likely also has an impact on their penetration-enhancing effects (20). A larger number of SC molecular segments are affected when PG or glycerol is added to SC as a cosolvent together with water compared with when they are added to the dry SC. Similar response to changes in SC hydration has been also previously shown for other polar and apolar chemical penetration enhancers (13). One remarkable observation from the data in Fig. 5 is that PG has a stronger effect on inducing mobility in the amino acids in the terminal domains of keratin filament compared with a large number of other polar and apolar compounds investigated with this method (13, 14). The addition of water to SC leads to increased mobility in both lipid and protein domains (Fig. 1 C–E). The added cosolvent can therefore more easily dissolve in the SC matrix and further fluidize these structures. Previous studies have shown disturbance of lipid chain packing (3, 53) and a decrease in lipid transition and keratin denaturation temperatures in hydrated SC pretreated with glycerol and PG (3). The effects on the thermal transitions have been shown to be more prominent in skin treated with PG compared with glycerol (3, 5).

Implication for the Use of Solvent in Skin Applications. In this study it is illustrated how different types of solvents influence SC molecular components. All solvents investigated induce mobility in SC components. The changes in the proportion between fluid SC and solid lipids and proteins can have a large impact on the macroscopic properties of the SC, including transport barrier function, flexibility, and softness (2, 6, 13, 16, 17). The observed effects vary with the polarity of the solvent, its concentration, and SC hydration conditions.

The findings can be directly related to practical situations where solvents are used in pharmaceutical, cosmetic, and cleansing skin formulations. When the skin is exposed to a formulation the SC molecular and macroscopic properties may be altered due to interactions between SC components and solvents, active substances, humectants, penetration enhancers, and other excipients (12, 13, 20). The effects of the formulation also depend on skin hydration, the state of the skin, and the occurrence of skin diseases (13, 57–60). The exposure time also varies between different applications in, for example, dermal patches, creams, salves, and washing formulations. During the application period, the amount of solvent in the formulation will change due to evaporation and skin absorption for small molecules such as those studied here [the limit for SC penetration has been reported to be 500 Da (61)].
penetration of the solvent molecules into the skin will depend on the skin’s permeability, which is in turn altered by molecular interactions between the diffusing molecules and SC components (13). For all systems investigated here the solvents induce fluid SC lipid structures, and are therefore expected to act as penetration enhancers. In most practical situations the distribution of foreign molecules in SC is not considered to be uniform. Upon exposure to a formulation the thermodynamic activity of the foreign molecules will be highest in the upper layer of SC, which is generally also the layer with the lowest water activity, which motivates the investigations of solvents in dry SC. The herein observed effects in homogenous samples of SC and neat solvent can be used to make predictions of the effects due to penetration of molecules from complex formulations.

In many experimental studies of skin permeation and molecular organization, samples of skin or isolated SC are washed in solvents, and compounds or probes of interests are added to SC from a solvent solution (62). In the analysis of experimental data and in the evaluation of the effects of skin formulations it is crucial to distinguish the effects caused by the solvents and by other components. This analysis may be complicated by synergistic effects due to interactions between solvents and other molecules (5, 63). For most applications, the solvent molecules are generally in large excess to other components in the formulation, and the effects observed in SC-neat solvent systems are likely also present in situation when skin is treated in more complex solutions. In applications where the SC is exposed to excess solution of solvents one also needs to consider the possibility that some lipid components are extracted from the SC as clearly demonstrated herein, which may also alter SC integrity and consequently SC permeability (1, 7).

Materials and Methods

Materials. DMSO, 1-decanol, OMTS, NaCl, NaHPO₄·2H₂O, and KH₂PO₄ were purchased from Sigma-Aldrich. Methanol, ethanol, 2-propanol, and hexane were obtained from Merck, PG was from Acros Organics, and hexadecane was from Alfa Aesar. Deuterated hexadecane was purchased from Lordan Fine Chemicals AB. PBS contained 130.9 mM NaCl, 5.1 mM NaHPO₄, and 1.5 mM KH₂PO₄, pH 7.4. All solutions were prepared using Milli-Q water.

Preparation of Dermal Skin and SC. Porcine ears were obtained from a local abattoir and stored at −80 °C until use. Hair was removed by a trimmer, and the skin from the inner ear was dermatomed (TCM 3000 BL; Nouvag) to a thickness of ∼500 μm. To separate SC from tissue, the dermatomed skin strips were placed on filter paper soaked in PBS solution with 0.2 wt % trypsin at 4 °C overnight. Sheets of SC were removed by forceps, washed with PBS five times, and further dried under vacuum. The dry SC sheets were then pulverized with the use of a mortar and pestle to facilitate the mixing and equilibration. Pulverized SC was dried again in vacuum and stored in a freezer until further use. To reduce complications related to the biological variation between the different individuals, batches consisting of pulverized SC from several individuals (∼15) were prepared, and all internal comparisons were performed with samples from the same batches. Two different batches were used for the present experiments, and control experiments were performed with both batches in same conditions to confirm consistency between the results (SI Appendix, Fig. S7). Corenocytes were prepared by extracting lipids from SC as described in ref. 12. The NMR experiments for different selected samples with the same composition were reproduced.

To study the effects of pure solvents on SC, ∼30 mg of dry SC powder was mixed with different amount of solvent (5, 10, 15, 20, and 40 wt %, based on the total weight of SC and solvent). For the effects of solvent on SC at hydrated conditions, ∼25 mg of dry SC powder was mixed with 5 wt % solvent (based on the total weight of SC and solvent) and 40 wt % water (based on the total weight of SC and water). These samples were then transferred into tight ssNMR inserts and incubated at 32 °C for 1–2 d before the NMR measurements. We have not observed significant difference between the samples incubated for 1 and 2 d. All experiments were done in triplicates in terms of hydration and concentration, and not to mimic a certain practical situation.

To examine the effects of excess exposure to solvents, ∼30 mg of dry SC powder was immersed and stirred in 20 mL of solvents for 2 h. The SC powder was then filtered and washed with the pure solvent. The solvents were removed under vacuum and the SC samples were then incubated at RH 84% for 2 d at 32 °C and then transferred into tight ssNMR inserts.

In a previous study we showed that there is no detectable differences in the PT ssNMR spectra between pulverized SC and intact SC sheet in same hydration conditions (11), indicating that these samples do not differ on the molecular scale. It was here also confirmed that there is no significant differences in the PT ssNMR spectra between pulverized SC and intact sheets of SC treated with methanol (SI Appendix, Fig. S6 B and E).

Solid-State NMR Experiments. All NMR experiments were performed on a Bruker Avance II 500-MHz NMR spectrometer equipped with a Bruker Efree 4-mm MAS probe and were carried out at a spinning frequency of 5 kHz. In PT ssNMR experiments, the following setup was used: spectral width of 248.5 ppm, acquisition time of 0.05 s, 2,048 scans per experiment, recycle delay of 5 s, and 13C hard pulse at ω1/2τc = 80.6 kHz. The power of 1H was ramped from 72 to 88 kHz during the contact time of 1 ms in the CP experiment. The INEPT experiments were performed with delay times of 1.8 ms and ω′ of 1.2 ms. All experiments were recorded under 68-kHz two-pulse phase modulation 1H decoupling (64). The 13C spectra were externally referenced to the methylene signal of solid n-glycerine at 43.7 ppm (65). The temperature was calibrated by methanol (66). The data were processed with a line broadening of 20 Hz, zero-filling from 1,024 to 8,192 time-domain points, Fourier transform, and phase and baseline correction by in-house MATLAB code partially from matNMR (67).

To estimate the detection limit of a fast isotropic segment, the signal-to-noise ratio of the INEPT peak from carbon C3 of PG in a sample of SC with 5 wt % PG and 40 wt % water (Fig. S4) was calculated to be ∼100 based on the ratio of the intensity of the INEPT peak and the SD of the intensities of the noise in the INEPT spectrum. Assuming that the signal is directly proportional to the concentration and the noise remains constant, the detection limit corresponding to the concentration where the signal-to-noise is above the value 3 is ∼0.15 wt % PG. If the amount of PG in the NMR insert is 1.32 mg, corresponding to 25 mg SC powder, the detection limit of the fast isotropic segment of PG is about 2 μg.

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