PROCEEDINGS

11th International Wool Research Conference

4 - 9th September 2005

LEEDS, UK

Published by

The Department of Colour and Polymer Chemistry

The University of Leeds

Leeds LS2 9JT

LAMELLAR REARRANGEMENT OF INTERNAL WOOL LIPIDS

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Abstract

The lipid composition and thermotropic behaviour of internal wool lipids (IWL) considerably resemble those observed in lipids from more studied keratinised tissues, such as the stratum corneum. In this substrate, X-ray diffraction techniques have been applied extensively, suggesting their suitability for the study of lipids from the intercellular wool matrix. Our study presents the results obtained by applying small angle X-ray scattering (SAXS) with different radiation sources to a new lipid model arrangement achieved by concentrating internal wool lipid liposome suspensions close to their "native" water content. The influence of the water content in the lamellar structure is also presented.

Furthermore, topical application of internal wool lipid liposomes on both intact and disturbed skin has been shown to improve skin barrier properties. These results suggest a certain level of interaction between these liposomes and the lipid domains of the stratum corneum (SC). To demonstrate the ability of internal wool lipids to rebuild lipid bilayers of the stratum corneum, lamellar structures, such as liposomes formed by SC lipids and IWL are applied Lo delipidised SC. Changes brought about by treatinent with the aforementioned liposomes on the structure of delipidised SC were observed using transmission electron microscopy of cryosubstituted samples. The results show that SC and IWL liposomes are able to reorganize the lipid lamellae of SC samples that were previously delipidised. These results offer ncw opportunities in the study of skin disorders associaied with the lack of lipids or with an alteralion in the lipid lamellar structure.

Introduction

Internal wool fibre contains about 1.5% of lipids, consisting mainly of free fatty acids (FFA), sterols and ceramides (1). These lipids, together with other minor wool components, constitute the Cell Membrane Complex (CMC), whicb is known to govern the perineability of the wool fibre. In fact, intercellular wool lipids (IWL) are assumed to be arranged in a bilayer of 5.0-8.0 nm in width (β layer) and serve as the main barrier against the penetration of hydrophilic compounds into the fihre. Moreover, they are essential for the dyeing and diffusion propeities of the wool fibre (2).

Despite the advances in characterising the lipid composition of the cell membrane complex (3, 4), little progress has been made in furthering our understanding of the arrangement and thermotropic behaviour of these components. However, the bilayer forming capability (5)and some physicochemical propcrties have been studied in an attempt to yield further insight into the lipid structure (6). DSC, FTIR and EPR methodologies have been applied to IWL extracts structured in liposome vesicles as a inodel of a wool lipid membrane, demonstrating that the membrane is less permeable at the surface hydrophilic ends than in its hydrophohic core. In

terms of thermotropic behaviour, the IWL membrane presents two phase transitions: a main phase transition at 40ºC (Tm), produced by a loss of the conformational order, increasing the fluidity of the membrane with the disorder; and a second minor and broader irreversible transition phase from 60-65ºC (*6*).

In terms of composition and thermotropic behaviour, the IWL resemble those from more studied keratinised tissues, such as the stratum corneum (SC) (*7*). In this substrate, the X-ray diffraction techniques have been applied extensively, suggesting their suitability for the study of the lipids from the intercellular wool matrix (*8*). However, the low lipid content and the high amount of β-keratin in wool fibre hinder their study by X-ray diffraction techniques. A study has been recently published applying wide- and small-angle X-ray diffraction techniques (WAXD & SAXS) to a new lipid model arrangement achieved by concentrating IWL liposome suspensions close to their "native" water content (*9*). Structurally, internal wool lipids are arranged in crystal orthorhombic states separately, and in a liquid crystal state when mixed together. At 40ºC there is a reversible phase transition produced by the melt of the crystal orthorhombic states, whereas the liquid crystal conserves its state until 65ºC (*9*). In our study SAXS results are presented using liposome suspensions at different hydration levels to determine the influence of the water content in the lamellar structure of the wool fibres.

These lamellar lipid bilayers are similar to the ones formed with the intercellular lipids of the SC (*6*). This arrangement plays an important role in the barrier function of the skin, the prevention of penetration of external agents and the control of transepidermal water loss to maintain physiological skin hydration levels (*10, 11*). Formulations that contain similar skin lipids and in particular some ceramide supplementation could improve disturbed skin conditions, provided they penetrate. The utilization of physiological lipids according to these parameters has envisaged new forms of topical therapy for some dermatoses (*12-14*). As stated above, the IWL composition, similar to the one present in the SC (*3, 4*) has been shown to be capable of forming liposomes with this similar lamellar structure (*5, 6*). Furthermore, topical application of IWL liposomes on intact and disturbed skin has been shown to improve barrier skin properties (*15, 16*). Accordingly, Transmission Electron Microscopy will be used to assess the modification of the lamellar structure of the SC when subjected to delipidisation and to evaluate its reorganization when the delipidised tissue undergoes treatment of liposomes made up of SCL and IWL. Internal wool lipids could be regarded as a new natural extract, beneficial for topical application and suitable for incorporation into pharmaceutical or cosmetic formulations in the treatment and care of skin.

Materials and Methods

Preparation of the IWL and SCL Liposome Samples. Raw industrially scoured Spanish Merino wool supplied by CORCOY S.A. (Terrassa, Spain) was used to obtain the internal wool lipids. The internal wool lipids were soxhlet extracted from cleaned wool with chloroform/methanol azeotrope (79:21 v/v) for 5 hours, as previously reported (*3*). Sample composition was checked by thin-layer chromatography (TLC) coupled to an automated ionisation detection (FID) system (Iatroscan MK-5, IATRON LAB. INC. Tokyo, Japan) with a sample spotter SES 3202/IS-01 (SES GmbH, Nieder-Olm, Germany) using an optimised TLC/FID protocol to analyse ceramide content (*4*).

The lipid extracts were dried with nitrogen and suspended in water, attaining a final concentration of 20% in weight. Suspensions were then sonicated in a Labsonic 1510 sonicator

(B. BRAUN, Melsungen, Germany) at 70 Watt with a thermostatic bath Ultraterm 6000383 (SELECTA, Barcelona, Spain) at a temperature of 65ºC for about 15 min, until the suspensions became homogeneous. The preparations were then annealed at the same temperature for 10 min and incubated at 37ºC in a nitrogen atmosphere until further use. Liposome suspensions were diluted with water or concentrated under nitrogen flux to achieve sample suspensions of 10%, 20%, 40%, 60% and 80% in weight of lipid in water.

Liposomes modelling the stratum corneum (SCL liposomes) were prepared from the following mixture (%wt): 40.0 Ceramide III, 25.0 cholesterol, 25.0% palmitic acid and 10.0 cholesteryl-sulphate using the same methodology as described before for the IWL liposomes.

Small Angle X-ray Scattering (SAXS). The small angle X-ray scattering measurements were carried out using a Siemens KF760 generator (working in the Cu Kα band) coupled to a Peltier temperature controller. The radiation was registered by a PSD-OED 50 linear detector coupled to a Kratky small angle camera (M Braun GmbH, Graching, Germany). Myler® chambers of 1mm in width were filled with the IWL liposome samples and maintained 10 minutes at every working temperature (25, 30, 35, 40, 45 and 50ºC) to reach thermal equilibrium before irradiation. The irradiation was performed under vacuum for a period of 30 min each sample. The SAXS intensity data were processed by software (Visual Basic v.5.0) to obtain the highest possible resolution. The X-ray diffraction data were also collected at the SAXS beam line at the Synchrotron radiation source Elettra (Trieste, Italty) containing 1024 channels and 2 GeV electron storage ring. The radiation wavelength was 1.542 Å. The SAXS measurements were performed by triplicate with acquisition times of 100 sec and 20ºC. The q value and intensity of the peaks were the same for every one of the triplicate. The data were collected on a linear position-sensitive Gabriel detector, which enabled simultaneous detection of the whole resolution range.

The scattering intensity was plotted as a function of the scattering vector Q, defined as $Q = (4 \pi \sin \theta) / \lambda$, in which λ and θ are the wavelength and scattering angle, respectively. The positions of the diffraction peaks are directly related by Bragg's law $2d \sin \theta = n\lambda$, in which n and d are the order of the diffraction peak and the repeat distance, respectively. In the case of the lamellar structure, the various peaks are located at equidistant positions, which are given by the equation $Q_n = 2 n \pi/d$, in which Q_n is the position of the nth order diffraction. Experiments were carried out in duplicate in order to check their reproducibility.

Stratum Corneum Isolation, Lipid Extraction and SCL and IWL Liposomes Application
Fresh unboiled pig skin was cut into strips of about 10x40cm with a butcher's knife. Most of the hair was removed from the skin surface with animal clippers followed by an electric shaver. Sections of fresh pig skin were placed in water at 65ºC for 4-5 min and the epidermis was scraped off in sheets. The epidermal sheets thus obtained were placed in 100 mL of 0.5% trypsin in phosphate-buffered isotonic saline (PBS) at pH 7.4 and kept at 4ºC overnight. The SC pieces were then collected on a coarse sieve, rinsed with distilled water, and suspended in a large volume of distilled water. The pieces were then individually transferred to a round flask, to which 100mL of fresh trypsin/PBS solution was added, and the flask was rotated by means of a rotary evaporator at 100 rpm to provide gentle agitation at room temperature. After 2h the tissue pieces were again collected on a sieve, washed with distilled water and blotted dry with filter paper (*17-19*).
The sheets were extracted twice for 2h with each of the three mixtures of chloroform/methanol (1gr SC/100mL of 2:1,1:1, and 1:2 vol/vol) and then for 1h with the

same mixtures. The SC was finally extracted with methanol overnight to remove traces of polar lipids (*19-21*). The stratum corneum sheets were incubated with 10mg/mL SCL and 10mg/mL IWL liposomes at 20ºC for 18h in a thermostated bath.

Freeze-Substitution Electron Microscopy (TEM) This technique in combination with ruthenium tetroxide (RuO_4) fixation provides a different visualization of the SC allowing us to observe cross sections of the SC (from the surface to the deeper layers), not only a few layers as is the case of the freeze fractured samples.
The methodology used was based on that described by Van den Bergh et al. (*22*). The SC was cut into small ribbons approximately 2x1 mm in size. The ribbons were fixed in 5% (w/v) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, and postfixed in 0.2% (w/v) RuO_4 in sodium cacodylate buffer, pH 6.8 with 0.25% (w/v) potassium ferrocyanide ($K_4Fe(CN)_6$). After 1 h, the RuO_4 solution was replaced by fresh RuO_4 in order to establish an optimal fixation. After rinsing in buffer, the tissue samples were cryofixed, by rapid freezing on a liquid nitrogen-cooled metal mirror (Cryovacublock, Leica) at –196ºC prior to freeze-substitution.

The freeze-substitution procedure was carried out in an AFS (Automatic Freeze Substitution) system (Leica). The tissue samples were cryosubstituted at –90ºC for 48 h using 100% methanol, containing 1.0% (w/v) osmium tetroxide (OsO_4), 0.5% (w/v) uranyl acetate and 3.0% (w/v) glutaraldehyde. After the 48 h substitution period, the temperature was raised to –50ºC, the samples were washed 3 times in 100% methanol, and subsequently the methanol solution was gradually replaced by the embedding medium, Lowicryl HM20 (100%). This resin was replaced after 24 and 48 h by freshly made embedding medium. Finally the samples were transferred to a mould containing Lowicryl, and were incubated for 48 h at –50ºC under UVA-radiation, to allow polymerisation. Ultrathin sections were cut (Ultracut UCT, Leica), transferred to formvar-coated grids and examined in a Hitachi 600 transmission electron microscope. For each sample, 10 overview and approximately 30-40 detail electron micrographs were taken.

Results and Discussion

The substrate studied is the internal wool lipids (IWL) extracted from wool and analysed by TLC/FID as described in the Materials and Methods Section. The major lipid classes quantified were cholesterol esters (3%), glycerides (4%), free fatty acids (12%), cholesterol (15%), ceramides (34%), glycosylceramides (5%) and cholesterol sulphate (10%) in weight. A number of works on biological membranes have demonstrated the usefulness of the study of structural changes in determining the morphological function of the structures involved. To this end, the thermotropic behaviour of IWL was previously studied by applying polarised optical microscopy and X-ray diffraction techniques (*9*). Liposome suspensions concentrated down from 60 to 20% of water content are arranged as lamellar structures of 5-8nm with an ordered phase crystal orthorhombic state and a liquid crystal state (*9*). These structural domains differ from other similar membranes such as SC, especially in the absence of a wider lamellar structure (the SC has a long periodicity phase of about 13nm attributed to the presence of Cer 1 (*23*)) and in the dependence of the main diffraction distance on the water content (hydration levels of the SC lipids from 6 to 60% in weight were published to allow the arrangement of the lipid bilayers without any significant effect in the X-ray results (*8*)). Therefore, the concentrated liposome suspensions were used as a model to study the internal wool lipid membrane structure by small-angle X-ray scattering technique (SAXS) at different hydration levels in order to determine the possible influence of the water content in the lipid structure of the β-layers and

its relationship with the particular behaviour of the wool fibre under different hydration conditions.

SAXS was employed to study the structural lamellar arrangements and the thermotropic behaviour of the IWL liposomes prepared at the concentrations between 10 and 80%. The diffraction pattern of each sample was obtained at the following temperatures 20, 30, 40, 50 60, 70 and down to 25ºC. Diffraction patterns of the different concentrated samples at 20 and 50ºC are shown in Figure 1.

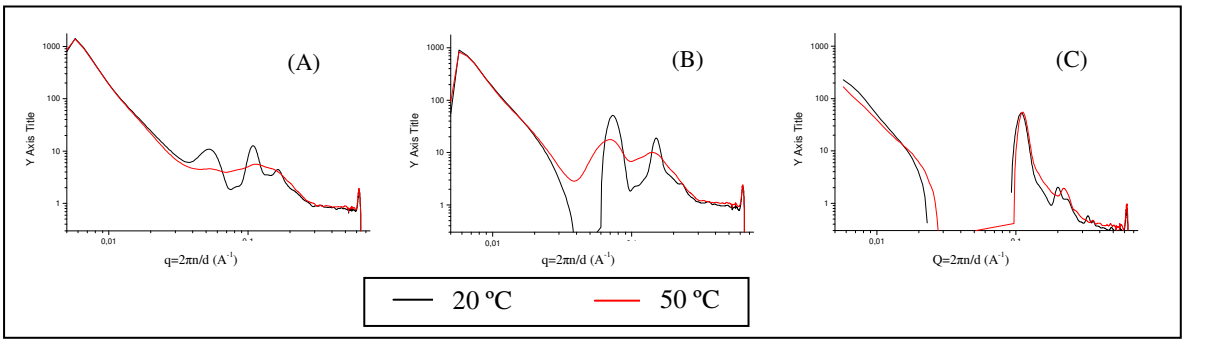

Figure 1. SAXS patterns of 20.1% (A), 40.2% (B) and 80.2% (C) IWL liposomes at 20º and 50ºC.

At room temperature, the main diffraction peak varies from 15.6nm at a diluted liposome solution of 10.1% of lipids to 12.0nm, 8.9nm, 7.6nm and 6.3nm at the increasing lipid concentrations of 20.1%, 40.2, 53.3% and 80.2%, respectively (See Table I). In all cases a second diffraction peak is obtained and in some cases the third one can also be detected. In all cases, a lamellar structure of the lipids can be deduced with an inclusion of water molecules, which induces a marked increase in the diffraction distances as the dilution increases. The unique lamellar structure obtained for the diluted samples is split into two smaller lamellar structures of similar distances obtained for each of the two samples at higher lipid concentrations. These two lamellar structures are more readily observed in the differences of the second order diffraction peaks. Moreover, the intensities are higher with the higher lipid concentration and some differences are clearly detected when the temperature is increased. In the case of the diluted samples of up to 40% of lipids, the lamellar structure disappears at 50ºC. For the more concentrated samples (53% and 80% of lipids), the two lamellar structures transform into one single lamellar structure of an intermediate distance and a similar intensity. In all cases these changes are reversible, obtaining similar diffraction patterns at low temperatures before and after heating at 70ºC. The main diffraction distance of the sample previously subjected to the heating process (Table I) is smaller. This could be attributed to a dehydration of the samples after the heating process.

Table I. Main diffraction distance of the differently concentrated IWL liposome samples obtained by SAXS

Liposome samples	20ºC	30ºC	40ºC	50ºC	60ºC	70ºC	25ºC
10.1 % lipids	15.6	15.6	15.4	--	--	--	15.6
20.1% lipids	12.0	12.1	12.3	--	--	--	11.3
40.2% lipids	8.9	9.2	8.7	--	--	--	7.7
53.3% lipids	7.6 6.0	7.5 6.0	7.2 6.1	6.8	6.5	6.2	6.2 5.5
80.2% lipids	6.3 5.8	6.2 5.5	6.0 5.5	5.8	5.5	5.3	5.3

These results highlight the importance of the presence and amount of water in the lamellar structure of the lipids from the wool fibre, which gives rise to a different lamellar pattern. This indicates that a large amount of water can be retained in the lipid wool membrane affecting the lamellar distances. Moreover, the absence of the ordered lamellar structure at 50ºC, when more than 50% of water is present in the bilayer structure, could account for the increase in the permeability of the wool fibres when these are soaked in water at temperatures exceeding 40ºC.

To confirm these results SAXS patterns of two liposome samples at different lipid concentrations were obtained using a Synchrotron radiation source (See Figure 2).

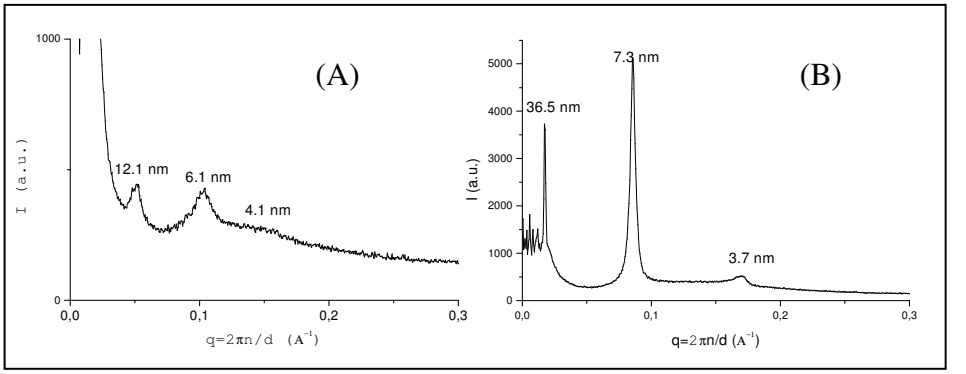

Figure 2. SAXS patterns using Synchrotron of 20% (A) and 45% (B) IWL liposomes at 20ºC

A larger lamellar structure was again obtained when more water was present in the liposome solution, which contrasts with the published reports of similar structures with lipids from the stratum corneum of mammals. In this case, water molecules are present in the lipid bilayer from the wool structure, affecting the lamellar distances.

The main diffraction distance obtained at 20ºC for each liposome sample is plotted against the percentage of water (See Figure 3).

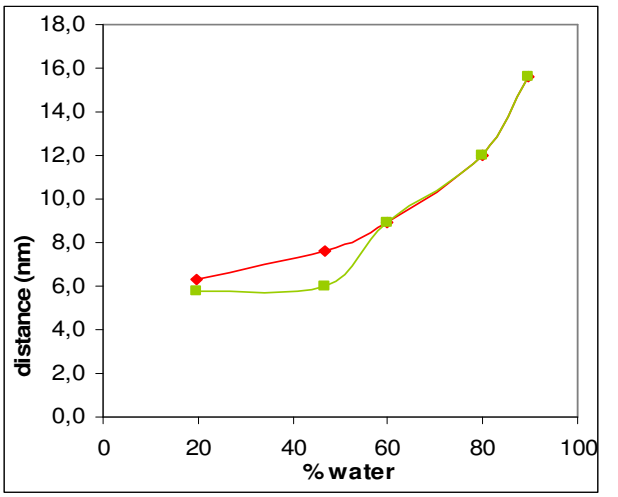

Figure 3. Bilayer distance of IWL liposomes obtained by SAXS versus water content

The distance of the lamellar structure in the absence of water (5.3nm) can be deduced by extrapolating the curve to 0% of water. Therefore, the approximate percentage of lipid related to water in volume can be obtained with the main diffraction distance: d dry x 100/ d real= percentage of lipid (in volume) in the ordered lamellar structure.

Table II. Percentage of lipid in the ordered lamellar structure obtained for the IWL liposome samples at different temperatures.

Liposome samples	20ºC	30ºC	40ºC	50ºC	60ºC	70ºC	25ºC
10.1 % lipids	34	34	34	--	--	--	34
20.1% lipids	44	44	43	--	--	--	47
40.2% lipids	60	58	61	--	--	--	69
53.3% lipids	70 88	71 88	74 87	80	82	86	86 96
80.2% lipids	84 91	86 96	88 96	91	96	100	100

It should be pointed out that these lamellar structures can retain different amounts of water, achieving in the case of the most diluted sample a high percentage of water (about 66%). At high lipid concentrations, the two different lamellar structures can be assumed to have different affinities for the water content at low temperatures, and at temperatures exceeding 40ºC, they form an single lamellar structure with an intermediate water content. The larger lamellar structure could be enriched in charged compounds such as free fatty acids and/or cholesterol sulphate with a greater affinity for water. All the lipids can be fused together at higher temperatures with an intermediate water content. Since the sample container is not hermetically closed, despite the existence of reversibility in the lamellar structure reformation, there is always a loss of water due to the heating process which in the case of the most concentrated sample prevents splitting of the lamellar structure.

The relationship obtained between the amount of water and the distance of the lamellar lipid structure of the IWL liposomes could be correlated with the distances of the β-layers of the

CMC obtained by TEM of the wool fibres. Thus, the water content and its capacity for changing the lamellar structure could be assumed; this could account for the different permeability of the fibres due to their water content.

Topical application of internal wool lipid liposomes and model stratum corneum lipid liposomes on intact and disturbed skin has been shown to improve skin barrier properties (*15, 16, 24*). These results suggest a certain level of interaction between these liposomes and the lipid domains of stratum corneum (SC). To demonstrate the ability of IWL to rebuild lipid bilayers of stratum corneum, lamellar structures such as liposomes made up of IWL and SCL were applied to delipidized SC sheets (see the experimental section). Transmission Electron Microscopy of the different cryosubstituted samples was performed and some examples are shown in Figure 4.

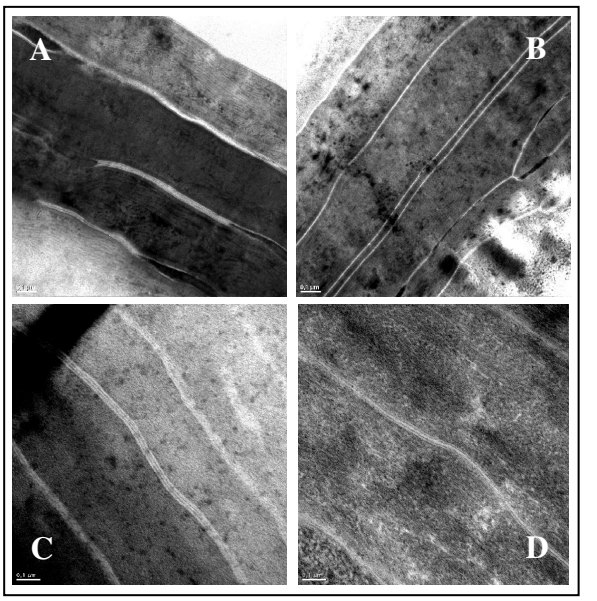

Figure 4. **TEM of: A) Native SC sample, B) Delipidised SC sample, C) SCL liposome treated sample of delipidised SC and D) IWL liposome treated sample of delipidised SC.**

The native SC (Picture A) shows the different corneocytes and the typical lamellar image of the intercellular lipids. Some corneosomes are also observed. The delipidised SC sample (picture B) shows the empty spaces of the intercellular region. This indicates that the chloroform/methanol mixtures are able to extract SC lipids, which is in agreement with other authors (*18-20*). Some corneocytes and corneosomes can also be seen. Moreover, the delipidised SC was treated with SCL liposomes (picture C). In these samples, the effect of the SCL liposomes on the SC intercellular spaces was clearly observed. Although these samples had been subjected to an exhaustive extraction with chloroform/methanol mixtures, the lamellar structure was reconstructed by subsequent treatment with SCL liposomes. The delipidised SC treated with IWL liposomes can be observed in picture D. In some cases a lamellar structure was observed in the intercellular spaces, which resembles not only the original lamellae present in the native SC but also the results of the treatment with SCL liposomes. This could indicate a certain restructuring effect of the IWL on the SC intercellular spaces, which had been previously delipidised.

Conclusions

The presence of water in the lamellar structure of the lipids from the wool fibre gives rise to a completely different lamellar pattern related to the water content. This indicates that large amounts of water can be retained in the lipid wool membrane, affecting the lamellar distances. Moreover, the absence of the ordered lamellar structure detected at 50ºC, when more than 50% of water is present in the bilayer structure, could account for the increase in the permeability of the wool fibres when these are soaked in water at temperatures exceeding 40ºC. A relationship was obtained between the amount of water and the distance of the lamellar lipid structure of the IWL liposomes, which could be correlated with the distances of the β-layers of the CMC obtained by TEM of the wool fibres. The modification of the lamellar structure of the SC when subjected to delipidisation and the reorganization of the structure when the delipidised tissue was treated with liposomes consisting of SCL and IWL were visualized by TEM .

Acknowledgements

We acknowledge the expert assessment and technical assistance of Jaume Caelles in the SAXD experiments and Carmen López in the TEM experiments. We also thank Dr. Ramón Pons for his comments on the SAXS results. Finally, the authors wish to thank G. von Knorring for improving the final version of the manuscript.

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