Micron-scale assessment of molecular lipid organization in human stratum corneum using microprobe X-ray diffraction

Jean Doucet¹,², Anne Potter³, Carine Baltenneck³ and Yegor A. Domanov³

¹Laboratoire de Physique des Solides, Université Paris-Sud, Orsay, France
²Phi-Axis, Antony, France
³Skin biophysics laboratory, L’Oréal Research & Innovation, Aulnay-sous-Bois, France

Short title: microprobe X-ray scattering of stratum corneum lipids

Correspondence address:
Yegor DOMANOV
L’Oréal Recherche Avancée - Biophysique de la peau
BP 22
1, avenue Eugène Schueller
93601 AULNAY-SOUS-BOIS
France

E-mail : ydomanov@rd.loreal.com
Tel.: +33 (0)1.58.31.73.28, Fax : +33 (0)1.58.31.71.96
Abstract

Lipid and protein components of the stratum corneum (SC) are organized in complex supramolecular arrangements. Exploring spatial relations between various possible sub-structures is important for understanding the barrier function of this uppermost layer of epidermis. Here, we report the first study where micro-focus X-ray scattering was used for assessing fine structural variations of the human skin barrier with micrometer resolution. We found that the scattering profiles were unchanged when scanning in the direction parallel to the SC surface. Furthermore, small-angle scattering profiles did not change as a function of depth in the SC, confirming that the lipid lamellar spacings remained the same throughout the SC. However, the wide-angle scattering data showed that the orthorhombic phase was more abundant in the middle layers of the SC, whereas the hexagonal phase dominated in the surface layers both at the external and the lowest part of the SC, i.e. the lipids were most tightly packed in the middle region of the SC. Taken together, our results demonstrate that the microprobe X-ray diffraction provides abundant information about spatial variations of the SC lipid structure and thus may be a promising tool for assessing the effects of topical formulations on the barrier function of skin.

Key words: x-ray diffraction, intercellular lipid lamellae, stratum corneum, barrier function, microdiffraction, heterogeneity, domains, ceramide, imaging

Abbreviations: SC – stratum corneum; SANS – small-angle X-ray scattering; WAXS – wide-angle X-ray scattering.
INTRODUCTION

The outermost layer of epidermis, the stratum corneum (SC), consists of keratin-rich corneocytes surrounded by lipid matrix. Despite the fact that they only represent around 10% of the total mass of the SC, the intercellular lipids play a major role in the barrier function of the skin. For example, lipid removal leads to a loss of the water diffusion barrier (1). The barrier function results from the complex and specific architecture of the inter-corneocyte lipid matrix (2) composed of a mixture of ceramides, cholesterol and long-chain fatty acids. Ultrastructural studies, using electron microscopy and diffraction techniques, have revealed that these lipids are organized in stacked bilayers that are predominantly parallel to the skin surface (3-5). Part of the lipids is covalently bound to the corneocytes whereas another part represents the “free lipids” removable by solvent extraction.

X-ray diffraction is an excellent technique for studying the spatial organization of the intercellular matrix because the lipid molecules are highly ordered and oriented in a crystalline structure. This technique has provided a number of important results with regard to the SC structure. In particular, small-angle X-ray scattering (SAXS) gives information on the inter-bilayer distance in the multi-lamellar lipid structures, whereas the wide-angle data (WAXS) contain information about the in-plane crystalline arrangement of lipids (lattice type).

Most of the SAXS studies of human SC find reflections corresponding to ~4.5-nm and ~6.5-nm inter-bilayer spacings (4;6;7). Some studies have also detected a reflection corresponding to a spacing of 11-13.5 nm (6;8), referred to as the “Long Periodicity Phase” (6). These data indicate that the SC lipid matrix has a complex organization into multiple sub-layers or even phase-separated domains with different lamellar repeat distances.

Similar complexity is found at the molecular level using WAXS, where two sets of peaks are found, corresponding to orthorhombic (peaks at 0.41 and 0.37 nm) and hexagonal (peak at 0.41 nm) packing of the lipid chains. In addition, a fraction of lipids might exist in a fluid or amorphous state, which would then contribute to the broad band corresponding to an average distance of 0.46 nm.
These observations raise the obvious question of location and relative spatial arrangement of these different sub-structures. Bulk X-ray scattering studies provide highly complex patterns where individual contributions of different substructures are difficult to resolve. For example, it is not clear whether different types of organization coexist in the same region of the SC or occur in different environments, e.g., at different depths or lateral locations. To address these questions, we performed combined SAXS and WAXS experiments with micrometer resolution making it possible to determine structural parameters at different depths or different locations in the same SC sample. We used this method to study single sheets of human SC that were obtained ex vivo and – unlike previous ultrastructural studies – were kept under near-native conditions at the time of data acquisition (no staining or fixation, measurements at ambient temperature and pressure). Two types of samples were analyzed: untreated (control SC) and after lipid removal by a solvent (delipidized SC).
MATERIALS AND METHODS

Stratum corneum

Human abdominal skin residues from plastic surgery were supplied in frozen form. Stratum corneum was isolated using a standard protocol (4). Subcutaneous fat was removed and the dermis was separated from epidermis by immersing skin into hot water (56°C). Remaining viable epidermis was removed by tryptic digestion at 37°C overnight. The SC was dried and stored at room temperature and ambient humidity.

The above SC preparation protocol is currently a common method of SC isolation in dermatological research (e.g., see refs. 7-8 and 26). However, there might be concerns about possible effects of the sample thermal history (skin stored at -20°C before separation, then heated to 56°C), trypsin treatment and possible contamination from subcutaneous fat. Therefore, we have performed additional micro-beam X-ray scattering measurements on freshly isolated ex-vivo SC that was neither frozen nor subjected to heating or trypsin treatment (fresh shave biopsies). The SAXS and WAXS profiles from all ex-vivo sources tested were very similar to those obtained earlier on isolated SC that is used in the present study (with temperature and trypsin treatment). In particular, the SAXS profiles displayed the usual 4.5-, 6.0- and 11.5-nm bands, and the WAXS profiles – the 0.95- and 0.46-nm bands as well as the sharp 0.41- and 0.37-nm peaks. The anisotropy and the depth-dependent variations were also very similar to isolated SC samples. Therefore, our sample preparation method did not cause any artefacts in the X-ray data and these samples are representative of the real skin surface.

Delipidized SC was obtained by immersion in a 1:1 chloroform/methanol mixture for 2h at ambient temperature. This procedure ensures only partial extraction of SC lipids (see Results); however, using more drastic extraction conditions may further alter other components of the SC and its overall structure.
Sample preparation

Narrow strips of SC (≈0.1×10 mm²) were cut with a razor blade. Both ends were then fixed on two horizontal notches 3 mm apart in such a way that the incident beam of X-rays was parallel to the plane of the SC (see Fig. 1a). The SC film was rigid enough to be supported by the notches without any other support, thereby allowing collection of the diffracted beams in a wide angular range. The identification of areas to be analyzed was made using a microscope with its optical path coaxial with the X-ray beam. Relatively flat and horizontally oriented zones of SC were visually identified for X-ray scattering analysis. Experiments were performed at room temperature (~21°C) and a relative humidity of 30-40%.

X-ray diffraction set-up

The experiments were performed at the European Synchrotron Radiation Facility (Grenoble, France) at the microfocus beamline ID13 (9). The high intensity monochromatic beam (wavelength λ) obtained from an in-vacuum undulator and a Si(111) double-crystal monochromator, was pre-focused with an ellipsoidal mirror (focal spot 20(h)×40(v) µm²) and focused down to a 1 or 2 µm diameter section by a Kirpatrick-Baez optics. A two-piece guard aperture (Pb, about 10 µm square aperture) was used to reduce the diffuse scattering from the exit of the collimator. Samples were mounted on a motorized gantry coupled with a microscope used for positioning the sample with a resolution close to 0.1 µm. The sample-detector distance F was calibrated using silver behenate (first order spacing 5.838 nm). Using a ≈200-µm diameter beam-stop, two-dimensional X-ray scattering patterns were recorded in the range of scattering vectors q = 2π/d = 4π(sin θ)/λ from 0.3 to 25 nm⁻¹ on a CCD detector (16-bit readout; window; 2048×2048 pixels). This setup therefore allowed simultaneously recording two-dimensional SAXS and WAXS signals from 20 nm periodicity down to 0.25 nm. The data collections were carried out during two measurement campaigns, which allowed to check the reproducibility of the results and to refine the measurement procedures. However, all the experimental data reported here comes from a single campaign to facilitate comparisons. The experimental conditions were as follows: λ=0.09621 nm, F=157.5 mm, detector MAR-CCD camera (pixel size 78.94×78.94
µm²). The measured angular resolution in 2θ was 0.075°, which corresponds to resolutions Δq of 0.1 nm⁻¹ in the SAXS region and of 0.01 nm⁻¹ in the WAXS region.

The data collection procedure consisted of a series of transverse scans (perpendicular to the SC plane), at least in triplicate for each sample, with a step size of 2 µm. Two-dimensional scans (transverse and lateral) were also performed to check the homogeneity of the structure at the micrometer scale. The step size for lateral scans was 7 µm. Time exposure for recording a pattern was limited to 1 or 3 s in order to collect data prior to the first radiation damage effects, which were observed after 5- to 10-s exposure. Data analysis was carried out using the equatorial and meridional profiles obtained by integration of the intensity along rectangular thin strips, as a function of q.

Micro-beam experiments have been performed on a total of 9 SC samples from at least 6 different batches/donors of SC (abdominal skin of Caucasian and dark-skin female donors aged between 30 and 40 years). For clarity, the X-ray data presented in the paper correspond to a single representative batch of stratum corneum isolated from a Caucasian female donor between 30 and 40 years old. The main spectral features and the conclusions made here are common to all samples studied. The X-ray data from different measurement campaigns and different donors revealed only small variations in relative intensity of some scattering peaks (for example, the shoulders at q ≈ 0.55 nm⁻¹ and q ≈ 1.4 nm⁻¹ were more or less pronounced in different donors but were invariably present. The shifts in peak position (scattering vector) did not exceed δq ≈ 0.05 nm⁻¹.
RESULTS

Fig. 1 shows a diagram of the scattering geometry and a series of patterns collected in a transverse scan of the control SC. The sample is oriented with the SC surface parallel to the incident X-ray micro-beam (Fig. 1a). Such a configuration is necessary to obtain valuable information about the predominant orientation (and the spread of orientations) of periodic lipid and protein structures within the SC. The scattering patterns at different depths across the SC were collected with a step of 2 µm (Fig. 1b). The intensity of scattering is weaker at the two extremes (toward the external side and toward the epidermal side) because when scanning close to the surface, only a fraction of the incident X-ray beam passes through the sample (cf. Fig. 1b). In the following analysis, scattering intensities will be normalised to take into account variations of the scattering volume and to facilitate comparisons of the profile shape; the normalisation is done by matching intensities in the wide-angle part of the spectrum where the lipid peaks are absent.

For clarity, the SAXS and the WAXS data are presented separately even though they were collected on the same pattern. SAXS features along the meridian (Figs. 2-3) depend on the layer stacking, whereas WAXS equatorial features (Figs. 4-5) provide information about the in-plane molecular packing of lipids inside the bilayers.

SAXS patterns

The SAXS features are well oriented in the meridional plane (Fig. 2a), which is consistent with periodic lamellar structures predominantly oriented parallel to the SC surface. The intensity profile $I(q)$ of the untreated SC sample along the meridional plane (Fig. 2b, black curve) contains several broad peaks attributed to intercellular lipid lamellae (4). The major peak contains two components, a main one at $q \approx 1.1 \, \text{nm}^{-1}$ (corresponding to a lamellar repeat $d = 5.8 \, \text{nm}$) and a shoulder at $q \approx 1.4 \, \text{nm}^{-1}$ ($d = 4.5 \, \text{nm}$). Another small-angle peak is detected at $q \approx 0.55 \, \text{nm}^{-1}$ ($d \approx 11.5 \, \text{nm}$), although it is visually reduced by a logarithmic scale. Finally, a broad and weak peak is seen around $q \approx 2 \, \text{nm}^{-1}$ ($d \approx 3.1 \, \text{nm}$), which is a superposition of the peak at $q \approx 1.85 \, \text{nm}^{-1}$.
often attributed to cholesterol microcrystals (6) and the higher-order reflections of the main peaks.

All these features are superimposed on a continuously decreasing background signal that is due to other (non-lipid) constituents of the SC. This signal is similar to that observed for hair, another keratinous tissue (10). The background scattering can be conveniently approximated by a power function of scattering vector \( I(\text{background}) \sim q^{-\beta} \), where the exponent \( \beta \) is of the order of 2 to 4 (10). Subtracting the background thus allows better visualisation of weaker scattering peaks in the small-angle region. This subtraction procedure is robust; small modifications of the power function do not lead to any significant modifications of the position or intensity of the peaks (Fig. S1). The scattering profile after the background subtraction \( (I \sim q^{-3}) \) plotted on a linear scale (Fig. 2c, black curve) confirms the peak positions given above.

The intensity profile of the delipidized sample along the equator (Fig. 2, panels b and c, red curves) was different from that of the control sample. A weaker peak corresponding to 5.8-nm spacing was observed, whereas the 4.5-nm shoulder was hardly detectable. This peak may be attributed to residual intercellular lipids (non-bound) that were not removed by solvent extraction (see below). The inner peak at \( q \approx 0.55 \text{ nm}^{-1} (d \approx 11.5 \text{ nm}) \) disappeared; instead, a weaker peak at \( q \approx 0.7 \text{ nm}^{-1} (d \approx 9 \text{ nm}) \) became visible. Such decrease of the overall layer thickness after lipid extraction is expected since a part of the lipids is removed. At the same time, the 9-nm peak may in fact be present also in the control sample, but hidden in the background of the main peak at 12 nm because of its relatively low intensity (note that the delipidized profile in Fig. 2c is exaggerated by the normalisation of profiles). If this is the case, one can attribute this new lamellar spacing either to keratin filaments (filament diameter) or to the juxtaposed layers of the corneocyte-bound lipids in the intercellular spaces that were depleted of the non-bound lipids (see Discussion).

The profiles considered so far represented the bulk scattering of the whole SC sample. We will now analyse the micro-diffraction data obtained at different depths in the SC on a micrometer scale. The series of SAXS meridional profiles collected every 2 \( \mu \text{m} \) along a line perpendicular to the SC surface of the control sample did not show any significant differences (Fig. 3a, only every
other profile is shown for clarity). The lamellar stacking is therefore fairly independent of the depth. Indeed, all the major SAXS features discussed above, including the peaks at \( q \approx 0.55 \text{ nm}^{-1} \) \((d \approx 11.5 \text{ nm})\), \( q \approx 1.1 \text{ nm}^{-1} \) \((d \approx 5.8 \text{ nm})\) and the shoulder at \( q \approx 1.4 \text{ nm}^{-1} \) \((d = 4.5 \text{ nm})\), are present at all depths (Fig. 3a). The lack of depth dependence of the SAXS profiles is even more evident after background subtraction (Fig. S2a). This implies that the alternating arrangement of 5.8-nm and 4.5-nm sub-layers in intact SC samples is conserved throughout the SC thickness. On the contrary, the profiles for the delipidized sample collected in the inner and outer zones of the sample (close to the surfaces) are different from those collected in the middle zone. The intensity of the main peak at \( q \approx 1.1 \text{ nm}^{-1} \) \((d \approx 5.8 \text{ nm})\) vanishes close to the two surfaces (Fig. 3b, see the inset with a linear intensity scale for clearer comparison of the intensity; the background-subtracted data are presented in Fig. S2b). Therefore, the lamellar structures with the 5.8-nm repeat distance are only present in the middle zone of the SC and are completely removed by lipid extraction in the outer zones of the delipidized SC. With the mild solvent extraction procedure used here, it is likely that full extraction of non-bound lipids only occurred in the zones most accessible to the solvent, i.e., the layers of the SC that are close to the two surfaces. For fuller extraction in the middle layers, harsher extraction conditions would be necessary. Indeed, in an additional experiment, where the lipid extraction was done for longer time, with agitation and with sequential changes of solvent mixture, we observed a virtually complete disappearance of the 5.8-nm peak in the bulk scattering profiles (Figure S.1b).

Similarly to the transversal scans, we performed lateral scanning in the direction parallel to the SC plane to verify that the molecular arrangement of periodic structures in the SC was also independent of the lateral position. Both SAXS and WAXS profiles obtained with a 7-µm step-size were unchanged throughout the control SC sample. The corresponding scattering patterns are shown in Fig. S3 a and b, respectively.

**WAXS patterns**

The WAXS microdiffraction patterns of the control sample (Fig. 4a) exhibited two broad diffuse rings at 0.95 nm and around 0.46 nm. The former ring is insensitive to lipid extraction and is
therefore attributed to protein component of SC, in particular, the soft keratin (4;16). This repeat distance is likely related to the molecular arrangement of keratin in the filament structure, for example, to the inter-chain distance (4;16-18). The scattering signal corresponding to the 0.95-nm spacing is noticeably polarised, the intensity in the meridional plane being about 65% higher than in the equatorial plane (Fig. 4 and Fig. S4). The anisotropy can be explained by relative preference of the keratin filaments to be oriented closer to the plain of corneocytes, i.e., parallel to the SC surface (19). The second diffuse ring around 0.46 nm is a superposition of the scattering signal from the secondary structure elements of keratin (4;16;18) and the diffuse contribution from the fluid-phase lipids characterized by an average inter-chain distance of 0.46 nm.

Superimposed on the diffuse ring around 0.46 nm are two sharp lipid arcs at 0.413 and 0.37 nm (Fig. 4). The latter two distances are typical of a lateral organization of lipid chains into hexagonal and orthorhombic lattices and the reinforcement of the arcs along the meridian indicates that the molecular axes are perpendicular to the SC plane, consistent with the orientation of the layers parallel to the SC plane.

The equatorial WAXS profiles averaged over the whole thickness of the SC are presented in Fig. 4c. This scattering pattern was approximately reproduced in individual profiles obtained at different depths in the SC (Fig. 5a) with the overall scattering signal diminishing at the two surfaces of the SC owing to reduced volume of the scattering material when scanning close to the surfaces. The keratin bands remained intact, as well as the anisotropy of the 0.95-nm ring (Fig. S4). At the same time, the relative intensity of the two sharp arcs corresponding to crystalline lipids was not constant. The orthorhombic packing gives rise to two reflections corresponding to 0.41 nm and 0.37 nm, whereas the hexagonal packing provides a single peak at 0.41 nm. We used this fact to estimate the relative abundance of the two lipid packing types by comparing the intensities of the reflections at 0.41 and 0.37 nm. The ratio of the intensities of the reflections at 0.41 and 0.37 nm evolved with respect to depth in a symmetrical way (Fig. 5c). In the control sample, this ratio is about three times higher in the middle zone than towards the two edges, indicating that the orthorhombic phase is more abundant in the middle part of the SC than at the
edges. Indeed, the 0.37-nm peak that is uniquely assigned to the orthorhombic phase virtually disappears towards the two surfaces of the SC.

Interestingly, owing to high spatial resolution of the experimental set-up, we found that the arc at 0.413 nm was in fact split into two arcs corresponding to the distances of 0.412 and 0.414 nm (splitting close to the angular resolution). This observation confirms the model of two crystalline phases, one orthorhombic and the other hexagonal which would be responsible for the two arcs, respectively (16;20). There is actually no reason for the reflexions of the two crystalline phases to be perfectly superimposed.

The major effect of lipid extraction in the WAXS pattern is the fading of the 0.37-nm arc (Fig. 4c and Fig. 5b,c). It disappears completely in the outer parts of the SC whilst persisting with a weakened intensity in the very central part. The residual peak at 0.41 nm and the part of the diffuse band around 0.46 nm are likely a contribution from the corneocyte-bound lipids and the part of free lipids that had not been removed by solvent extraction.
DISCUSSION

Lipid structural variations across the SC

The stratum corneum comprises 15-30 alternating layers of flat corneocytes and intercellular lipid lamellae (21), the number of cell layers depending on anatomical site, subject etc. The variation of lipid composition and structural arrangement as a function of depth in the stratum corneum has been explored using electron paramagnetic resonance (22), electron diffraction and microscopy (20) and Fourier-transform infrared spectroscopy (23). Fourier-transform infrared and Raman imaging has also been used to assess the depth-dependent alterations in the concentration of the natural moisturizing factor (24). All these studies used tape-stripping to separately analyse different layers of the SC. Our results represent, to the best of our knowledge, the first attempt to characterize the depth-dependent structural variations in the intact stratum corneum kept at near-native conditions at the time of data acquisition.

The overall SAXS profile observed here at different depths in the SC is similar to those previously reported for bulk human SC (4;6;8) and does not correspond to a single well-defined lamellar spacing. It has been generally interpreted assuming a two-period organization, the largest one at around 11-13.5 nm corresponding to the stacking of a few asymmetric lipid sub-layers that are likely to be responsible for the other periods (≈4.5 nm and ≈6 nm) observed by SAXS (5;11-13). For example, these data can be readily explained by a model of molecular lipid arrangement proposed recently by the group of Norlén (5). Based on comprehensive cryo-electron microscopy analysis and simulations, they proposed that the intercellular lipids are organised in two-band asymmetric 11-nm repeat units consisting of alternating 4.5-nm and 6.5-nm bands. This electron density pattern fits well with the arrangement where ceramides are in stretched conformation with sphingosine tail pointing to the sphingosine tail in the next layer and the fatty acid tail pointing to the fatty acid tail in the layer on the other side, so that two adjacent fatty acid tail layers form the 6.5-nm band and two adjacent sphingosine layers form the 4.5-nm band. Cholesterol would be enriched in the 4.5-nm band, and the long-chain free fatty acids would be in turn concentrated in the 6.5-nm band due to tight packing between the free fatty acid
chains and the long-chain fatty acid tails of ceramides. This model, however, does not explicitly address the conformation of the acylceramides, which are known to be necessary for proper arrangement of the SC barrier. Some authors have in fact proposed that the strength of the 4.5-nm scattering peak of SC is linked to the content of the acylceramide EOH (7). A number of other models of lipid organisation have been also proposed earlier to explain scattering patterns (11-15). The main focus of this study is to follow possible variations in scattering profiles as a function of the microscopic location within the SC.

We have not detected significant differences in the lamellar stacking periodicity as a function of depth in the SC. The scattering profile with 11.5-, 6- and 4.5-nm reflections is reproduced in all layers across the SC. This is in line with the earlier electron microscopy studies of vitreous epidermis, where the characteristic electron-dense line pattern with the same interline spacing was observed throughout the cornified layers (25). The lamellar stacking is therefore independent of the maturation processes accompanying the progression of the corneocyte layers from stratum granulosum to stratum disjunctum.

The lipid lateral packing, however, was not uniform across the SC. We found that the orthorhombic lattice was more abundant in the middle layers of the SC and disappeared towards both the external and the epidermal sides. Looser packing of lipids near the skin surface can be related to progressive disorganisation of the intercellular spaces as corneocytes loose cohesion in approaching the desquamation state. This disorganisation can also be induced by sebaceous lipids naturally present at the skin surface; these short-chain and partially unsaturated lipids could intercalate in the upper layers of SC lipids thereby loosening their lateral arrangement. Lower degree of lipid ordering in the outermost SC layers has indeed been observed by Fourier-transform infrared spectroscopy (23). Furthermore, electron diffraction experiments with grid-stripped SC layers have shown that hexagonal structure dominated in the outer region of the SC, whereas the orthorhombic structure was prevalent in the deeper layers, including the epidermal side (20). This discrepancy with our results for the epidermal side of the SC can be attributed to differences in sample preparation procedures.
Taken together, our results illustrate the potential of the microprobe X-ray diffraction technique to assess depth-dependent variation of SC structure in various conditions. This method is now successfully applied in our laboratories to track structural alterations following treatment with cosmetic ingredients or formulations. The depth-dependent effects are especially interesting in the latter case due to the differences in the permeation kinetics for different formulation ingredients and the complexity of physicochemical transformations of the formulation at the SC surface.

Finally, the X-ray microprobe diffraction is equally well suited to follow the structural variations in the protein component of the SC such as keratin filaments. Protein folding type and supramolecular arrangement can be inferred from the orientation and periodicity information available in the X-ray patterns. In this study, we observed diffuse reflections at 0.95 and 0.46 nm typically attributed to keratin filaments (4,18). For the control SC samples discussed here, we did not find significant variations of these reflections. However, treatment of SC with cosmetic ingredients can lead to depth-dependent alterations in the protein structure that are easily detectable with this technique.

**Lateral homogeneity of SC lipids**

Possible lateral variations of the structure, lipid composition and lateral packing (crystalline, gel or fluid) have been put forward as a way to explain the complex behavior and properties of the SC barrier. For example, Forslind proposed the “domain mosaic model” where liquid crystalline and gel phase domains could coexist laterally in the bilayers (15). Other studies propose or imply the existence of separate coexisting phases or domains with different molecular organisation of lipids (26,27). Our measurements, however, did not detect any significant lateral variations of either the lamellar spacing or the lipid packing at the scale comparable with the corneocyte size. This complements the morphological studies by cryoelectron microscopy showing uniform multilamellar morphology of the intercellular spaces throughout the cornified layers (25).
Amorphous vs. crystallized lipids

In an attempt to estimate the relative amount of the lipids in amorphous state, we subtracted the WAXS meridian profiles of delipidized samples from those of control SC (Fig. 4c). The resulting profile corresponds to the lipids that have been removed by solvent extraction. It consists of a broad peak due to a non-crystalline phase (amorphous) with superimposed small peaks at 0.41 and 0.37 nm. Despite the low statistics of the signal, the relative amounts of crystallized lipids in the control SC versus the amorphous lipids that have been removed by solvent extraction can be roughly estimated from the ratio of the two sharp peak areas in the control profile / broad peak area in the subtracted profile. Based on the method described by Vonk (28) the proportion of crystallized lipids in relation to all the extracted lipids could be estimated at 20% ± 10%. This estimation therefore suggests that the majority of lipids SC therefore are non-crystalline. This result, however, contradicts the general notion that the majority of intercellular lipids are in solid state as well as the recent NMR data for porcine SC (29). This may be related to incomplete lipid extraction in our experiments leading to possible preferential extraction of more fluid lipids. In addition, a contribution from fluid intercellular lipids cannot be excluded. Additional measurements with an optimised WAXS setup and harsher lipid extraction conditions will be necessary to confirm this result.

ACKNOWLEDGMENTS

We are grateful to Manfred Burghammer and the staff of the ID13 beamline of the ESRF for providing valuable assistance with the X-ray data collection. We would like to thank Jean-Luc Lévéque and Claude Bouillon for reading the manuscript and for their valuable suggestions. The help of Emilie Leccia with the supplementary measurements at ESRF is gratefully acknowledged.
REFERENCES


Figure 1. Scattering geometry and the patterns collected from different layers of the stratum corneum using a scanning X-ray microbeam. (a) Set-up geometry and orientation of equatorial and meridional planes of the scattering pattern relative to the plane of the SC. Scanning across the SC layers was performed by vertically displacing the sample with 2-µm step (marked with black dots). Both small-angle and wide-angle reflections are visible in the scattering pattern. (b) A series of nine scattering patterns obtained by transversal scanning of the SC. Left-to-right then top-to-bottom reading direction corresponds to sequential shifts from the inner (epidermal) to the outer (external) SC side.
Figure 2. Small-angle scattering data for native and delipidized human stratum corneum.
(a) Typical SAXS pattern with symmetrical arcs corresponding to the 6-nm and 4.5-nm spacing. Note the orientation of the scattering features in the meridional plane. (b) Scattering profiles in the meridional direction for native (black) and delipidized (red) samples. Calculated background scattering profile corresponding to non-ordered components is shown with dashed line. Abscissa corresponds to the scattering vector \( q = 2\pi/d = 4\pi (\sin \theta)/\lambda \) and ordinate – to the scattered intensity on a logarithmic scale. Lamellar repeat distances corresponding to scattering peaks are indicated with arrows. (c) Scattering profiles after background subtraction (note the linear intensity scale).
Figure 3. Depth-dependent small-angle scattering profiles for native and delipidized stratum corneum. The rainbow colour code corresponds to scanning the SC from the outermost layer (violet) to the innermost one (wine red). (a) Control SC sample. Only every other profile is shown for clarity, which corresponds to 4-µm steps. (b) Lipid-extracted SC sample. The smaller overall thickness of the scans for the delipidized sample is explained by local variations of the SC thickness, lack of perfect alignment of the scan direction with the normal to the control SC sample (see Fig. S3b) and the lipid extraction itself. The inset shows a magnified fragment of the scattering profiles on a linear intensity scale to demonstrate the attenuation of the 5.7-nm peak at the SC sides.
Figure 4. Wide-angle scattering data for native and delipidized human stratum corneum.

The microdiffraction patterns correspond to a single depth position in the middle part of the SC samples. (a) Control SC sample. Sharp arcs in the equatorial plane are visible, as well as two diffuse rings: nearly isotropic one located around $2\pi/0.46\,\text{nm}^{-1}$ and the other one centred at $2\pi/0.95\,\text{nm}^{-1}$ and enhanced in the meridional plane. The inset shows magnified detail of the pattern with sharp equatorial arcs where the splitting of the 0.41-nm arc is evident. (b) Corresponding WAXS pattern for the delipidized sample. Note the decreased intensity of the equatorial arcs. (c) Averaged equatorial profiles of the control sample (black) and the delipidized one (red) are shown along with the result of subtraction of the latter from the former (black dotted curve). The resulting profile therefore corresponds to the lipids removed by solvent extraction. Note that the WAXS profiles were not normalised and therefore correspond to actual scattering intensity observed.
Figure 5. Variation of the equatorial WAXS profiles with the depth in the stratum corneum. The rainbow colour code corresponds to scanning the SC from the outermost layer (violet) to the innermost one (wine red). (a) Control SC sample. Only every other profile is shown for clarity, which corresponds to 4-µm steps. (b) Lipid-extracted SC sample. (c) Depth dependence of the ratio of scattering intensities of the 0.37-nm and the 0.41-nm arcs representing relative abundance of the orthogonal packing in different SC layers. Note that the WAXS profiles were not normalized and therefore the scattering is more intense from the middle portions of the SC due to the larger scattering volume (the entire X-ray microbeam is inside the SC thickness).