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Penetration mechanism of dimethyl sulfoxide in human and pig ear skin: An ATR–FTIR and near-FT Raman spectroscopic *in vivo* and *in vitro* study

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Abstract. The penetration mechanism of dimethyl sulfoxide (DMSO) in human skin *in vivo* and *in vitro* and pig ear skin *in vitro* was studied using attenuated total reflectance (ATR) Fourier transform (FT) infrared (IR) and near-FT-Raman spectroscopy. The results showed changes in the conformation of the skin keratins from an α-helical to a β-sheet conformation. These changes were proved to depend on the concentration of free water in the sample as DMSO tended to bind to free water before the protein-bound water was replaced and the protein conformational changes were induced. The induced conformational changes were shown to be completely reversible as the proteins are returned to their original state within 20 h after the treatment with DMSO. The penetration depth of DMSO was shown to depend on the time of exposure – however, after only 15 min DMSO has penetrated the *stratum corneum*, which is the skin barrier.

Keywords: Penetration, human skin *in vivo*, pig ear skin *in vitro*, attenuated total reflectance infrared spectroscopy, near Fourier transform Raman spectroscopy, dimethyl sulfoxide

1. Introduction

Understanding the mechanisms and properties of skin penetration is of crucial importance in the development of drugs for topical use – either for local therapeutic effect or for systemic therapy.

Human skin is a complex multi-layered organ which acts as a barrier to prevent water loss and to protect the body from chemical and microbial attack. The skin essentially consists of three layers – the epidermis, dermis and the subcutaneous fatty layer. The barrier function of skin is mainly dependent on the stratum corneum (SC) which is a part of the epidermis and is the outermost layer of the skin. SC is only about 10–20 µm thick and consists of approximately 75% protein, 15% lipids and 10% water [1].

One approach to improve the therapeutic efficacy of topically applied drugs is the use of penetration enhancers which are chemicals that can reversibly decrease the barrier properties of the SC. Dimethyl sulfoxide (DMSO) is a well-established skin penetration enhancer [2–7] and has been shown to improve the permeation of steroids, antibiotics, narcotics [8] and a wide range of compounds with molecular weights below 3000 [9]. It is the earliest and most widely studied skin penetration enhancer, but despite

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the wide ranging of studies that has been performed on DMSO, its mechanisms of action still remain unclear [2]. Thus to understand the enhancing effect, the penetration of the pure solvent must be studied.

In the study presented here, attenuated total reflectance–Fourier transform infrared (ATR–FTIR) spectroscopy and near-FT-Raman spectroscopy was used to probe the interactions between DMSO and human skin *in vivo* and *in vitro* as well as pig ear skin *in vitro*. Pig ear skin is a well-accepted and readily available model for the human barrier and is often used to assess topical and transdermal pharmaceuticals *in vitro* [10]. We also used the techniques to study the penetration depth of DMSO.

2. Materials and methods

2.1. Skin samples

In vivo spectra from one male and two females were recorded from the left ventral forearm. No pretreatment was performed on the sites. Informed consent was obtained from all subjects.

In vitro spectra were recorded on 19 pig ears and 3 human skin biopsies. The pig ears were obtained from a local abattoir within a few hours *post-mortem*. The skin surface was washed and dried. The IR measurements were performed without further treatment of the membrane (i.e., the skin remained on the isolated ear). Before Raman measurements the epidermal membrane was prepared by heat separation [11]; a 3×3 cm skin biopsy was cut off the ear, subcutaneous fat was removed and the skin was placed in a desiccator over condensed water steam at 60°C. After 5 min the intact epidermis inclusive SC was gently teased off with forceps which gives epidermal samples of approximately 200 μm thickness. During the spectral measurements all samples were placed on stainless steel plates, which do not give rise to any bands in the Raman spectra. Human skin samples were obtained from female breast biopsies, which were performed during breast reduction surgery, within a few hours after the operation.

2.2. Chemicals and procedures

DMSO was obtained from Merck KGaA (Darmstadt, Germany) with a purity of 99.9%.

At each skin sample approximately 1 ml of DMSO was evenly applied to a circular area with a 2 cm diameter. The solvent was left to penetrate the skin surface for 15 min. Thereafter excess solvent was gently removed using a soft paper tissue. These samples are referred to as DMSO treated samples.

For studies of penetration depth a sequential tape stripping procedure was used [12]. This technique uses standard adhesive tape (type Q-connect, from Interaction, Belgium) to remove a layer of stratum corneum with each sequentially applied piece of tape leaving the skin approximately 0.5 μm thinner [13]. An ATR–FTIR spectrum was recorded of the untreated skin, then the DMSO treatment was performed and another spectrum was recorded. Hereafter a piece of tape was applied to the skin for 20 s then stripped off and an ATR–FTIR spectrum was recorded again. This sequence was applied for 4 tape-strips. Subsequently, spectra were recorded every two strips up to a total of 16 strips. The time delay between each sequentially recorded spectrum from DMSO treated skin are thus 3 min and 20 s for the first 4 tape strips and 3 min and 40 s for the following strips. This procedure was also used for 1 min and for 3 h initial DMSO exposure to the skin – for *in vitro* pig ear skin samples only.

2.3. ATR–FTIR spectroscopy

A Bruker Equinox 55 spectrometer was used equipped with a DLATGS detector. The spectrometer was coupled with an MKII Golden Gate™ Single Reflection ATR System (Specac), which have a diamond crystal of approximately 1 mm in diameter. The spectral range of the acquired spectra was from 4000 to 650 cm^{-1}. For each sample 200 spectra at 5 cm^{-1} resolution were collected for 3 min and averaged. Spectra were recorded of the untreated and DMSO treated skin. The penetration depth (d_p) of the IR radiation is given by the following equation [14]:

$$d_p = \lambda \Big/ \left(2\pi * n_2 * \left(\sin^2\theta - \left(\frac{n_1}{n_2}\right)^2\right)^{1/2}\right),$$

where λ is the wavelength; θ is the angle of incidence of the IR beam into the sample; n_1 is the refractive index of the sample and n_2 is the refractive index of the ATR crystal. The specification for the Golden Gate gives an incident angle of 45° and a refractive index of 2.4. The refractive index of skin is 1.55 [15] and hence the penetration depth in the spectral range is 0.6–3.5 μm. This means that only vibrations from the molecules in the outermost layers of the stratum corneum can be seen in the spectra. So by using the tape stripping procedure each spectrum contains information about new skin layers.

2.4. Near-FT Raman spectroscopy

A Bruker FRS 100/S Raman spectrometer was used, equipped with a Nitrogen cooled Nd:YAG laser providing a power of 1000 mW. The spectral range of the acquired spectra was from −2000 to 3500 cm^{-1}. For each sample 500 scans at 4 cm^{-1} resolution were collected for 15 min and averaged. Spectra were recorded of untreated and DMSO treated epidermal membranes – no tape stripping was performed on these samples. The measurement depth of the Raman spectrometer is up to several hundred micrometers [16], which is beyond the boundary of epidermis. Hence each spectrum is in essence the average spectrum across the various epidermal layers.

2.5. Curve fitting

The appropriate number of bands for the curve fitting procedure were determined from the second derivative of the amide I region (1720–1580 cm^{-1}) in the spectra. This revealed that this spectral region consists of four overlapping bands.

Prior to curve fitting the data were converted to JCAMP files and curves were then fitted using Origin Pro 7.5 software (OriginLab Corporation). We used the Lorentzian band shape as this gave the best fit. The program fitted a linear baseline to each spectrum and then used iterations to find the best combination of heights, positions and widths that fitted the data.

3. Results

3.1. ATR–FTIR spectra of DMSO

The IR spectrum of pure liquid DMSO possesses very characteristic bands of which the strongest is assigned to the S=O stretching mode [17]. The S=O stretching frequency has been reported at

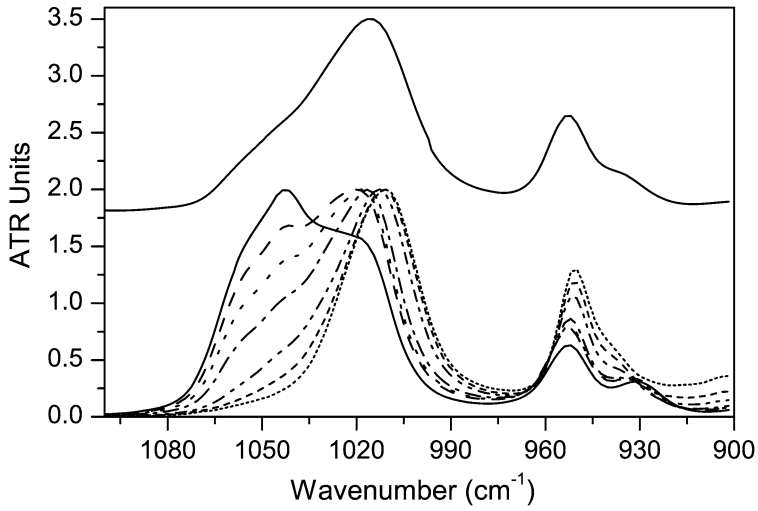

Fig. 1. Top: ATR–FTIR spectra of DMSO in human *Stratum Corneum*. Bottom: Pure DMSO (solid) and DMSO/water mixtures with the following concentrations (v/v); 50:1 (dash), 10:1 (dot), 5:1 (dash-dot), 2:1 (dash-dot-dot), 1:1 (short dash) and 1:2 (short dot).

1050 cm^{-1} for pure DMSO and at 1020 cm^{-1} for DMSO–water hydrogen-bonded complexes – measured at a DMSO concentration of 0.2 mole fraction [18]. A contemporary study also reported that the S=O stretching mode shows a downward shift in frequency when hydrogen bonded to water [19] and in a later report the band at $\sim$1008 cm^{-1} was assigned to the S=O stretching mode of a DMSO–water complex [20].

In our study the S=O stretching vibration mode for the pure DMSO liquid was found at 1043 cm^{-1} but even here a shoulder at 1015 cm^{-1} is seen. A small contribution of water is also seen in these spectra and it is found that DMSO absorbs atmospheric water and hence a completely pure spectrum cannot be recorded using the standard ATR technique which is used for skin measurements. We have found that in a DMSO/water mixture, the S=O stretching mode shifts from the 1043/1015 cm^{-1} doublet in the "pure" DMSO solvent to a single band at 1010 cm^{-1} in a 1:2 v/v DMSO/water mixture (Fig. 1). Looking at these changes graphically (Fig. 2) shows a large frequency shift when going from 100 to 99% DMSO but from 98 to 67% DMSO there's a linear dependency in the downward frequency shift. Then the frequency shift slowly decreases till the lowest value of 1010 cm^{-1} is reached at $\sim$33% from which point adding more water does not change the spectrum further.

3.2. ATR–FTIR spectra of human skin in vivo

In the spectra of human skin *in vivo* – following the described DMSO treatment – the ν (S=O) band frequency for DMSO is found at 1015 cm^{-1}. According to the previous section this indicates a hydrogen-bonding between DMSO and the component of water within the SC.

To further investigate the modes of action for the DMSO penetration the induced changes in the skin is resolved. Two ATR–FTIR spectra from our study of human skin *in vivo* are given in Fig. 3 – recorded before and after DMSO treatment.

The amide I band at approximately 1650 cm^{-1} contains information about the secondary structure of the skin proteins as the vibration frequencies change for the different structures [21]. Hence an α-helical

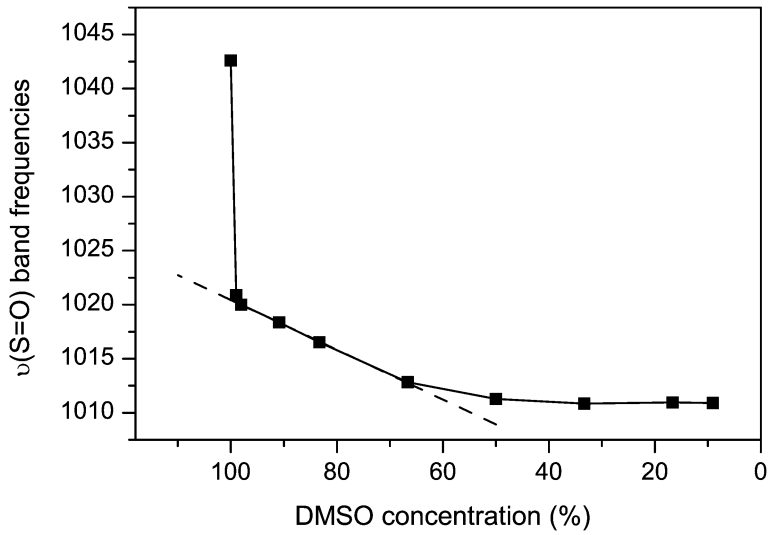

Fig. 2. Measured ν (S=O) band frequencies as a function of the DMSO percentage in a series of DMSO/water mixtures (solid) and the trend line (dash), $y = 0.23x + 997$, $R^2 = 0.9996$.

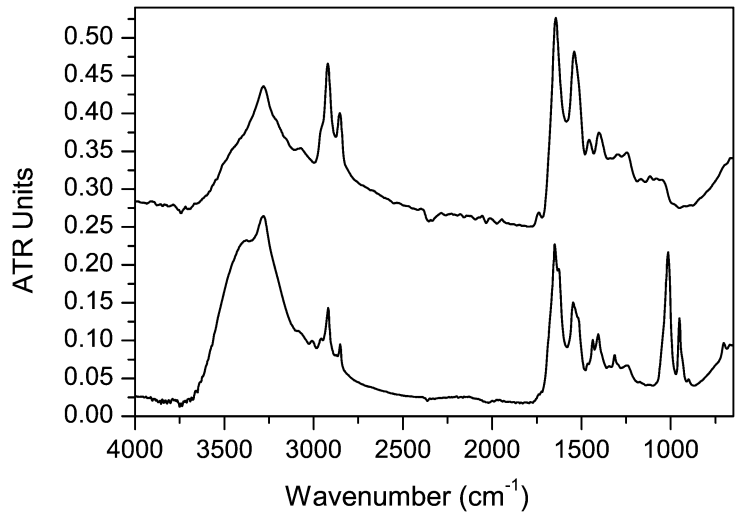

Fig. 3. ATR–FTIR spectra of human skin *in vivo*, before (top trace) and after (bottom trace) DMSO treatment.

conformation gives rise to a band in the 1647–1657 cm^{-1} area whereas a β-sheet conformation gives rise to bands in the 1621–1640 cm^{-1} and 1671–1679 cm^{-1} areas.

To detect a more accurate relationship between the α-helix and β-sheet conformations, the amide I bands are being curve fitted and the peak areas of the fitted peaks are compared. Prior to the curve fitting a water spectrum is being subtracted from the skin spectrum since the O–H bending vibration band at 1640 cm^{-1} interferes with the amide I bands. The spectrum subtraction is performed as to diminish the largest contribution from water – meaning that the O–H stretching vibration mode around 3300 cm^{-1} is suppressed. A subtracted spectrum is given in Fig. 4 along with an example of a curve fitted amide I band for untreated skin.

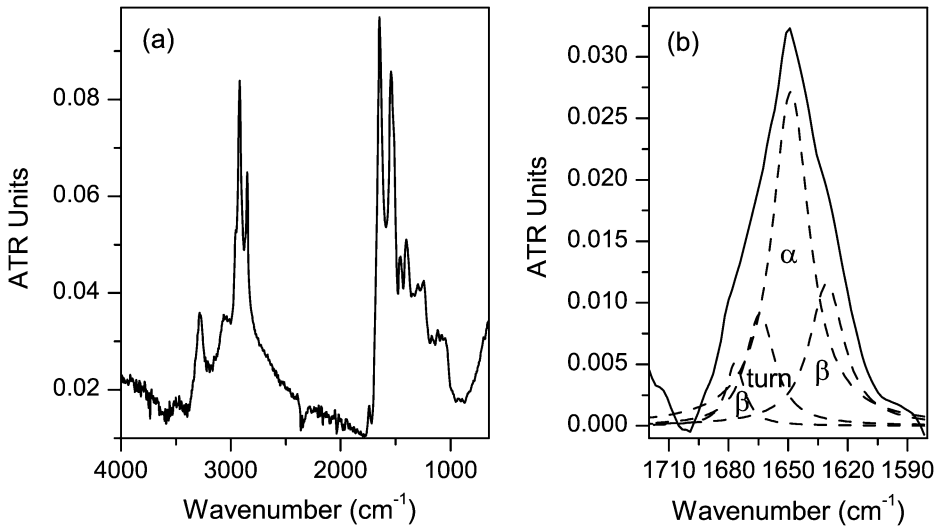

Fig. 4. (a) ATR–FTIR spectrum of untreated human skin after subtraction of water spectrum. (b) Curve fit of amide I band.

Table 1
Frequencies, FWHM values and percentage areas for *in vivo* human skin samples before and after DMSO treatment

Assignment	Wavenumber (cm^{-1})	FWHM (cm^{-1})	Area (%)
Before DMSO treatment			
β	1629 ± 2	22 ± 5	21 ± 2
α	1647 ± 1	24 ± 2	59 ± 3
Turn	1662 ± 1	18 ± 0	14 ± 4
β	1677 ± 1	15 ± 1	6 ± 2
After DMSO treatment			
β	1624 ± 1	25 ± 2	35 ± 2
α	1650 ± 1	21 ± 0	40 ± 2
Turn	1668 ± 1	23 ± 2	17 ± 5
β	1672 ± 0	19 ± 3	7 ± 1

The relative areas of the fitted bands are proportional to the amount of the secondary structures they represent [2,21] using an average of the areas from the two β-sheet bands. Hence the curve fit of the spectra from the three volunteers shows that the skin proteins are approximately 68% α-helix, 16% β-sheet and 17% bends/turns prior to the DMSO treatment (Table 1).

The protein content of stratum corneum is often described as "predominantly α-helix" [1,22–26]. A few attempts have been made to characterize a more accurate description of the protein secondary structure, though: Based on X-ray diffraction on extracted proteins from stratum corneum it has been reported that SC proteins are primarily α-helical with only 5–10% in the β-sheet conformation [27,28]. However, Raman spectroscopic studies on isolated human stratum corneum membranes has shown a relationship of 68% α-helix, 13% β-sheet and 19% unidentified protein residues [2]. The latter is thus in very good agreement with our results; except that they assign the remainder to unidentified protein residues, whereas we have assigned the non-α-helix–nor-β-sheet bands to the protein turns and bends – following a general assignment [21].

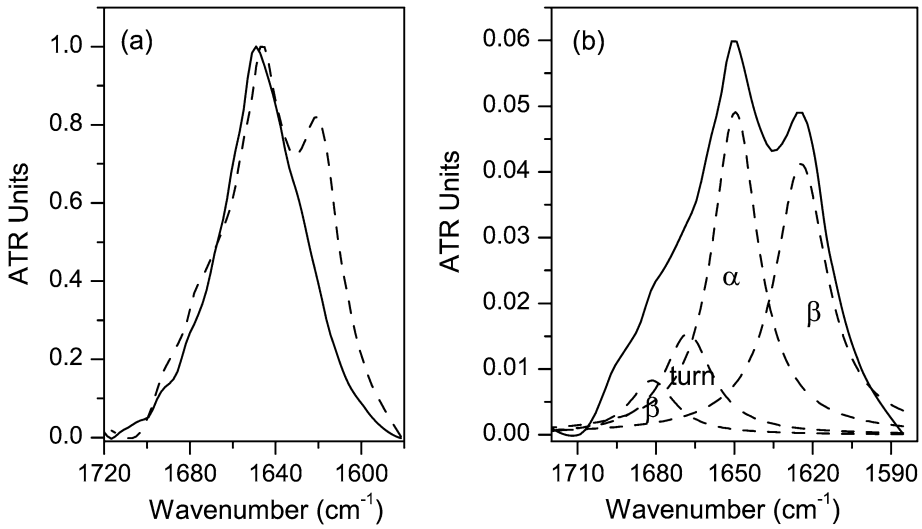

Fig. 5. (a) Amide I band from ATR–FTIR spectra of untreated (solid) and DMSO treated (dash) human skin *in vivo*. (b) Curve fit of amide I band for DMSO treated skin.

After the DMSO treatment the secondary structure of the proteins clearly changes (Fig. 5) as some of the α-keratin is modified to a β-sheet conformation. Using the curve fitting procedure shows that proteins in human skin *in vivo* after DMSO treatment are approximately 53% α-helix, 28% β-sheet and 19% bends/turns. Measuring over time shows that this conformational change is completely reversible as spectra recorded 20 hours after DMSO treatment are similar to the spectra of the untreated skin; meaning that there is no solvent left in the uppermost layers and the conformational changes are reversed. This result is supported by reported findings on *in vitro* stratum corneum samples [4] or isolated corneocytes [13].

However, Van de Weert *et al*. [29] has described that in the performance of FTIR analyses of secondary structural changes of proteins using the amide I region, one must be aware that protein secondary estimation can be affected by sample physical state. Hence spectral changes can be solely the result of water removal as is the case for lyophilized lysozyme [29].

3.3. ATR–FTIR spectra of in vitro skin samples

For human and pig ear skin *in vitro* (3 and 10 samples, respectively) the reaction seems to be somewhat different as the β-sheet bands do tend to get more intense after DMSO treatment but at the same time the band width decreases. Hence, using the curve fitting procedure the areas of the β-sheet bands after DMSO treatment remain the same as before or even slightly decreases (Table 2). This indicates that there might be some conformational changes of the proteins but, moreover, the surrounding components seem to be altered which affects the bonding of the proteins.

The DMSO S=O stretching vibration band is found at 1013 cm^{-1} which is a bit lower than in the *in vivo* spectra. As the change of this band from its position in the pure DMSO solvent to the position in a 66% DMSO/water mixture is somewhat linear, the lower frequency found for *in vitro* skin than for *in vivo* indicates that the solvent reacts or binds to a higher amount of water in the former case. Since the intensity of the ν (O–H) mode from water at 3300 cm^{-1} is in general more intense in the spectra of *in vitro* skin than in those of *in vivo* skin, more water is present in the *in vivo* case (Fig. 6). Hence it

Table 2
FWHM values and percentage areas for *in vitro* human and pig ear skin samples before and after DMSO treatment

Assignment	Wavenumber (cm^{-1})	FWHM (cm^{-1})	Area (%)
Before DMSO treatment			
β	1630 ± 2	23 ± 2	30 ± 4
α	1646 ± 1	22 ± 2	50 ± 3
Turn	1662 ± 1	19 ± 1	14 ± 1
β	1678 ± 1	16 ± 2	7 ± 1
After DMSO treatment			
β	1629 ± 2	18 ± 1	18 ± 2
α	1648 ± 1	23 ± 1	55 ± 5
Turn	1664 ± 1	20 ± 2	16 ± 3
β	1679 ± 1	18 ± 3	10 ± 2

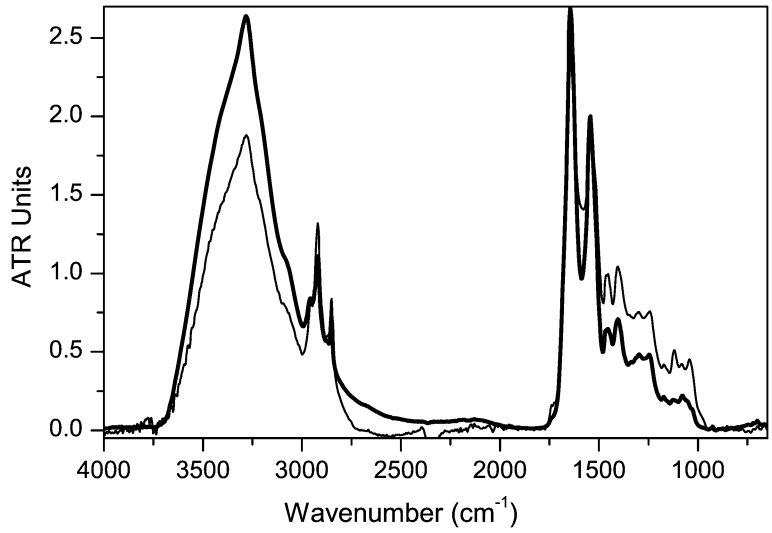

Fig. 6. Averaged ATR–FTIR spectra of human and pig ear skin *in vitro* (thick line) – 13 samples – and human skin *in vivo* (thin line) – 3 samples.

seems reasonable that a larger amount of water is available for DMSO bonding. So as long as there is sufficient water to get monolayer coverage of the protein – which seems to be the case for the *in vitro* skin samples – its structure will not change. But at lower concentrations of water – as in the *in vivo* skin samples – DMSO starts removing this monolayer. Hence structural changes do occur, and the protein will attempt to both reduce its surface and improve interprotein hydrogen bonds to compensate for this loss of water. The end result is formation of intermolecular beta-sheets.

A similar reaction has previously been reported for a number of purified α-helical and β-sheet proteins in various DMSO/^2H_2O mixtures [30]: in this report it is stated that at DMSO concentrations above 33% and below 75% an increase in absorption at 1621 cm^{-1} is indicating intermolecular hydrogen bonding or aggregation – below these concentrations no structural changes occur and above these concentrations the aggregate becomes increasingly dissociated by the DMSO solvent and the intensity of the band at 1621 cm^{-1} is decreased. Hence the results of Jackson and Mantsch [30] are in good agreement with our results: if the DMSO concentration is sufficiently low compared to that of water, no protein structural

changes will occur, as the DMSO solvent is hydrogen bonded to a fraction of the water molecules, whereas the rest of the water molecules are protein-bound or remain 'free'. However, at greater DMSO concentrations where protein structural changes occur, Jackson and Mantsch assign this to the formation of DMSO–protein hydrogen (or deuterium as their research is performed in ^2H_2O solutions) bonds which begin to unfold the polypeptide chain. Hence the formation of intermolecular β-sheets may be a general change in protein secondary structure following DMSO treatment and the dependence of water concentration also seems to be common, although this has not previously been reported for skin keratins.

3.4. Depth of penetration

In all cases – *in vivo* and *in vitro* – after 15 min of DMSO treatment, DMSO had penetrated the skin to a depth below the stratum corneum as the ν (S=O) mode was seen in all spectra following the tape stripping procedure (Fig. 7).

Lowering the time of exposure to 1 min (3 samples) decreases the depth of penetration to half the depth of SC as the ν (S=O) mode is only seen for the first 7–8 tape strips. Increasing the time of exposure to 3 h (3 samples) slightly increases the intensity of the ν (S=O) mode in all layers of SC and hence a larger amount of DMSO has penetrated and permeated the skin barrier. No significant differences in the penetration depth for *in vivo* and *in vitro* skin samples were found, and hence the penetration profiles, as can be seen in Fig. 8, are based on an average of all spectra at each tape stripping level.

3.5. Near-FT Raman spectra

Additional information on the DMSO–water interactions can be derived from the Raman spectra when focusing on vibration bands arising from the water molecules: A ν (O–H) mode around 3300 cm^{-1} arises from all water in the skin sample, i.e. water bound to biomolecules and water with a structure like the one in bulk liquid water. Because the latter is not bound to biomolecules it is sometimes referred to as "free" water although it is still hydrogen bonded in liquid water [31]. A 180 cm^{-1} band is characteristic of bulk

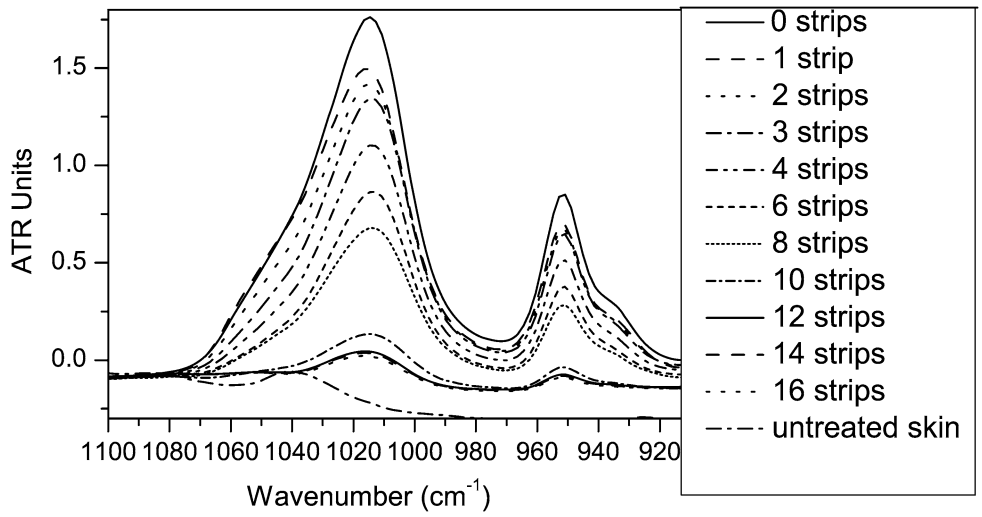

Fig. 7. ν (S=O) mode in ATR–FTIR spectra of DMSO in human skin *in vivo* following sequential tape stripping (15 min DMSO exposure time).

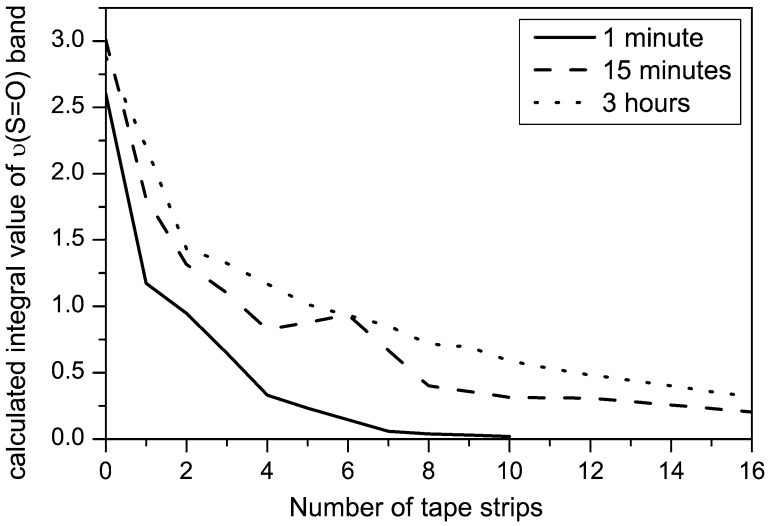

Fig. 8. Calculated integral values of ν (S=O) stretching vibration band for averaged skin spectra at subsequent tape strip levels.

water [32] but a serious problem in this low-wavenumber region is the high intensity of the Rayleigh line. By use of the so-called $R(\nu)$-representation the band due to Rayleigh scattering is converted to a weakly declining plateau, and weak vibrational features of water are more easily seen [32,33]. Hence the 180 cm^{-1} band can be used to detect changes in the amount of free water in the skin samples following DMSO treatment.

As the measurement depth is deeper for the Raman measurements than for ATR–FTIR the epidermis of 3 pig ear skin samples were separated off prior to recording the Raman spectra and measurements were performed on the epidermal tissue only. This procedure does induce some water evaporation and hence the amount of water is decreased.

In the Raman spectra α-helical structures shows an amide I band around 1645–1657 cm^{-1} and the β-sheet structures shows an amide I band around 1665–1680 cm^{-1} [34]. Hence the Raman spectra obtained in this study of untreated and DMSO treated pig ear skin samples confirm the conformational change from an α-helical to a β-sheet state (Fig. 9) which has also been reported for isolated stratum corneum membranes [2] and on isolated corneocytes [22]. The 3300 cm^{-1} band showed no changes in the total amount of water before and after DMSO treatment but looking in the low-wavenumber region and using the $R(\nu)$-representation reveals a decrease in the amount of free water in the DMSO treated sample (Fig. 10). Hence it is stated that DMSO does in fact bond to the free water within the skin samples.

Unfortunately this $R(\nu)$-representation cannot be calculated for *in vivo* skin samples as the fiber optic probe contains a filter which absorbs all light below 200 cm^{-1}.

4. Conclusions

Penetration and reaction mechanisms of DMSO in human skin *in vivo* and *in vitro* and pig ear skin *in vitro* was studied using ATR–FTIR and near-FT Raman spectroscopy. Analysis of the spectra showed that DMSO first of all binds to the free water but if there is not sufficient free water present within the sample DMSO probably removes the protein-bound water molecules. Hence structural protein changes

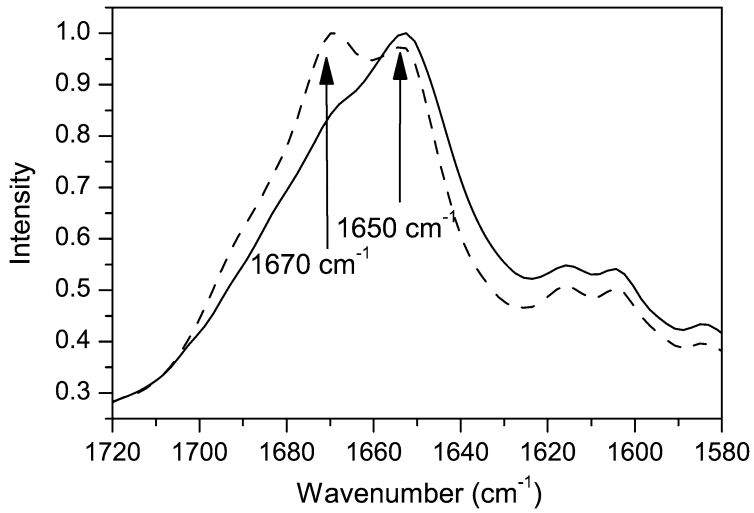

Fig. 9. Near-FT Raman spectra of epidermal tissue from pig ear skin before (solid) and after (dash) DMSO treatment.

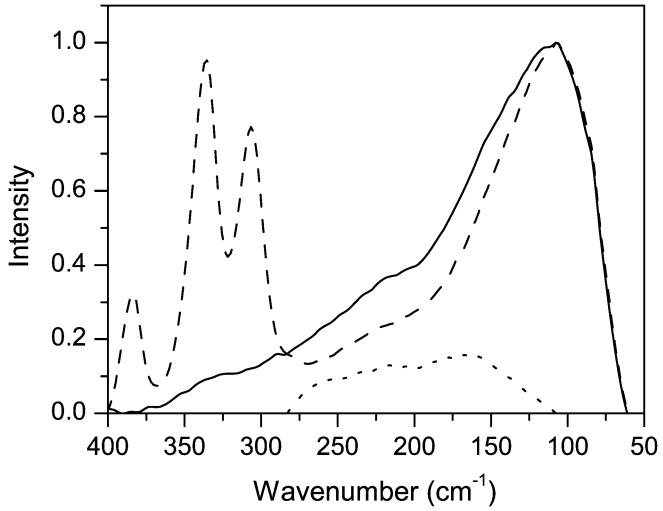

Fig. 10. $R(\nu)$-representation in the low-wavenumber Raman region. Epidermal tissue from pig ear skin before (solid) and after (dash) DMSO treatment – including the difference spectrum (dot).

occur as the protein will attempt to improve intermolecular hydrogen bonding to compensate for this loss of water. Removal of protein-bound water has previously been suggested as a possible reaction mechanism of DMSO penetration [35] – but that report does not comment on the dependency of the water concentration.

It has also been suggested that DMSO induces changes in lipid-composition [2,36,37]. The IR- and Raman spectra before and after DMSO in the present study showed no changes in the CH-stretching region supporting the results by Caspers et al. [3] that no lipid structural changes occur by DMSO treatment of skin.

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