Tracking heavy-water-incorporated confocal Raman spectroscopy for evaluating the effects of PEGylated emulsifiers on skin barrier

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Abstract
The class of PEGylated emulsifiers finds broad application in the pharmaceutical and cosmetic industry. We target on one of the categories of polyethylene glycol (PEG) alkyl ethers with different lipophilic and hydrophilic chain length and aim to examine their effects on the skin comprehensively. In this study, we employed confocal Raman spectroscopy for skin depth profiling and imaging. A unique probe of heavy water (D₂O) was incorporated, which can be tracked percutaneously and simultaneously monitor the effects caused by emulsifiers. According to the results, most of the PEGylated emulsifiers caused changes in skin lipid content/organization and induced the alteration in relative water content/hydrogen bonding structure. The results obtained from the depth profiling analysis provided the possibility to estimate the least penetration depth of emulsifiers. Among them, PEG-20 ethers displayed the most penetration ability. Meanwhile, it is interesting to find that the treatment of emulsifiers also affected the spatial distribution of D₂O whose differences were in line with the molecular skin variations. In particular, the isotopic H/D substitution in the skin was highlighted in detail. This result supports the possibility to use D₂O as an excellent and cost-effective probe to evaluate the skin barrier function.

KEYWORDS
confocal Raman spectroscopy, heavy water, PEGylated emulsifiers, skin barrier function, skin molecular properties

1 | INTRODUCTION

In everyday life, our skin is facing contact with sanitary, cosmetic, or pharmaceutical products, gaining the potential of external irritations [1–3]. Establishing a significant barrier against environmental influences, stratum corneum (SC) makes up the outermost layer of the skin, providing mechanical protection with commonly described “bricks and mortar” model [4, 5]. Primary protection of SC is provided by the intercellular lipids (the mortar) [6, 7].
At ambient conditions, skin lipids are highly structured and mainly assembled in densely packed orthorhombic lamellar phases. Nevertheless, with the skin exposed to foreign substances, physical and chemical interactions may happen to each other, changing the molecular properties of skin components. When the skin is highly irritated, the lipid structures will tend to be a more fluid state [8–10]. Except for the lipids, the skin water-related property is also considered as an attractive property due to the common observation of trans-epidermal water loss in skin research [11]. In particular, in recent, the tight relationship of skin water regulation with intercellular lipids was introduced. Although the role of the skin lipids and water-related properties has been highlighted in some studies, the deepened understanding of their molecular variation is still limited [12–14].

There is extensive literature describing the irritation triggered by topically applied compounds in which emulsifiers are the main considering targets, owing to their intrinsic surface activity and intensive applications [15, 16]. However, conflicting data are emerging about their adverse effects on the skin [17, 18]. Walters et al stated that PEG alkyl ethers based on a C12 alkyl chain were effective in disrupting the lipid monolayer [19]. Our previous study also found reduced skin lipid content and altered lipid structures after applying the PEG-20 glycerol monostearate [20, 21]. Recently, we evidenced the different influence of a series of PEG alkyl ethers and PEG sorbitan esters on skin lipids [22]. Compared with the previous study, our current research focuses more on the depth-dependent comparison of molecular skin properties due to different penetration depth of emulsifiers. Furthermore, the relative water content and hydrogen bonding state are taken into consideration to gain insight view of interactions with lipid properties.

The insights into the mechanisms of molecular skin alterations gain complete attention in skin research. Considerable studies have been evaluating the skin barrier function based on the electrical, microscopic, and scattering approaches, which provide limited information on the insights of molecular skin interactions [23–25]. The fluidity and mobility of skin components regarding their structural changes were recently announced by using nuclear magnetic resonance, which is more precise but challenging to elucidate the skin depth variations [26, 27]. For a more comprehensive look, confocal Raman spectroscopy (CRS) is introduced. It is sensitive to identify minor changes in molecular skin components and available to detect skin from different depth [28].

Skin lipid analysis has been well grounded using CRS. Lipid content and lateral packing structure analysis have been comprehensively discussed by selecting useful Raman signals [29, 30]. However, skin water regulation and the associated hydrogen bonding state still need to be well elucidated. Recently, the hydrogen bond water molecular types were suggested by Choe et al using CRS to analyze the skin water-related properties of intact skin. Although it is an effective way, the shortage was uncovered when treating the skin with moisturizing solutions or formulations. In this case, extra water will penetrate the skin and subsequently induce a variation of OH bond signal on CRS spectra [31, 32]. To minimize the effects of external water penetration, we introduced a probe of heavy water (D2O) to dissolve the model substances applied to the skin. With this probe, the effects of external penetrated water can be eliminated due to different Raman spectral features of OD bond and inherent skin OH bond [33]. Thus, skin water-related properties can be effectively detected. Meanwhile, as the CRS spectra recorded, D2O distribution can be monitored and visualized across skin depth. As is known that water penetration dynamics can be regulated by the skin barrier integrity [34, 35], the D2O penetration depth can be expected to differ following the effects of emulsifiers. Therefore, we assume the application of D2O as a promising approach for evaluating the skin barrier state. The isotopic substitution and water diffusion behavior are also taken into adequate account.

2 MATERIALS AND METHODS

2.1 Materials and animal

Deuterium oxide (D2O, 99.9 atom % D) is obtained from Sigma Aldrich (St. Louis, Missouri). PEG alkyl ethers including PEG-2 oleyl ether (O2), PEG-10 oleyl ether (O10), PEG-20 oleyl ether (O20), PEG-2 stearyl ether (S2), PEG-10 stearyl ether (S10), PEG-20 stearyl ether (S20),
PEG-2 cetyl ether (C2), PEG-10 cetyl ether (C10) and PEG-20 cetyl ether (C20) were purchased from Croda GmbH (Nettetal, Germany) (Figure 1 shows their chemical structures with different oxyethylene groups; Figure S1 represents the CRS spectrum of pure substances). Sodium lauryl sulfate (SLS) was achieved from Cognis GmbH & Co. KG (Düsseldorf, Germany). Sodium chloride, disodium hydrogen phosphate, potassium dihydrogen phosphate and potassium chloride were of European Pharmacopeia grade. Porcine ear skins (German landrace; age: 15 to 30 weeks; weight: 40 to 65 kg) were provided by the Department of Experimental Medicine at the University of Tuebingen and local butcher.

### 2.2 Dermatomed porcine skin

Porcine ears were achieved on the day of slaughter and cleaned with isotonic saline. The skin was removed from cartilage, cleaned from blood, and cut into strips of approximately 3 cm width. The strips were stretched onto a Styrofoam plate with pins. Skin hairs were trimmed to around 0.5 mm with electric hair clippers. Thereafter, the skin was dermatomed to a thickness of 1 mm (Dermatom GA 630, Aesculap AG & Co. KG, D-Melsungen), punched out for circles to a diameter of 25 mm, wrapped with aluminum foil and stored into the refrigerator at −30°C. Upon use, the circular skin samples were thawed to room temperature and washed clean with phosphate-buffered saline (PBS). This procedure has been previously described in ref. [36]. Besides, two donors were used for the depth profiling analysis of PEGylated emulsifiers, which were slaughtered from the same slaughterhouse on the same day. The first donor was used for the studies of PEG-2 ethers and PEG-10 ethers. The second donor was used for investigations of PEG-20 ethers and SLS. D2O only treatment was performed in both donors and used as a reference to normalize the slight data differences and make the emulsifier-treated samples comparable, which meant to decrease the inter-experiment variations.

### 2.3 Franz diffusion cells

D2O dissolved PEGylated emulsifiers were applied on porcine skin using Franz diffusion cells. Degassed and prewarmed (32°C) PBS was used as receptor fluid. A circular skin sheet was placed on the cell with SC facing up. The donor compartment was put on top of the skin and clamped tightly. The equipped cell was put into a water bath with a temperature of 32°C and stirring speed of 500 rpm. After 30-min equilibrium, 1 mL of each sample was applied to the skin (D2O or D2O dissolved). PEGylated emulsifiers used in this study are shown in Table S1. A piece of parafilm was then capped onto each donor compartment to prevent evaporation. After 2 hour incubation, the skin samples were removed from cells and cleaned with isotonic saline and cotton swabs for 30 times. The actual involved skin area was punched out and mounted onto the device for CRS measurement. The cross-sectional view of the device is depicted in Figure 2. A full description was provided in supporting information (Figure S2) and has been reported in the previous publication of our group [37].

### 2.4 Confocal Raman spectroscopy

#### 2.4.1 CRS setting

Skin spectra were acquired with an alpha 500R confocal Raman microscope (WITec GmbH, Ulm, Germany) which was equipped with a 532 nm excitation laser, UHTS 300 spectrometer and DV401-BV CCD camera. The optical grating was 600 g/mm for recording the spectra in the range.
of 0 to 3800 cm\(^{-1}\). Herein, 100\(\times\)/1.25 NA (numerical aperture) oil immersion objective (E Plan, Nikon, Tokyo, Japan) in combination with a 50 \(\mu\)m pinhole was utilized [38]. Laser power was adjusted to 5 mW by the optimal power meter (PM100D, Thorlabs GmbH, Dachau, Germany) to avoid skin sample damage. For non-biological samples (eg, \(\text{H}_2\text{O}, \text{D}_2\text{O}\) and their mixtures), 40x/0.6 NA objective was used (EC Epiplan-neofluor, Carl Zeiss, Germany).

2.4.2 | CRS depth profiling

Line scans were conducted to collect skin spectra in-depth with the laser spot recording from 10 \(\mu\)m above the skin down to 50 \(\mu\)m inside the skin. The step size was 1 \(\mu\)m and the exposure time was 2 seconds for one measurement followed by 2 accumulations for each spectrum. The complete acquisition time was around 4 min for each depth analysis. The obtained spectra were preprocessed by the WITec Project Software (WITec GmbH, Ulm, Germany) including the smoothing process (Savitzky-Golay filter with third polynomial order and nine smoothing points) and background subtraction (automatic polynomial function was fitted to the spectrum and subtracted). Principal component (PC) analysis was then employed to reduce the noise of spectra. The first three PCs are selected for gaining the reconstructed spectra, as they can mostly describe the dataset and obtain the critical characteristic information of skin and \(\text{D}_2\text{O}\). The skin surface was determined based on the detection of the boundary between SC and coverslip. The area under the curve (AUC) of keratin peak (\(\nu(\text{CH}_3)\), 2920-2960 cm\(^{-1}\)) was plotted against scan depth. The left point of the full-width of half-maximum of this plotted profile was identified as the boundary between the skin surface and coverslip. The AUC was calculated using the trapezoidal method.

2.4.3 | CRS spectral image

The third donor was used for CRS image scanning. Two-dimensional CRS maps were obtained by using image scans. A scanning area of 25 \(\times\) 50 \(\mu\)m\(^2\) (25 \(\times\) 50 pixels) in x-z direction was mapped with 1 \(\mu\)m step size in both directions and 0.05 s integration time per spectrum. The scan was started from above the skin surface to obtain a complete skin cross-sectional image. After a series of preprocedures including cosmic ray removal, smoothing and background subtraction, a color-coded image was generated. In these images, each pixel corresponds to one CRS spectrum. In order to visualize the skin layer and track the spatial distribution of \(\text{D}_2\text{O}\), the pure, untreated skin spectrum and \(\text{D}_2\text{O}\) spectrum were assigned, respectively, to different colors to indicate their location within the examined area.

2.5 | Evaluation of skin properties

2.5.1 | Gaussian-function based decomposition

The decomposition process in high wavenumber (HWN) region has been mainly introduced by Choe et al in skin research [31, 32]. In brief, in our work, the band of 2800 to 3800 cm\(^{-1}\) was decomposed into 10 subbands using Gaussian functions (see Figure 3). This process was automatically performed by applying curve-fitting toolbox on Matlab software (Math work, version R2019a). The goodness of fitting results was generated with \(R^2\) over 0.98. As shown in Figure 3A, the four subbands centered at around 2800 to 3000 cm\(^{-1}\) are assigned to lipids and keratin. Peaks at 2850 and 2880 cm\(^{-1}\) correspond to \(\nu(\text{C-H})\) symmetric and \(\nu(\text{C-H})\) asymmetric stretching mode of lipids. Peaks at 2930 and 2980 cm\(^{-1}\) are assigned to \(\nu(\text{CH}_3)\) symmetric and \(\nu(\text{CH}_3)\) asymmetric stretching mode of keratin. The other six subbands in the spectral range of 3000 to 3800 cm\(^{-1}\) represent the various hydrogen bonding types of the water molecule and the small contribution of keratin. Among them, the peaks centered at 3070 and 3325 cm\(^{-1}\) are assigned to the unsaturated methylene stretching vibration of keratin and the N-H vibration of keratin separately. The four peaks corresponding to the hydrogen bond of water molecules were located at around 3010, 3280, 3460 and 3600 cm\(^{-1}\), associated to tightly bound DAA-OH (single donor-double acceptor), strongly bound DDAA-OH (double donor-double acceptor), weakly bound DA-OH (single donor-single acceptor) and free water (superposition of very weakly DDA-OH bond, double donor-single acceptor and unbound OH). The hydrogen bonding configurations are represented in Figure 3B, which have been introduced in detail by Sun [39].

2.5.2 | Intercellular lipid properties

Lipid content could be analyzed in the fingerprint region. The peak at 1425 to 1490 cm\(^{-1}\) is composed of mostly the intercellular lipids. The peak at 1630 to 1710 cm\(^{-1}\) is corresponding to the \(\nu(\text{C}=\text{O})\)-mode for amide-I, representing the least variations from different donors. Thus, relative lipid content can be calculated according to the equation of normalized lipid = AUC\(_{1425-1490}/\text{AUC}_{1630-1710}\).
The detailed explanation has been described previously by our group [21].

The decomposed lipid and keratin related peaks in the HWN region have been demonstrated to be able to calculate the lipid content following the function of \( \text{Normalized lipid} = \frac{\text{AUC}_{2880} + \text{AUC}_{2850}}{\text{AUC}_{2930} + \text{AUC}_{2980}} \) [40]. In this study, skin lipid content analysis in fingerprint and HWN region can be mutually verified and provide a better comparison of these two signals in-depth profiling analysis.

For indicating the transition of skin lateral packing state from orthorhombic to hexagonal (more disorder state) phase, decomposed peaks at 2850 and 2880 cm\(^{-1}\) were utilized for calculation of \( \text{Ratio}_{\text{lat}} = \frac{\text{AUC}_{2880}}{\text{AUC}_{2850}} \) based on the findings from previous research [41]. The higher value of \( \text{Ratio}_{\text{lat}} \) represents a prevalence of highly crystalline and orthorhombic phases.

2.5.3 | Skin water-related properties

In this study, different hydrogen bound water types were used to calculate the relative water content. With the sum of AUCs of all OH-related bands normalized by keratin band (\( \nu(\text{CH}_3), 2920-2960 \text{ cm}^{-1} \)), the total water content can be calculated in different skin depths. Furthermore, the DDAA-OH and DA-OH bounds are mainly focused, as they account for over 90% of water molecular type in the skin. The hydrogen bonding state of water molecules can be represented by the ratio of AUCs of DA-OH- and DDAA-OH-related bands, which was firstly proposed by Choe et al [42].

2.5.4 | Heavy water as a probe in skin

By extracting the spectrum from each pixel in-depth, the relative O-D content was calculated by applying the spectral feature of the O-D stretching band (2250-2750 cm\(^{-1}\)) normalized by keratin band (\( \nu(\text{CH}_3), 2920-2960 \text{ cm}^{-1} \)). This ratio represents the relative intensity of the O-D bond and the D\(_2\)O distribution quantitatively.

3 | RESULTS

3.1 | Intercellular skin lipid content and lateral packing state

The findings of lipid-related differences were shown in Figure 4 and Figure 5. Lipid content variations against depth were plotted in both fingerprint and HWN regions based on the calculations mentioned above (Figure 4A-C and Figure 4D-F). It is visible from the both regions that skin lipid distribution is inhomogeneous and intercellular lipid content keeps decreasing from the skin surface. Comparing the variations of calculated content values, the analysis in the fingerprint region showed better selectivity and sensitivity, as shown in previous study [30]. It is apparent that the variations of the normalized ratio
(from about 0.8 to 1.3) are intensively larger than in the HWN region (from about 0.46 to 0.57). It indicates that the lipid signal in the fingerprint region can provide more information on smaller differences of lipid molecular changes, which could be a better choice for future studies. However, the lipid signal HWN region can also be a second verification in our study to investigate the effects of PEGylated effects.

The depth profiling results among the investigated emulsifiers revealed the different extent of lipid extraction in both regions. SLS, as the positive control, showed the most reduction of lipids, especially at the upper layer of the skin, which is line with previous reports [1, 43]. PEGylated emulsifiers presented different effects on extracting lipids from the skin. As both figures showed, O2, S2 and C2 treated skin only showed a slight reduction from the surface of the skin comparing with the result from reference (heavy water only treated skin). O10, S10 and C10 have similar effects on the levels of detected lipid contents, mainly located at the upper layer of approximately 8 μm. The trend is more evident in O20, S20 and C20 treated skin. Their ability to extract skin lipids increased remarkably from the depth analysis. Comparing the different extent of lipid reduction, the number of hydrophilic groups of PEGylated emulsifiers highlights their crucial role in lowering skin lipid content.

The variation of lipid lateral packing order state could be identified from the viewpoint of calculated Ratio\textsubscript{lat} vs skin depth. As shown in Figure 5A-C, the lateral packing states of lipids in the skin are inhomogeneous. The highest ordered lateral packing state of lipid structure is located in the intermediate part of the SC (approximately 4-10 μm), whereas less ordered lipid packing state was found on the skin surface. We could clearly see from Figure 5 that the SLS treated skin exhibits the most vital ability in decreasing the ordering state of lamellar lipids, and this influence spreads deep into the viable epidermis. Whereas for PEGylated emulsifiers treated skin, O2, S2 and C2 only show small variation by depth on their lipid packing state. Significant differences are hard to notice in

**FIGURE 4** Depth-dependent profiles of skin lipid content of D\textsubscript{2}O and emulsifiers treated skin, calculated by using the lipid signals in fingerprint region, A-C, and lipid signals in high wavenumber region based on the decomposition process, D-F, mean ± SD, n = 9

**FIGURE 5** Depth-dependent profiles of calculated Ratio\textsubscript{lat} of D\textsubscript{2}O and emulsifier-treated skin representing the order of lipid lateral packing structure, mean ± SD, n = 9
this category of emulsifiers, indicating more friendly effects of them on skin. O10, S10 and C10 lead to a modest decrease of Ratio_int compared with the reference, which can be observed at the upper part of the SC, indicating a slight increase of disordered lateral packing state. In comparison, the lateral packing orders are more disordered after the treatment of O20, S20 and C20. Clear decrease of Ratio_int can be observed in the intermediate layers (approximately 12 μm) of skin. The comparison among all the emulsifier effects on the lipid lateral packing order suggests nearly consistent results with the reductions of lipid contents.

3.2 Skin water-related properties

Figure 6A-C shows the relative skin water content by depth. It can be seen that the application of SLS on skin significantly reduced skin water content. The treatments of PEGylated emulsifiers reveal different extent on affecting the water content. O2 and S2 only slightly decrease the water content from the upper layer of SC, whereas C2 causes slightly more reduced compared with O2 and S2. O10, S10 and C10 have a similar tendency to cause relative more reduction in water content. The most substantial effect can be observed with the applications of O20, S20 and C20 on skin from skin surface to around 15 μm depth. By comparing the water content profiles with the effects of emulsifiers, it is not difficult to find that the trend of water content variation is generally consistent with the extraction of lipids and the disordering of lipid lateral packing order. Besides, the relationship between their distribution statuses is also noticeable that the skin layer with the most ordered state of lipids presents the highest water content. Meantime, the skin layer with the lowest water content on the uppermost layer and the substrate of the SC shows the more disordered state of lipid packing state.

Figure 6D-F presents the profiles of hydrogen bonding skin water molecular types calculated by the ratio of AUCs of DA-OH- and DDAA-OH-related bands. It can be seen that the water molecules on the skin surface have a higher content of weakly bound water, corresponding to the less ordered lamellar lipids on the skin surface, which may easily cause breaks of OH bonds and loss of water. With the treatment of emulsifiers, the transition state to a

![Figure 6](image)

**Figure 6** Depth-dependent profiles of the water content of D2O and emulsifier-treated skin calculated by integrating the signal intensities of O-H stretching vibrations normalized by keratin band after decomposition process, A-C, and the hydrogen bonding state of skin water molecules obtained from the ratio of weakly bound (DA)/strongly bound (DDAA) water molecule types, D-F, mean ± SD, n = 9

![Figure 7](image)

**Figure 7** Raman spectra of full-thickness skin and heavy water
weaker bound water type can be observed at different levels. The differences are not significant after applying O2 and S2 on the skin. Only slight differences can be observed on the skin surface layer when treating skin with C2 on the skin. The emulsifiers of PEG-10 ethers and PEG-20 ethers exhibit similar differences in DA/DDAA values. The influences are generally following the lipid-related properties, although the PEG-20 ethers revealed more potent effects on skin lipid properties. Based on these results, we may assume that when the skin lipid phases convert into a more disordered lipid packing state, the bonding state of skin water molecules might be more weakly bound. This correlation between skin water-related properties and lipid variations also complies with

**FIGURE 8** CRS analysis of the skin. Recorded CRS spectra associated with the red points representing each depth of the skin after incorporation of heavy water showing the basic principle for obtaining the skin depth profile by using spectral line scan and skin map of cross section by using spectral image scan

**FIGURE 9** Color-coded Raman images representing the x-z direction distribution of heavy water in skin samples with the treatment of D2O and different emulsifiers. The spectral characteristics of D2O was shown in red, while the blue region presented the spectral features of skin components
other studies when analyzing the distribution of human/porcine skin or age-related profiles, etc. [32, 44].

3.3 | CRS for monitoring heavy water within the skin

Deuterium is a heavier isotope compared with hydrogen, which shifts the OH stretching vibration into a lower wavenumber area in Raman spectra. As shown in Figure 7, the O-D stretching band is located at 2250 to 2750 cm\(^{-1}\), which completely stands out from the skin spectrum without any overlapping area. It indicates that the exogenous penetrated water (D\(_2\)O) can be easily identified and distinguished from the endogenous skin hydrogen bonds (O-H). The approach for CRS measurement has been shown in Figure 8.

Figure 9 depicts the images of D\(_2\)O distributions from x, z-directions. The D\(_2\)O alone treated skin shows the minimal distribution of D\(_2\)O, which is mostly located in the upper layer of the skin with relatively lower content. With the SLS treatment, D\(_2\)O shown in red penetrates the deepest inside the skin. It shows densely packed red color overlaid, indicating more presence of OD bond in each pixel. Among the PEGylated emulsifiers, O2 and S2 treated skin represent similar behavior of D\(_2\)O distribution with D\(_2\)O alone treated skin. O10, S10 and C2 slightly accelerated the penetration of D\(_2\)O in the skin with the observation of O-D signals in a relatively deeper layer. C10 seems to promote the penetration more compared with O10 and S10 even with some slight inhomogeneous spread in skin. The deeper penetration of D\(_2\)O followed to be affected by O20 and S20, which displayed similar depth location of D\(_2\)O. The strongest impact came from C20, which revealed the deeper distribution of D\(_2\)O compared to other PEGylated emulsifiers. In summary, the D\(_2\)O penetration depth is clearly influenced by selected PEGylated emulsifiers. The impact behaviors were mostly in accordance with the variation of lipid and water-related properties, which reflected the potential of tracking heavy water diffusion in skin to evaluate the influences of compounds on skin.

3.4 | H/D substitution in D\(_2\)O-incorporated skin

The application of D\(_2\)O on skin was mentioned in a few studies. However, the behavior of isotopic substitution that happened in skin was only briefly discussed [45, 46]. To better understand the distribution process of D\(_2\)O in skin, isotopic substitution cannot be simply ignored. First of all, the C-H/C-D substitution was taken into account. In order to have a clear view of C-H/C-D substitution, the mixture of methanol and deuterated methanol was detected to simulate the substitution process (full description can be found in supplementary information). As shown in Figure S3, the C-D stretching mode is located at 2000 to 2300 cm\(^{-1}\). However, there is no prominent peak appeared on the skin spectra in this region (Figure 8), indicating that the C-H/C-D substitution in the skin is not detectable.

O-H/O-D substitution is commonly highlighted in deuterated water applications [47, 48]. To elucidate the action between skin H\(_2\)O with penetrated D\(_2\)O, the mixtures of different volume ratios of H\(_2\)O and D\(_2\)O were used as models. As shown in Figure 9A-B, the intensity of the OH/OD bond increases linearly with the H\(_2\)O/D\(_2\)O
content, demonstrating a convincible spectra display. It is clear from Figure 10C-D that the signal intensity increases with the increased volume proportion and corresponding molecular vibration. However, the O-D bond has a greatly higher intensity than the O-H bond. When the H₂O molecule dominates the significant proportion, the O-H bond appears relatively similar intensity with O-D bond. In this case, the increase of H₂O volume (see $V_{\text{H}_2\text{O}}/V_{\text{D}_2\text{O}}$ of 2:1, 3:1, 4:1) only show a slight increase of O-H bond intensity. If we compare the dynamic spectra of D₂O in the skin (Figure S1), it is easy to find that the penetrated D₂O amount is less than the skin H₂O amount. It indicates that the proportion of D₂O molecule in the skin is less than the H₂O molecule. Thus, the actual O-H bond intensity will only be slightly underestimated due to the O-H/O-D substitution.

4 | DISCUSSION

The alterations of skin lipid properties are associated with the impairment of skin barrier function [14, 49]. According to the effects of PEGylated emulsifiers, the decrease of skin water content and the prevalence of weakly bound hydration state of skin water are parallel to the adverse effects on lipid properties. The mechanism can be speculated that the disorder of lipid packing and subsequently caused impairment of skin barrier function lowered skin water content and further reflected the decrease of water holding capacity. In specific, our results supported the finding that the disorders skin lipids will loosen some tight hydrogen bonds surrounded and reduce the ratio value of DA/DDAA [42].

By tracking the distribution of D₂O, emulsifiers appeared different effects on its penetration depth. It turns out that the extent of their effects on molecular skin segments correlates to the permeability of simultaneously applied D₂O. The potential mechanism might be triggered by the increase of SC lipids mobility and fluidity, which facilitated the interactions of water molecules with lipid polar head groups and making the D₂O easily to be penetrated. With these correlated results, it demonstrates that the application of D₂O on the skin enables the assessment of skin barrier function. Meanwhile, the research by Wang et al also provided support to our study which found out that the treatment of glycerin on skin promoted the penetration of D₂O [46]. Besides, the advantage of using D₂O is clearly highlighted in our study to enable the detection of skin water-related properties. Even with the effects of isotopic substitution, the variation of skin water content and hydrogen bonding state of skin water can also be identified.

Overall, the tendency for skin interactions mainly follows the sequence: PEG-20 alkyl ethers > PEG-10 alkyl ethers ≥ PEG-2 alkyl ethers. The hydrophilic chain length seems to be the primary determinant in the activity of skin interactions. Many reports also underlined the importance of alkyl chain length in the potency of skin interactions [18, 50]. However, it is still debatable in our study, as its influence is not clearly highlighted when comparing the results of PEG oleyl ethers, PEG stearyl ethers and PEG cetyl ethers. In addition, little differences were found when comparing to the PEG oleyl ethers (C₁₈, unsaturated), and PEG stearyl ethers (C₁₈, saturated). It indicates that the effects of PEGylated emulsifiers with unsaturated hydrocarbon chains are similar to those with saturated chains. The more information provided in this work referred to the penetration depth of them. The spectral signal of lipid content in the fingerprint region appeared to be the most sensitive one. With this result, we could have a clue about the least penetration depth of each emulsifier due to their effects on different depths of the skin.

5 | CONCLUSION

In this work, we investigated the effects of a class of PEGylated emulsifiers using CRS to monitor their effects on molecular skin properties. D₂O was simultaneously incorporated to visualize its distribution in the skin. The comprehensive depth-dependent profiling results illustrated the changes in the skin lipid and skin water-related properties. They clearly showed the prevalence of disordering skin molecular properties by the treatment of PEGylated emulsifiers with longer hydrophilic chain length. Besides, the least penetration depth of emulsifiers in the skin can be estimated based on their depth of influences. The application of D₂O allows the differentiation of endogenous and exogenous hydrogen bonds, which enables the detection of skin water-related properties. The overall results stressed the correlation data of different D₂O penetration depth with different extent of molecular skin effects, supporting the idea of using D₂O as a convenient and inexpensive target in the skin for multiple roles. The H/D substitution as a frequently missing part has been considered to compensate for the incomplete description of their occurrence and behavior in the skin. Finally, this study performed a multifactorial analysis of emulsifiers on skin and enhanced our understanding in skin molecular variability with topically applied compounds. It would be of interest in the future for us to deepen the understanding of mechanisms of emulsifiers used as penetration enhancers for evaluating drug penetration and permeation behaviors.
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CONFLICT OF INTEREST
The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT
Research data are not shared.

ETHICS STATEMENT
Porcine ears were achieved from Department of Experimental Medicine of University Hospital Tuebingen. Live animals used were kept at Department of Experimental Medicine and sacrificed in the course of their experiments, which are approved by the Ethics Committee of University Hospital Tuebingen. Those ears were received directly after the death of the animals. Prior to study, Department of Pharmaceutical Technology has registered for the use of animal products at the District Office of Tuebingen (registration number: DE 084161052 21).

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of this article.

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