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Physical and chemical perturbations of the supramolecular organization of the stratum corneum lipids: In vitro to ex vivo study

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ABSTRACT

Background: FTIR spectroscopy is classically used to study the supramolecular organization of the stratum corneum lipids. Exposure to UV_A is responsible for a small decrease in packing observed on cutaneous lipid films. *Methods:* Lipid films and human skin biopsies were either exposed to UV_A irradiation of 120 J/cm^2, UV_B irradiation of 0.15 J/cm^2 or put in contact with ethanol. Using FTIR in vitro and IR microspectroscopy ex vivo provided information on: i) the precise localisation of the stratum corneum in the skin, ii) its thickness, and iii) the organization of its constituted lipids. *Results:* Different action modes were observed for UV irradiation and the contact with ethanol with a certain destabilisation of the lipidic layer. Ethanol was also found to be responsible for the creation of pores. The destabilisation of the lipid cement was mainly observed ex vivo. *Conclusion:* The barrier function of the skin is affected by the action of physical and chemical external agents at the molecular level. The increased laxity of the lipid packing could enable the percutaneous penetration velocity of actives.

1. Introduction

The skin is the part of the body the most exposed to environmental attacks (UV irradiation, pollution [1], toxics…). UV irradiations are known to be very dangerous for the skin and are directly responsible for grave diseases. For instance, they are involved in the formation of wrinkles [2], erythema and melanoma [3]. Researches over the past few years, have shown different irritation effects on the skin depending on the irradiation level. UV_B act at the level of the epidermis [4,5] and are responsible for sunburns and the formation of reactive oxygen species (ROS) in the epidermis, whereas the UV_A damages occur at the level of the dermis [4] and DNA [6], and are therefore much more dangerous for health.

The stratum corneum (SC) is the outmost layer of the skin and so the most affected by the environment. It is composed of corneocytes embedded in lipids and its thickness ranges from 10 to 20 μm. Due to its particular composition, the SC plays an important role of barrier function. The lipids present in this layer (fatty acids (FA), ceramides (CER) and cholesterol (CHOLE)) are organized into two liquid crystal phases of different periodicity: orthorhombic (13 nm), and hexagonal (6 nm) [7]. The cement formed by these lipids protects the body from external attacks and also from water loss.

It is then of great interest to understand the effects of UV_B or UV_A irradiations and of the contact with a penetration enhancer (PE) such as ethanol (EtOH), on the molecular organization of the SC lipids. PE increases the permeation of molecules or formulations through the skin [8]. EtOH was chosen due to its recurrent use in cosmetics or the pharmaceutical fields. PE acts at the molecular level of the SC. Several action modes can be observed: a decrease in the intermolecular interaction between polar headgroups, or a laxity of the carbon chain [9], depending on the structure of the PE. The disorder induced by these perturbations permits an increase of the percutaneous penetration of actives.

It has already been proved by in vitro studies [10–12], that UV_A are responsible for i) β scission, ii) hydrogenation of double bond in unsaturated compounds, and iii) formation of oxygenated entities in lipids. Despite these chemical modifications, the conformational order of the CER, CHOLE and FA remains globally organized in an orthorhombic state, even if a decrease of the packing is locally observed.

In a previous work, the effects of UV_A irradiation of 60 or 120 J/cm^2 were studied on lipids films [12]. We have extended this work by studying the effects of a single UV_B irradiation of 0.15 J/cm^2 and the contact with EtOH on the supramolecular organization of the lipids using Fourier Transformed infrared spectroscopy (FTIR). Over the last years, this technique has been classically employed to study the supramolecular order of lipids [13,14], through the shift in frequency of particular bands.

Secondly, these experiments were transferred to ex vivo studies on human skin biopsies, comparing UV_A and UV_B irradiation and contact of EtOH. In this case, IR imaging was used. This technique enables the simultaneous analysis of the conformational order of the

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molecules as a function of the depth, and the thickness of the different layers of the skin. The effects of UV irradiation were compared as a function of the lagtime between the irradiation and the IR measurements.

2. Experimental

2.1. Material

The CER III, CER IIIa and CER IIIb with a purity of 95% were a gift from Cosmoferm. CHOLE and nervonic acid (C_{24}:1) were purchased from Sigma-Aldrich (St Quentin Fallavier, France) with a purity of 99%. Phosphate buffer saline pH 7.4 (PBS) also comes from Sigma-Aldrich. Ethanol (EtOH), methanol (MeOH) and chloroform ($CHCl_3$), with a purity of 95%, 98% and 99.3% respectively, come from VWR (Fontenay-sous-Bois, France).

Full thickness human skin biopsies were obtained from abdominoplastic surgery of 35 years old women. The skin was cleaned of the hypoderm with a scalpel and the final thickness was approximately 400 μm. The biopsies were then stored at −80 °C until use.

2.2. Irradiation protocol

2.2.1. In vitro experiments

Each lipid (CER, FA, or CHOLE) was dissolved in a $CHCl_3$/MeOH mixture (2:1, v/v). Lipid stock solutions of 10^{-3} mol/l were prepared. 150 μl of each solution was disposed on a CaF_2 window. At room temperature, the solvent evaporates, forming a "film" of lipids. The term "film" indicates that the lipids are auto organized in a solid form. In parallel, a mix of each class of lipids (lignoceric acid/CER III/CHOLE 33:33:33, v/v/v) was also studied to mimic the SC composition.

UV_B irradiation of 0.15 J/cm^2 at a wavelength of 321 nm was applied on the films using a Vilber Lourmat lamp RMX 3W (Marne La Vallée, France).

2.2.2. Ex vivo experiments

Skin biopsies were disposed in a phosphate buffer pH 7.4 (PBS) before the irradiation.

UV_B irradiation of 0.15 J/cm^2 at a wavelength of 321 nm was applied for 32 s using a Vilber Lourmat lamp RMX 3W (Marne La Vallée, France). This amount of irradiation is equivalent to 8 min of exposure to the sun at a latitude of 40 N (South of France).

For the UV_A irradiation of 120 J/cm^2 an Oriel lamp (Stratford, United States) 91292–1000 with UV_B and UV_C filters was used. The wavelength of the lamp spans from 315 to 380 nm. 2 h 27 min were required for this irradiation corresponding to 6 h of exposure to the sun at a latitude of 40 N (South of France).

We selected two levels of irradiation assumed to have a comparable action on the skin at an erythema level. For this reason the amount ratio of UV_A/UV_B irradiation was chosen to be around 800 because the effect of 1 J/cm^2 of UV_B is approximately equivalent to a dose of 800 J/cm^2 of UV_A [15].

The biopsies were stored at +5 °C for various durations after the irradiation (2 h, 1, 2, 3 or 4 days) in PBS before to be frozen at −80 °C, and cut with a Leica cryomicrotom CM 3050S, at 5–6 μm of thickness and disposed on a CaF_2 window. The windows were kept at room temperature prior to the IR experiments.

2.3. Chemical perturbation protocol

2.3.1. In vitro experiments on films

The influence of the application of two concentrations of EtOH on the supramolecular order of the lipids was studied: pure EtOH and a mixture of EtOH/CHCl3/MeOH (3:2:1, v/v/v). Each lipid was diluted in these two solutions. The results were compared to the blank, diluted in CHCl3/MeOH (2:1, v/v), a solvent mixture which has no influence on the organization of the molecules. 150 μl of each solution was disposed on a CaF_2 window. At room temperature, the solvent evaporated, forming a "film".

2.3.2. Ex vivo experiments: penetration experiments

Biopsies with a diameter of 3.5 cm were disposed on Franz cells with a diameter of contact with the donor solution of 1 cm. The receptor part was filled with PBS. A stirring of 400 rpm was applied and the system was thermostatted at 32.5 °C. 1 ml of H_2O, H_2O/EtOH (1:1, v/v) or pure EtOH was disposed on the donor part and was maintained in contact with the skin for 24 h. Semi-oclusive environment was chosen to avoid the evaporation of the EtOH without accelerating the penetration. After 24 h, a part of the donor solution remained present. After elimination, a tissue is softly applied on the surface to remove possible droplets of solvent, taking care not to damage the skin.

2.4. FTIR spectroscopy measurements

2.4.1. FTIR: in vitro experiments

Infrared spectra were acquired using a Perkin Elmer Spectrum 2000 spectrophotometer equipped of a microscope, used simply to locate the samples on the CaF_2 window. We worked on a transmission mode. Two spectra of each sample were acquired right after the irradiation and a second time 3 days after the irradiation to study the evolution over

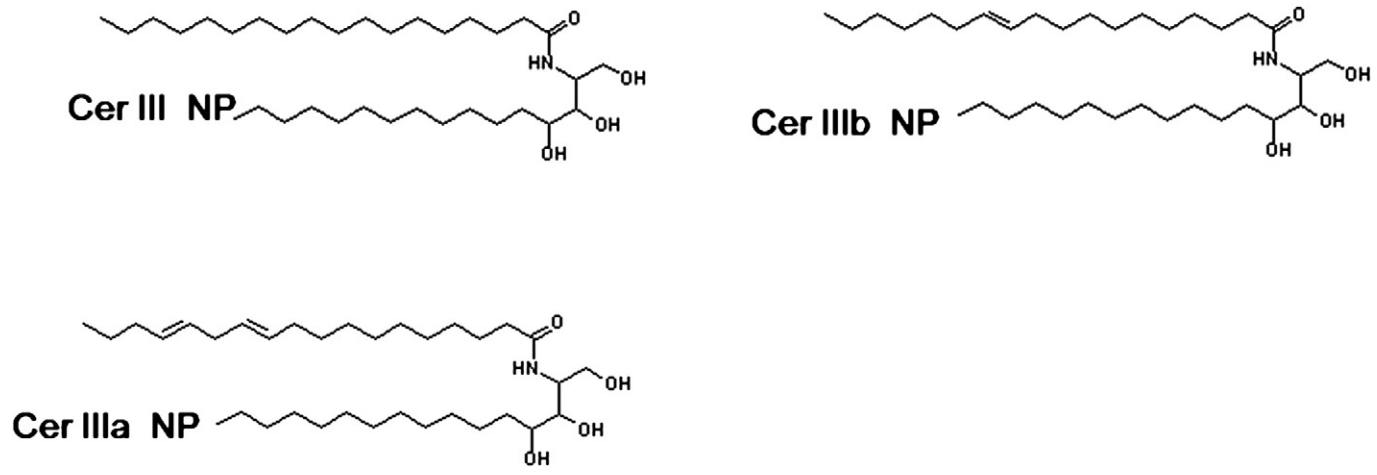

Fig. 1. Structure of the different ceramides studied. P indicates the phytosphingosine and S the sphingosine headgroup. N indicates that the molecules are non-OH FA.

Table 1
Effects of the initial dilution of lipids in pure EtOH on the supramolecular organization of the lipids after evaporation, through the modification of several confirmation IR description

	Cer III	Cer IIIa and Cer IIIb	(C_{24}:1)	(C_{24}:0)	Mixture
ν–C=O amide	1613*	1613<1645→1645	–	–	Decrease of the hydrogen bonding strength
ν–C=O acide	–	–	1694→1620–	1694→1701+1686	1711+1700
νCH_2 stretching	2850*	2850→2851	2850→2851	2849.5→2850.2	2849.8*
δCH_2 scissoring	1466*	1466→1468	1472*	1472*	1472*
$\rho\omega CH_2$ wagging	–	–	No bands	8 bands*	8 bands*

The * indicates the values that remain constant between an initial dilution in $CHCL_3$/MeOH (2:1, v/v) and in 100% of EtOH. All the band frequencies are indicated in cm^{-1}.

time. The spectra were recorded between 4000 and 700 cm^{-1} at 2 cm^{-1} resolution with 32 accumulations. All the spectral measurements were obtained at ambient temperature. All the data were studied using Spectrum 2000 software. The spectra were automatically smoothed.

2.4.2. IR microspectroscopy: ex vivo experiments

IR images of human skin biopsies were obtained with a Spotlight 3000 imaging system at a resolution of 4 cm^{-1} between 4000 and 700 cm^{-1} with 16 accumulations and a pixel size of 6.25 μm × 6.25 μm. The background was recorded with a resolution of 4 cm^{-1} and 256 scans. As previously, the spectra were automatically smoothed. The data were analysed using the spotlight software.

3. Results and discussion

3.1. In vitro experiments on lipid films

3.1.1. Conformational order descriptors

Several conformational descriptors are already known to describe molecular modifications at the level of the SC lipids [16]. νCH_2 (stretching mode), ρwCH_2 (wagging mode), δCH_2 (scissoring mode) and ρCH_2 (rocking mode) are used to study the apolar chain behaviour. For the polar headgroup, the amide I bands at 1632 cm^{-1} (weak hydrogen bonding) and 1613 cm^{-1} (strong hydrogen bonding) as well as the νC=O acid band (1740 cm^{-1}) were chosen as descriptors. Moreover, a chemical descriptor was selected [12]: the ratio of the intensity of νCH_2 asymmetric stretching band (2919 cm^{-1})/ν=CH stretching band (3005 cm^{-1}) (noted $\nu CH_2/\nu$=CH) was chosen to study the chemical effect of UV irradiation on unsaturation.

CER III, IIIa, and IIIb (Fig. 1) were essentially covered as well as lignoceric acid (C_{24}:0) and nervonic acid (C_{24}:1). According to the literature, the lignoceric acid (C_{24}:0) is the most abundant in the stratum corneum [17]. As it was proved that UV has no effect on saturated FA, the corresponding monounsaturated fatty acid, i.e. the nervonic acid (C_{24}:1) was thus selected. The Cer III was chosen as it plays a crucial role in the properties of the SC [7]. Unsaturated lipids were selected due to their high sensitivity to UV irradiation.

3.1.2. Chemical perturbation: EtOH

The evolution of the different IR descriptors observed in films obtained from $CHCl_3$/MeOH and pure EtOH solutions is shown in Table 1.

The Cer III is not sensitive to the presence of EtOH at the level of either the polar or the apolar part. All the conformational descriptors described above remain constant. There is no interaction between the CER III and the EtOH.

On the contrary, the influence of EtOH on the headgroup, more precisely on the hydrogen bonding is relatively significant in the case of CER IIIa and IIIb. A disappearance of the strong hydrogen bonding (1613 cm^{-1}) is observed for the CER IIIa and IIIb when films were formed from 100% as well as 50% EtOH solutions. New hydrogen bonding are formed between EtOH and the polar headgroup of the CER. These new intermolecular interactions are weaker than the interactions between two vicinal CER. The cohesion between the CER is then less strong in the presence of EtOH.

The supramolecular organization of the CER IIIa and IIIb were found previously to be very more sensitive to temperature and to UV_A exposure, due to the presence of double bond [12,17]. In the case of the presence of EtOH the same observation is made. The space between two carbon chains is a bit more important when unsaturated, the area of the polar headgroups is then also a bit bigger. EtOH can insert more easily between the polar headgroup.

In the case of the FA, the νC=O band at 1694 cm^{-1} disappears when films were formed from 100% EtOH solution and a new band rises at 1620 cm^{-1} for the (C_{24}:1), while two bands appear at 1701 and 1686 cm^{-1} for the (C_{24}:0). The intermolecular hydrogen bondings between two vicinal molecules are destroyed, but new bondings are created between acidic groups and EtOH. The type of hydrogen bonding formed between the acidic groups and EtOH is different for (C_{24}:0) and (C_{24}:1). Like in the CER case, the area between two acids is higher in the presence of double bond on the carbon chain which requires more space than a saturated one.

With films formed from 50% EtOH solution, the hydrogen bondings between the acids remain the same. The effect of EtOH on the organization seems to be concentration dependant.

This insertion of EtOH between the polar groups creates an indirect partial disorder at the carbon chain level. The only difference observed

Table 2
Effect of UV_A or UV_B irradiation of 120 J/cm^2 or 0.15 J/cm^2, respectively, on the supramolecular organization of the lipids though the modification of several confirmational IR description

Band unit	νCH_2 cm^{-1}	δCH_2 cm^{-1}	$\nu CH_2/\nu$=CH Intensity ratio	Amide I cm^{-1}	νC=O cm^{-1}
CER III					
Standard	2850	1467	–	1612	–
UV_A	2849.9	1467	–	1612	–
UV_B	2850	1467	–	**1632**>1613	–
CER IIIb					
Standard	2850	1467	30	**1633**>1619	–
UV_A	2850.2	1471	Disappearance of the unsaturation	1633	–
UV_B	2850.5	1470	Disappearance of the unsaturation	1631	–
CER IIIa					
Standard	2848.9	1467	27	**1638**>1613	–
UV_A	2850	1471	Disappearance of the unsaturation	1638	–
UV_B	2850	1469	Disappearance of the unsaturation	1631	–
(C_{24}:1)					
Standard	2850	–	9	–	1694
UV_A	2850	–	51	–	1694
UV_B	2851	–	2.75	–	1719+1694
(C_{24}:0)					
Standard	2849.8	1472	–	–	1694
UV_A	2850	1472	–	–	1694
UV_B	2849.9	1472	–	–	1694
Mix					
Standard	2849.6	1468	–	11613	1711
UV_A	2850.3	1467	–	1614>1645	1711
UV_B	2850.4	1472+1464	–	1614	1711

The > indicate the difference of intensity of the two bands observed.

for the (C_{24}:0) concerned the νCH_2 increase from 2849.5 to 2850.2 cm^{-1}; that prove that the acids are a bit less packed in presence of EtOH. For the (C_{24}:1), several modifications were observed in the presence of EtOH: an increase of the νCH_2 frequency and the disappearance of the wagging bands between 1180 and 1350 cm^{-1}. The wagging bands, characteristic of an organized phase, were observed for FA but not for CER. All these modifications prove a decrease of the packing between molecules. The (C_{24}:1) packing is more sensitive to EtOH than the (C_{24}:0), again due to the presence of an unsaturation.

The CER IIIa and IIIb show the same behaviour. An increase of the νCH_2 (2851 cm^{-1}) and of the δCH_2 (1468 cm^{-1}) gives evidence of a decrease in the packing even if the lipids remain in an orthorhombic state, due to the insertion of EtOH between the molecules.

In the case of the (C_{24}:1) as well as for the unsaturated CER, the modifications of the supramolecular order are only partial. The molecules are still organized into an orthorhombic phase after contact with EtOH.

A mix of each class of lipids (CER III/(C_{24}:0)/CHOLE, 33:33:33, v/v/v) was also studied to mimic the SC composition. Firstly, when mixed with CER, the disappearance of the CO band of the FA at 1694 cm^{-1} was noticed, and a new band arose at 1711 cm^{-1}, proof of intermolecular interactions between the polar headgroups of FA and CER. In films formed from 50% EtOH solutions no modification of the supramolecular organization of the lipids was induced. In films formed from 100% EtOH, a small decrease of the ratio intensity strong hydrogen bonding/weak hydrogen bonding of the CER is observed in addition to the appearance of a new band at 1700 cm^{-1}. These modifications result from the creation of new hydrogen bondings between the different lipids and EtOH at the polar headgroup level. However, these changes seem to be minimal because they have no influence on the apolar chain packing; the νCH_2 frequency remains 2850 cm^{-1}.

The supramolecular organization of the lipids mixture is less sensitive to the presence of EtOH than the lipids studied separately. The only influence was observed at the level of the intermolecular bonding, but there was no significant modification of the apolar chain organization.

IR spectra of the same samples were carried out 3 days later. The results were similar. The partial disorganization of the lipids, when observed, occured almost instantaneously after the evaporation of EtOH and did not change over time.

3.1.3. Perturbation induced by a physical agent: UV irradiation

To complete a previous work [12] on the effects of UV_A exposure on the supramolecular order of the SC lipids, UV_B irradiation of 0.15 J/cm^2 was applied on lipid films. Table 2 compares the results obtained after UV_B irradiation with those obtained previously with UV_A.

Concerning the FA, the (C_{24}:0) undergoes no modification after UV_B irradiation. The same observation was made after UV_A exposure [12], from which we conclude that saturated FA are not sensitive to UV. In the case of the (C_{24}:1), a small destabilisation of the apolar chain is observed through the modification of the νCH_2 band, which shifted for 1 U. Despite this destabilisation, the FA remains in an orthorhombic state, which is confirmed by the presence of the wagging bands between 1185 and 1334 cm^{-1} present only in organized systems [18]. A small decrease of the $\nu CH_2/\nu$=CH ratio is observed after UV_B irradiation. This could be in agreement with the formation of hydroperoxide which decreases the number of CH_2.

Taking into account the polar headgroup behaviour, the νC=O band at 1694 cm^{-1} remains present after the irradiation. However a new band at 1719 cm^{-1} arises, showing new intermolecular interaction, probably due to the formation of oxidative entities.

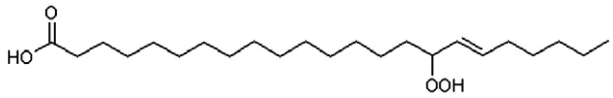

Fig. 2. Example of a hydroperoxide molecule that could have been formed on the nervonic acid during an UV_B irradiation of 1.5 J/cm^2.

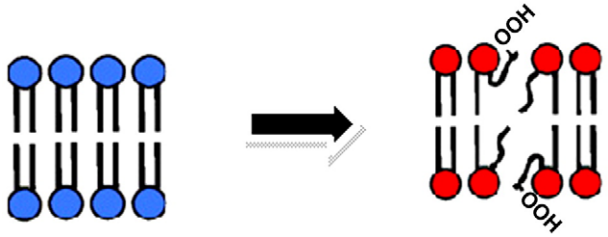

Fig. 3. Schema of hypothetical modification of the supramolecular organization of the lipids after the formation of oxidative compounds such as hydroperoxyde.

A hypothesis might be that UV_A irradiation favour the hydrogenation of the double bond whereas UV_B create new oxygenated entities such as hydroperoxide. An example of molecules potentially formed is represented in the Fig. 2. The formation of such entities would create a lack of packing within the carbon chain order observed though the small shift of the νCH_2 and would explain the appearance of new hydrogen bonding at the polar headgroup level.

The CER III, IIIa and IIIb have the same length but differ in the presence or not of unsaturation on their FA moiety. A laxity of the carbon chain order is noted for the CER IIIa and IIIb through the δCH_2 band that is shifted from 1467 to 1470 cm^{-1} after an UV_B irradiation of 0.15 J/cm^2. These results are similar to those after an UV_A irradiation of 120 J/cm^2. The νCH_2 and ρCH_2 frequencies remain unmodified. The global conformation of the lipids remains orthorhombic, accompanied with a small decrease in packing.

On the unsaturated CER, the disappearance of the double bond, observed by the disappearance of the band at 3005 cm^{-1} occurs in the same way after both UV_A and UV_B irradiation.

Concerning the polar headgroup behaviour, the three molecules display a decrease of the hydrogen bonding strength. A band of weak hydrogen bonding (1633 cm^{-1}) appears in the case of CER III after UV_B irradiation. This was not expected considering the insensitivity of this molecule to increases of temperature [17] and to UV_A exposure [12,19]. In the case of the CER IIIa and IIIb, UV_B irradiation led to the disappearance of the strong hydrogen bonding as observed after UV_A irradiation. This could be the result of the formation of new oxidative entities such as hydroperoxide. Despite the modification of the interactions, hydrogen bondings were still present between the amide headgroup of the CER and the oxidative entities. Because of the polarity of such molecules, a change in the lipids conformation occured as it is schematically represented on the Fig. 3.

A mix of each class of lipids (CER III/(C_{24}:0)/CHOLE 33:33:33, v/v/v) was also studied to mimic the SC. After UV_B irradiation, a

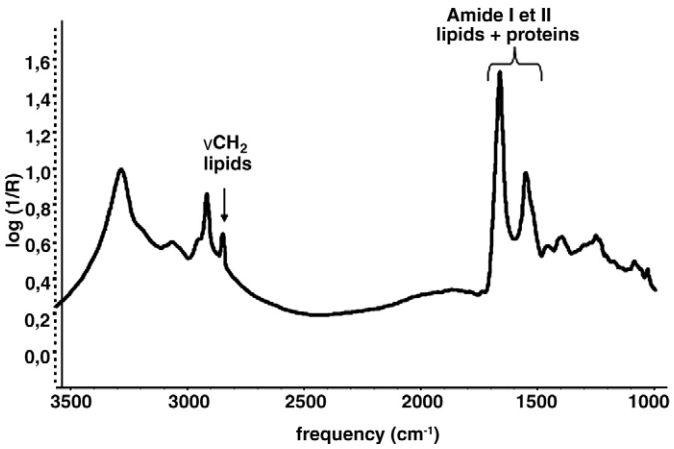

Fig. 4. IR spectrum of the SC from a human skin biopsy, obtained with a resolution of 8 cm^{-1}, 64 scans and a pixel size of 6.25 × 6.25 μm with a spotlight imaging system.

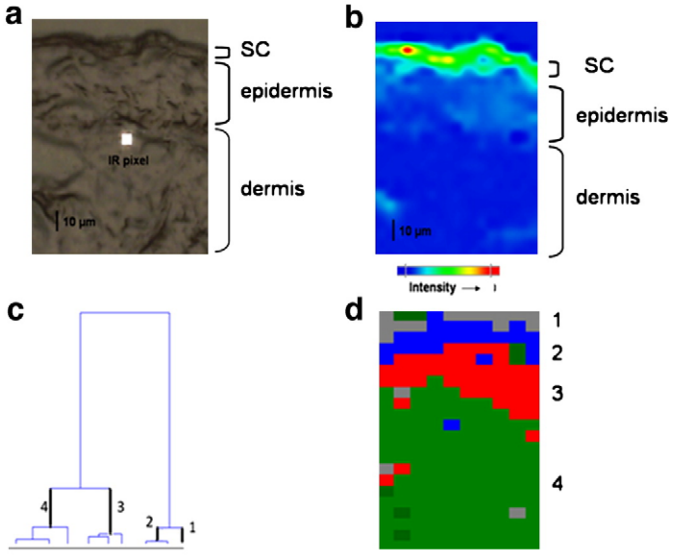

Fig. 5. Representation of human skin: (a) visible image of the skin. The white square represents the pixel size of 6.25 µm × 6.25 µm, (b) IR image realized between 2848 and 2852 cm^{-1} with a precision of 8 cm^{-1}, 64 scans and a pixel size of 6.25 µm × 6.25 µm, (c) dendrogram realized on human skin biopsy on the range of 2840 to 2870 cm^{-1} and (d) image realized from the HCA analysis. For c and d we can distinguish from 1 to 4: the background, the SC, the epidermis and the dermis.

small decrease of the lipid packing was noticed through a shift of the νCH_2 frequency from 2849.7 to 2850.4 cm^{-1} and thought the modification of the δCH_2 from one band at 1468 to two bands at 1472 and 1464. However, this had no impact on the hydrogen bonding inter-CER: the amide band at 1614 cm^{-1} remained present. The CO band of the FA present at 1711 cm^{-1} was not modified either.

UV_A irradiation had no effects on the apolar head group. Only a small decrease of the hydrogen bonding strength was observed by the appearance of a small shoulder at 1645 cm^{-1}.

As previously discussed, exposures to 0.15 J/cm^2 of UV_B or to 120 J/cm^2 of UV_A have similar action at an erythema level. According to the results, UV_B irradiation is more favourable to the formation of new oxidative entities such as epoxides or hydroperoxides than UV_A. The β scission of the carbon chain of the different lipids is comparable after both, UV_A or UV_B, irradiations. The decrease of the apolar chain packing is similar for isolated lipids after UV_A or UV_B irradiation. However, the apolar chain packing of the lipid mixture is more sensitive to UV_B than to UV_A, again maybe due to the formation of oxidative entities.

The IR experiments were repeated on the same samples three days after the irradiation. The results obtained were similar, displaying that time has no effect on the organization of the lipids within films.

Overall, these effects of UV irradiation on lipid films seem to be quite limited but only small amounts of irradiation have been used.

3.2. Ex vivo experiments on human skin biopsies

The experiments presented in this section were carried out on two donors. Moreover, they have been confirmed during further works, carried out using an IR synchrotron light source and focused on skin penetration of some molecules. The conclusions on supramolecular modifications after UV irradiation were similar to those described in this section.

3.2.1. Conformational order descriptors

As shown on the SC spectrum in Fig. 4, the only apolar conformational descriptor used in vitro which can be transferred to ex vivo studies is the νCH_2 band. The other CH_2 descriptors ($\rho(CH_2)$ rocking and $\delta(CH_2)$ scissoring) are not suitable for the skin spectra due to an overlapping with other bands. In addition, the amide I bands used in vitro to describe the polar headgroup behaviour cannot be used ex vivo to reflect the CER organization. Indeed, on the skin, these bands represent not only the vibrational signal of the CER of the lipidic cement, but the keratine contained in the corneocytes as well. For these reasons, the focus was put on the νCH_2 band at 2849 cm^{-1}, specific of the SC lipids, to study the evolution of the supramolecular organization ex vivo.

3.2.2. Localisation of the SC

The study of the IR images was focussed on the SC. It is of major concern to delimitate with precision the upper layer of the epidermis. In the visible image of the skin (Fig. 5a), the SC is well recognised by its dense aspect. This area appears darker than the rest of the skin. To corroborate this interpretation, IR images were realized at wavelengths between 2848 and 2852 cm^{-1}, specific of the SC layer (see Fig. 5b). A hierarchical clustering analysis (HCA) was carried out in the range 2840 to 2870 cm^{-1}, specific of lipids. Four major groups were found and are represented in Fig. 5c: the background, the SC, the epidermis and the dermis, differing by the presence or not of lipids, and their organization. Fig. 5d is an IR imaging representation of the previous dendrogram. These different representations show that the νCH_2 band at 2849 cm^{-1} permits to discriminate the SC lipids from the rest of the epidermis and the dermis. This was expected since the frequency of 2849 cm^{-1} is specific to an organized liquid crystal phase, characteristic of the SC lipids organization [16]. The thickness of the SC can then be measured from such IR images.

3.2.3. Perturbation induced by a chemical agent: EtOH

Firstly, the supramolecular organization was studied. After a contact time with pure water, only a small laxity of the lipid chain order occured, observed through a small increase (+1 cm^{-1}) of the νCH_2 band frequency. Indeed, water can interact with the polar headgroups of the lipids of the SC through the formation of hydrogen

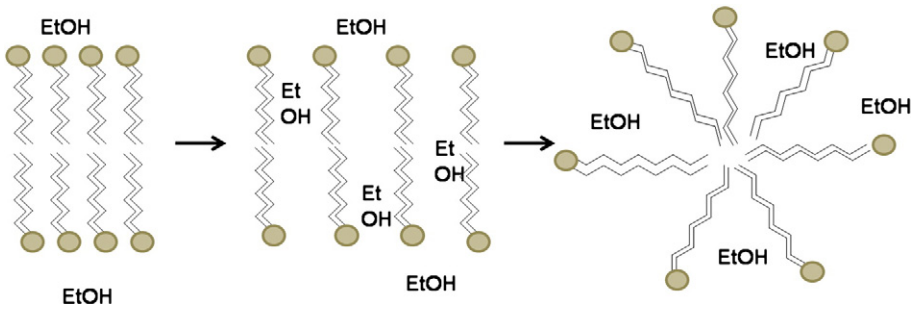

Fig. 6. Speculative schema of the evolution of lipids from lamellar phase to micelles in the presence of EtOH.

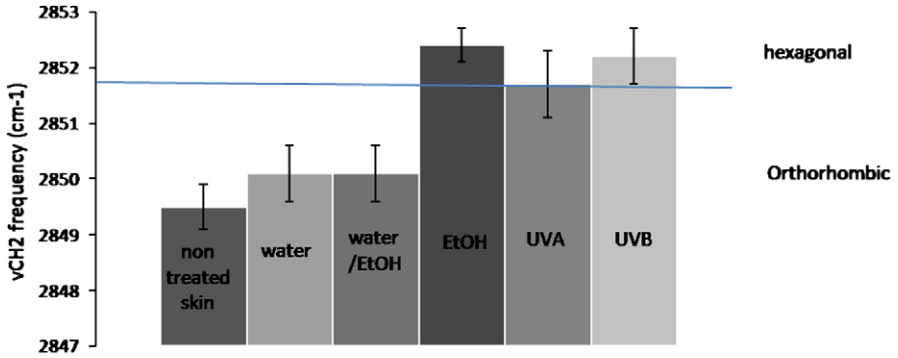

Fig. 7. Variation of the average values of $\nu CH_{2\ sym}$ frequency in the SC as a function of the external perturbation (24 h of contact with water, a mixture of H_2O/EtOH (1/1, v:v) or EtOH and 3 days after an irradiation of UV_A 120 J/cm^2 or UV_B 0.15 J/cm^2), carried out ex vivo on human skin biopsies.

bonding and then display of a small spreading of the polar headgroup and consequently on the carbon chains. However, the lipids remain in an orthorhombic phase. The same action on the supramolecular organization of the lipids is observed after the contact with the H_2O/EtOH (1/1, v:v) mixture.

With pure water or with the mixture of H_2O/EtOH (1/1, v:v), molecules of EtOH or water stay close to the polar headgroups without inserting in between the carbon chains. However, only a very small amount of PE (few percents cad.) is known to increase the percutaneous penetration kinetic [20]. Despite that, the orthorhombic packing is maintained. In the present case, other action modes must be at the origin of the penetration enhancement.

After contact with pure EtOH, a νCH_2 band with a frequency of 2852 cm^{-1} was observed, characteristic of a hexagonal phase [17]. The role of a PE, such as EtOH, is to induce a higher penetration of actives through the skin; more particularly through the lipid phase which is well known to be the major determinant of percutaneous transport rate [21]. As previously explained, EtOH, due to its structure, acts essentially at the polar headgroup level, through hydrogen bonding. However, ex vivo this phenomenon cannot be observed from spectral data because of the overlapping problem of the band amide I of the keratine and of the CER. An indirect consequence is a laxity of the carbon chain packing going to a hexagonal packing (not observed in the in vitro studies). A potential explanation for this phenomenon is that the liberty degree of the SC lipids is higher in the skin than in films (in a solid state).

Secondly, the evolution of the SC thickness was considered. The contact with water or with a mixture of H_2O/EtOH (1/1, v:v) does not change the SC thickness which is between 15 to 20 μm. A study carried out with SAX [19] also found a constant thickness of the SC even after hydration, on contrary with several studies that observed a thickening of the SC as a consequence of a hydration [22,23]. Prior to the penetration experiments, the skin was disposed for at least 2 h in a PBS solution. The skin explants were then fully hydrated, which can explain that no thickening was observed following the 24 hour-contact with water or with a mixture of H_2O/EtOH (1/1, v:v).

The application of pure EtOH led to a decrease of the SC thickness to 6 μm. Indeed, with pure EtOH, besides the destabilisation of the lipids, a part of the SC lipids seems to be extracted by its action. IR images showed this partial extraction, through the absence of signal in certain areas. From 50% of EtOH solution contact, holes appeared. This can be attributed to the formation of pores, as it was demonstrated by K.D. Peck et al. [24]. The EtOH extracts the lipids by solubilising them thought the formation of micelles (speculative representation in Fig. 6). By inserting between the lipids, EtOH spreads the polar headgroup and then the apolar chains. When the space starts to be too important, they rearrange into micelles. Consequently, the highest the EtOH concentration, the more lipids will be solubilised and then more pores will be formed.

According to the results, EtOH acts as a penetration enhancer through different actions, as a function of its concentration. For pure EtOH two phenomena take place: i) a spreading of the SC lipids, ii) the extraction of lipids at the origin of pores in the SC. A mixture of H_2O/EtOH (1/1, v:v) only acts through a partial extraction of the SC lipids; the lipids still present remain in an orthorhombic state. The effects of EtOH on the organization of the SC cement are concentration and contact time dependant and differ for in vitro and ex vivo experiments.

3.2.4. Perturbation induced by a physical agent: UV irradiation

After both UV_A and UV_B irradiation of 120 J/cm^2 or 0.15 J/cm^2 respectively, an increase of the νCH_2 frequency is noticed with the increase of the lagtime between the irradiation and the freezing. A maximum of νCH_2 is reached after three days, i.e. 2852 cm^{-1} and 2851.4 cm^{-1}, after UV_B or UV_A exposure respectively. The νCH_2 frequency comes back to the initial state at 2849 cm^{-1} four days after the irradiation. This was already observed by Jiang et al. [25] on hairless mouse SC, and the consequences of UV on water loss also found a maximal disruption of the barrier function three days after the irradiation before to reach a plateau and to come back to the

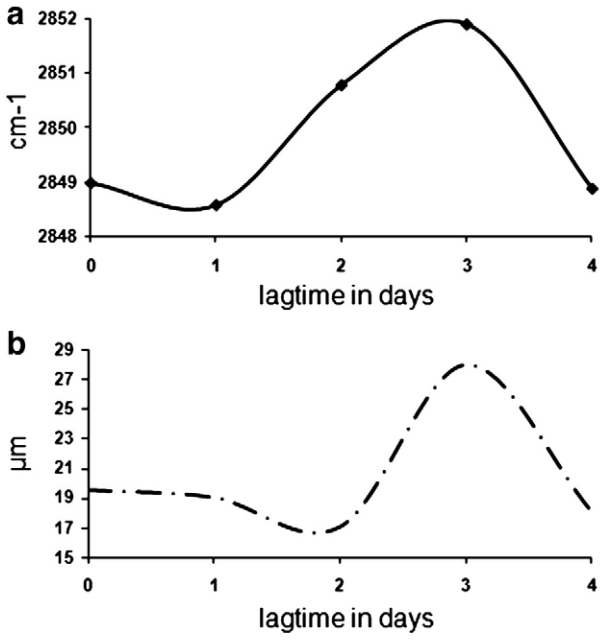

Fig. 8. (a) Evolution of the νCH_2 frequency in the SC as a function of the lagtime between the UV_B irradiation of 0.15 J/cm^2 and the IR measurement, (b) evolution of the thickness of the SC as a function of the lagtime between the irradiation and IR measurement.

initial state. The different average values of the νCH_2 frequency that we found after physical or chemical perturbations are gathered in Fig. 7.

By IR images realized in the νCH_2 range frequency between 2848 and 2852 cm^{-1} (found before to be characteristic of the SC lipids vibration), the evolution of the thickness of the SC was studied after the different irradiation. UV_A irradiation did not lead to a modification of the SC thickness. However, after UV_B irradiation a thickening of the SC was observed, in parallel to the variation of the organization as it is represented in Fig. 8. A comparable thickening was observed by R.M. Lavker et al. and H.C. Wulf et al. [26,27] after a mix of UV_A and UV_B irradiation. UV_B disorganized the liquid crystal phase going from an orthorhombic to a hexagonal state. When in a hexagonal state, the apolar chains are less packed. The space between two lipids is increased; this laxity is responsible for the thickening of the SC on the contrary of EtOH where a decrease of the thickness was observed as well as a formation of pores. The disorganization observed after UV_A exposure was less significant, which explains why no thickening was observed in this case.

The effects of both irradiations are comparable with the effect of certain PE, such as oleic acid [9], which provide a disorganization of the apolar chain. We can assume that a part of the UV effects also takes place on the polar headgroup of the lipids considering the in vitro study. Unfortunately, for the reason mentioned before, it was not possible to verify it on the skin spectra. We can then suppose that UV irradiation would modify the barrier function properties as well as PE and that the penetration of actives through the skin will be modified by an UV exposure, like it is modified by the application of a PE. To confirm this hypothesis, penetration studies are under way in our laboratory. These experiments could provide interesting information for the cosmetic industry.

As well as after the EtOH contact, the effects of UV irradiation seem more important ex vivo than in vitro, again because the degree of liberty of the lipids is more significant than on films. However, the UV effects observed ex vivo are reversible.

4. Conclusion

In this work the effects of physical (UV irradiation) and chemical (EtOH) perturbations on the SC organization and thickness were studied. FTIR spectroscopy and IR imaging microspectroscopy were found to be appropriate tools for in vitro and ex vivo studies respectively.

For both types of agent, the SC lipid order is more susceptible to damage ex vivo than in vitro, probably due to the higher liberty degree of the lipids. UV_B and UV_A irradiations are responsible for a disorganization and a thickening of the SC. As a consequence, one could expect a reversible modification of the cutaneous barrier and of the percutaneous penetration. Moreover, a modification of the supramolecular order of the lipids was observed over time after the irradiation ex vivo whereas in vitro the systems were fixed.

The results in vivo would probably be different, at least for the UV exposure effects. Indeed, in the skin, several anti-oxidative mechanisms and molecules (vitamin E, melanin…) are present to avoid the degradation of the lipids and proteins [28,29].

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