In Vivo Confocal Raman Microspectroscopy of the Skin: Noninvasive Determination of Molecular Concentration Profiles

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Confocal Raman spectroscopy is introduced as a noninvasive *in vivo* optical method to measure molecular concentration profiles in the skin. It is shown how it can be applied to determine the water concentration in the stratum corneum as a function of distance to the skin surface, with a depth resolution of 5 μm. The resulting *in vivo* concentration profiles are in qualitative and quantitative agreement with published data, obtained by *in vitro* X-ray microanalysis of skin samples. Semi-quantitative concentration profiles were determined for the major constituents of natural moisturizing factor (serine, glycine, pyrrolidone-5-carboxylic acid, arginine, ornithine, citrulline, alanine, histidine, urocanic acid) and for the sweat constituents lactate and urea. A detailed description is given of the signal analysis methodology that enables the extraction of this information from the skin Raman spectra. No other noninvasive *in vivo* method exists that enables an analysis of skin molecular composition as a function of distance to the skin surface with similar detail and spatial resolution. Therefore, it may be expected that *in vivo* confocal Raman spectroscopy will find many applications in basic and applied dermatologic research. ***Key words: natural moisturizing factor/stratum corneum/water. J Invest Dermatol 116:434–442, 2001***

Driven by medical, pharmaceutical, and cosmetic research questions a large number of methods have been developed to obtain information about the presence and concentration of molecular compounds in the skin. Many of these methods are *ex vivo* methods, which require material to be removed from the skin and analyzed *in vitro*. A drawback of these *ex vivo* methods is that, to a greater or lesser extent, they are invasive. They alter the system under investigation either by extraction of compounds from the skin or by physical disruption of cell layers.

A widespread *ex vivo* method is tape-stripping, whereby cell layers are removed from the stratum corneum with adhesive tape. Sequential tape-stripping has been employed in various studies to obtain information about the depth distribution of molecular compounds in the stratum corneum (Bommannan *et al*, 1990; Higo *et al*, 1993; van der Molen *et al*, 1997; Berardesca *et al*, 1998; Kalia *et al*, 1998; Pilgram *et al*, 1998). Rawlings and coworkers have developed a tape-stripping procedure that allows measurement of the concentration of natural moisturizing factor (NMF) at different depths in the stratum corneum (Rawlings *et al*, 1994). In a number of studies extraction techniques have been employed to determine concentration levels of free amino acids in the stratum corneum (Tabachnick and LaBadie, 1970; Pratzel and Fries, 1977; Koyama *et al*, 1984; Meyer *et al*, 1991).

A limited number of *in vivo* methods have been developed to study the skin. Tagami and coworkers described a noninvasive high frequency electrical impedance method, which evaluates skin surface hydration *in vivo* (Tagami *et al*, 1980). This technique enables indirect detection of changes in skin hydration, but not direct quantitative measurements of water content, and the information is not obtained from a well-defined location within the skin. Infrared (IR) spectroscopy has been used for *in vivo* studies of stratum corneum hydration and permeability (Potts *et al*, 1985; Bommannan *et al*, 1990; Wichrowski *et al*, 1995; Pirot *et al*, 1997; Lucassen *et al*, 1998). Due to the strong absorption of mid- and far-IR radiation by water, however, the penetration depth in a naturally hydrated tissue such as the skin is limited to a few micrometers. Therefore in an *in vivo* IR spectroscopy experiment only the outermost layer of the stratum corneum is sampled.

Raman spectroscopy, in contrast, can be applied to obtain information regarding the molecular composition of the skin down to several hundred micrometers below the skin surface. Raman spectroscopy is a vibrational spectroscopy, similar to IR spectroscopy but based on inelastic light scattering rather than absorption of light. In a Raman experiment the sample under investigation is illuminated by monochromatic laser light. Interactions between the incident photons and molecules in the sample result in scattering of light. In a Raman scattering event a sharply defined amount of energy is transferred from the photon to the molecule, in which a vibrational mode is excited. It follows that a small fraction of the scattered light (the Raman spectrum) is found at wavelengths

Manuscript received January 5, 2000; revised September 29, 2000; accepted for publication November 22, 2000.

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Abbreviations: NMF, natural moisturizing factor; PCA, pyrrolidone-5-carboxylic acid; UCA, urocanic acid.

longer than that of the incident light. The exact energy needed to excite a molecular vibration depends on the masses of the atoms that are involved in the vibration and the type of chemical bonds between these atoms and may be influenced by molecular structure, molecular interactions, and the chemical microenvironment of the molecule. This and the fact that molecules may have a great number of independent vibrational modes ($3N - 6$ for a molecule consisting of N atoms), many of which may be excited by a Raman scattering event, means that a Raman spectrum is highly molecule specific. Therefore the positions, relative intensities, and shapes of the bands in a Raman spectrum carry detailed information about the molecular composition of a sample and about molecular structures and interactions present (Koningstein, 1971).

Raman spectroscopy is widely used in biologic studies, varying from investigations of purified compounds to studies at the level of single cells (reviews by Carey, 1982; Tu, 1982; Parker, 1983; Puppels, 1999). More recently Raman spectroscopy investigations have been initiated aimed at tissue characterization and pathologic tissue classification, many of these targeting the skin (Barry *et al*, 1992; Schrader *et al*, 1997; Shim and Wilson, 1997; Caspers *et al*, 1998; Gniadecka *et al*, 1998). First *in vivo* (Fourier transform) Raman spectra of skin were published by Schrader *et al* (1997), Shim and Wilson (1997), and Williams (1993). Recently, Schallreuter and coworkers published results of *in vivo* Raman studies in which the concentrations of phenylalanine and hydrogen peroxide were measured in the skin (Schallreuter *et al*, 1998; 1999). In all these studies, however, there was little or no control over the actual tissue volume that was being sampled. Recently, Caspers *et al* (1998) introduced *in vivo* confocal Raman spectroscopy as a method to gain control over the actual skin layer from which the Raman signal is collected.

The stratum corneum is the skin's outermost layer and the main protective barrier against water loss, microorganisms, and toxic agents. A detailed knowledge of the concentration and distribution of water and free amino acids in the stratum corneum is of importance for a better understanding of the properties of this layer. Water greatly affects physical properties of the stratum corneum, such as permeability and flexibility, and is also thought to regulate the activity of specific hydrolytic enzymes that are important for normal desquamation of corneocytes at the skin surface (Rawlings *et al*, 1995). Hydration of the stratum corneum, which is exposed to the relatively dry environment, is maintained by NMF. NMF is a highly hygroscopic and water-soluble mixture of amino acids, derivatives of amino acids, and specific salts and is found exclusively in the stratum corneum (Rawlings *et al*, 1994). It is produced in the lower part of the stratum corneum by enzymatic degradation of the protein filaggrin (Scott *et al*, 1982; Dale *et al*, 1990).

In this paper we present confocal Raman microspectroscopy as a noninvasive *in vivo* optical method for obtaining detailed information about the molecular composition of the skin and for determining molecular concentration gradients in the skin. Water concentration profiles were determined for the stratum corneum, as well as semiquantitative concentration profiles of constituents of NMF and sweat.

MATERIALS AND METHODS

Sample preparations

Preparation of washed and delipidized ex vivo stratum corneum samples Sheets of human stratum corneum were obtained from the upper back and shoulders of a male volunteer (37 y of age) suffering from a mild sunburn. Water-soluble compounds and lipids were extracted by washing the stratum corneum samples in ultra-pure water for 60 min, followed by soaking in chloroform:methanol (2:1, vol:vol) for 48 h. Finally the samples were washed again in water to remove possible water-soluble compounds that had not been released before lipid extraction.

Preparation of solutions Chemicals were purchased from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands) and used without further purification. Protein solutions in water were prepared of pepsin (33 kDa), urease (480 kDa), lysozyme (14 kDa), and bovine serum albumin (68 kDa), in 20% concentrations (mass-%; for bovine serum albumin 20% and 40% were used). Solutions of pyrrolidone carboxylic acid (PCA, 0.26 M), arginine (Arg, 0.19 M), ornithine (Orn, 0.46 M), citrulline (Cit, 0.19 M), serine (Ser, 0.72 M), proline (Pro, 0.37 M), glycine (Gly, 0.96 M), histidine (His, 0.21 M), and alanine (Ala, 0.45 M) were prepared in ultra-pure water at pH 5.5. Solutions of urocanic acid (UCA, 0.013 M) were prepared at pH 4.5 and pH 6.5. These compounds represent the main free amino acids and amino acid derivatives that constitute NMF. Also solutions of the sweat constituents lactate (1.14 M) and urea (0.90 M) were prepared in pure water at pH 5.5.

Instrumentation *In vitro* Raman experiments described in this paper (measurements on amino acid solutions and on stratum corneum samples) were carried out on a custom-built near IR Raman microspectrometer that has been described in detail elsewhere (Caspers *et al*, 1998; Wolthuis *et al*, 1999). *In vivo* Raman experiments on skin were carried out on a dedicated inverted confocal Raman microspectrometer that was developed in our laboratory (see **Fig 1**). Raman spectra of the skin of hand and arm were obtained by placing the hand or arm on a CaF_2 window in a metal stage. A laser beam from a tunable titanium-sapphire laser (model 3900S, Spectra Physics, Mountain View, CA) pumped by an argon-ion laser (model 2020, Spectra Physics) is focused in the skin by a microscope objective (Leica PL Fluotar 63×0.7 corr) located under the CaF_2 window. Light that is scattered by the tissue is collected by the same objective and focused onto an optical fiber connected to the spectrograph. The core of this optical fiber (100 μm diameter) acts as a confocal pinhole, which rejects signal coming from out-of-focus regions of the skin (Puppels *et al*, 1991; Puppels, 1999). The microscope objective is mounted on a precision translation table. This enables the distance between the objective and the skin surface to be adjusted in order to focus the laser light at a selected depth below the skin surface. Raman spectra were thus obtained from different, well-defined depths, at a depth resolution of 5 μm. The depth resolution was measured as follows. The Raman signal of oil was recorded, as the laser focus was scanned across a CaF_2–oil interface. The step response function thus obtained was differentiated to obtain the impulse response of the system (i.e., the response of the system that would be obtained if it were scanned through an infinitesimally thin plane with Raman scatterers). The bandwidth (full width at half maximum) of this impulse response is a measure for the depth resolution. For both excitation wavelengths used in this study (730 and 850 nm) the depth resolution was 5 μm. Due to the fact that the hand or arm rests on the metal stage and is in contact with the CaF_2 window, artifacts caused by unwanted vertical movement of the skin during the measurements are avoided. No changes in Raman spectra occurred during prolonged measurements at the same focusing depth (30 spectra for up to 6 min), and repeated scans through the skin resulted in identical molecular concentration profiles. It is therefore estimated that such vertical movements are limited to 1-2 μm, i.e., well below the depth resolution of the setup.

Near-infrared (NIR) laser light is used in order to minimize the excitation of tissue autofluorescence, which severely hampers Raman experiments in which visible laser light is used (Frank *et al*, 1994; de Faria and de Souza, 1999). On the other hand the quantum efficiency of charge-coupled device detectors, which are the detectors of choice in multichannel Raman spectroscopy, rapidly decreases above 1000 nm. This puts an upper limit to the NIR excitation wavelengths that can be employed. Therefore different laser excitation wavelengths were used for measurements in the

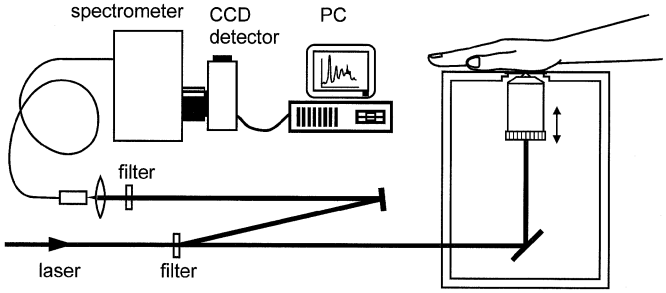

Figure 1. Confocal Raman setup for *in vivo* experiments. Laser light from a titanium-sapphire laser is transmitted by a short-pass filter and focused in the skin by a microscope objective. Raman scattered light is collected by the same objective, reflected by the short-pass filter, filtered by a laser rejection filter (either a notch filter or a color glass filter), and focused onto the core of an optical fiber. The fiber guides the light into a multichannel spectrometer equipped with a charge-coupled device camera.

so-called fingerprint region (400–1850 cm^{-1}, excitation wavelength 850 nm, Raman signal detected between about 880 and 1000 nm) and for measurements in the high wavenumber region (2500–3800 cm^{-1}, excitation wavelength 730 nm, Raman signal detected between about 890 and 1010 nm).

Data acquisition

Determination of concentration profiles of water and NMF *In vivo* Raman spectra were recorded of the thenar and the volar aspect of the arm of six healthy individuals (three male, three female; between 23 and 37 y of age). The spectra were measured at a range of depths below the skin surface, along a line perpendicular to the skin. Raman profiles of the thenar, where the thickness of the stratum corneum is of the order of 100 μm, were recorded with depth increments of 5 and 10 μm. Profiles of the arm (stratum corneum thickness 10–15 μm) were recorded with depth increments of 2 μm. By using depth increments of 2 μm the optical depth resolution of 5 μm (i.e., the dimension of the measuring volume in the direction perpendicular to the skin surface) is in fact oversampled by more than a factor of 2. In this way the resolution of the Raman scans is determined only by the optical properties of the system and not degraded by the step size of the depth increments. Measurement time was 30 s per spectrum in the 400–1850 cm^{-1} spectral region and 3 s per spectrum in the 2500–3800 cm^{-1} region. The laser power on the skin was 100 mW in all experiments. Other than removal of superficial dirt and excessive sebum with a tissue soaked in ethanol, no pretreatment of the skin was performed.

DATA ANALYSIS

Wavenumber calibration and sensitivity correction Raman standards with accurately known peak frequencies were used for wavenumber calibration of the Raman spectra. Recorded Raman intensities were corrected for the wavelength dependence of the detection efficiency of the setup using the emission spectrum of a calibrated tungsten band lamp. A detailed description of these procedures is given elsewhere (Wolthuis *et al*, 1999).

Determination of water concentration The ratio of the intensities of the Raman bands at 3390 cm^{-1} and 2935 cm^{-1} can be used to determine the water to protein ratio in tissue, as was successfully demonstrated by Huizinga and coworkers in their work on eye lenses (Huizinga *et al*, 1989) and more recently by Bauer and coworkers in studies of the cornea (Bauer *et al*, 1998). We have used this method to determine the water concentration in the skin. Water to protein ratios in the stratum corneum were calculated as the ratio between the Raman signal intensity of water (due to OH-stretching vibrations) integrated from 3350 to 3550 cm^{-1} and that of protein (due to CH_3-stretching vibrations) integrated from 2910 to 2965 cm^{-1}. These spectral ranges were chosen so as to maximize signal-to-noise ratio and to avoid overlap between the N-H vibration of protein at 3329 cm^{-1} (Leikin *et al*, 1997) and the water Raman signal. This is illustrated in **Fig 2**, which shows Raman spectra of pure water and of freeze-dried stratum corneum. A straight baseline between the spectrum points at 2500 and 3800 cm^{-1} was subtracted from the *in vivo* skin spectra in order to correct for the influence of slight variations in background (fluorescence) signal. Water content was calculated from the water:protein ratio using the following equations:

$$\frac{W}{P} = \frac{m_w}{m_p} R \qquad (1)$$

$$\text{water content } (\%) = \frac{m_w}{m_w + m_p} = \frac{W/P}{W/P + R} 100\% \qquad (2)$$

where m_w and m_p are the water and protein masses in the sampling volume, W is the integrated Raman signal of water, p is the integrated Raman signal of protein, and R is a proportionality constant describing the ratio between the Raman signals of water and protein of a 50% solution. The water content is expressed in grams of water per 100 g of wet tissue (water + dry mass).

Raman spectra of various solutions of protein in water (40% and 20% solutions of bovine serum albumin and 20% solutions of pepsin, lysozyme, and urease) were used to obtain an estimate of the proportionality constant R. Equation 2 was then used to convert ratios of water:protein Raman signal (W/p) to absolute water content in weight percentage. The proportionality constant R was found to vary with the type of protein used: $R = 2.0 \pm 15\%$.

(Attempts to use keratin for a calibration along this line failed. Keratin is water insoluble, so that inhomogeneous mixtures of protein and water were obtained that resulted in strongly varying and therefore unreliable values for R. Calibration experiments with hydrated delipidized stratum corneum samples failed for the same reason. Also here sample inhomogeneity caused the ratio between water signal intensity and protein signal intensity to vary strongly as a function of exact measurement location, but only one overall water:protein ratio can be determined for a sample by weighing it before and after hydration.)

Error estimation for Raman spectroscopic determination of water concentration The absolute error ΔR in R and the absolute error $\Delta(W/p)$ in the ratio W/p were used to obtain an estimation of the error in absolute water content as determined by Raman spectroscopy according to expression 3.

$$\frac{\Delta[m_w/(m_w + m_p)]}{m_w/(m_w + m_p)} = \frac{\Delta(W/P)}{W/P} + \frac{\Delta(W/P)}{W/P + R} + \frac{\Delta R}{W/P + R} \qquad (3)$$

The first two terms on the right-hand side of expression 3 represent random errors, and the third term represents a systematic error introduced by the error in the determination of the proportionality constant R. The error ΔR in the value of R that was obtained for the four calibration proteins was 15%. The error $\Delta(W/p)$ in the W/p ratio for a specific protein solution, as determined from independently prepared and measured samples, was $\leq 4\%$.

Determination of the concentration of free amino acids Raman spectra obtained at different depths below the skin surface were found to show consistent differences in the 400–1850 cm^{-1} spectral interval. Multiple least-squares fitting procedures were used to analyze these spectral differences. Both a qualitative analysis and a semiquantitative analysis were made. The qualitative analysis served to identify the molecular components of the skin that give rise to these spectral differences. The semiquantitative analysis yielded information about the semiquantitative concentrations of these components as a function of depth below the skin surface.

Qualitative analysis In a previous study we identified differences in the relative concentration of NMF and sweat constituents as the source of differences in skin spectra obtained at different locations of the body (Caspers *et al*, 1998). This led us to consider variations in the concentrations of NMF and sweat constituents as a possible source of the observed differences in this study. A set of *in vitro* Raman spectra of solutions of the dominant constituents of NMF (PCA, Arg, Orn, Cit, Ser, Pro, Gly, His, Ala, and UCA) (Tabachnick and LaBadie, 1970; Pratzel and Fries, 1977; Scott *et al*, 1982; Koyama *et al*, 1984) was collected. Also included in this set were spectra of solutions of the sweat compounds lactate and urea and spectra of ceramide 3 and of water. The spectrum of UCA was found to be strongly pH-dependent within the acidity range that may be encountered in the skin (pH 4.5–7). Because skin pH varies from person to person and changes as a function of depth (Berardesca *et al*, 1998; Oehman and Vahlquist, 1998), UCA spectra were obtained at pH 4.5 and pH 6.5 and included in the set of fit spectra. It was verified that the UCA spectrum at intermediate pH is a linear combination of these two spectra. The intensities of the spectra of the solutions of NMF and sweat components were normalized with respect to the concentration of the respective solutions. Hereafter this set of spectra will be referred to as the "model set" or "model spectra".

The qualitative analysis then proceeded as follows (illustrated in **Fig 3*a***). The *in vivo* Raman spectrum obtained at the skin surface

(i.e., the spectrum obtained at depth 0) was fitted with the model set, and the *in vivo* skin spectrum that was obtained at the next depth increment (depth 1). The results of this analysis are fit coefficients for each of the model spectra, and a fit coefficient for the *in vivo* skin spectrum obtained at depth 1. The fit coefficients for the model spectra reflect the changes in concentration of the NMF and sweat constituents between the two locations in the skin. The absolute Raman signal intensity of *in vivo* skin spectra decreased with increasing distance to the skin surface, however. In order to enable a direct comparison of relative changes in concentration at different depths in the skin, the absolute intensities of the *in vivo* skin spectra were normalized. This was done by multiplying the skin spectrum at depth 1 by the fit coefficient that was obtained for this spectrum.

The procedure described above was then repeated with the intensity-normalized skin spectrum obtained at depth 1 and the skin spectrum obtained at depth 2, in order to analyze the changes in molecular composition between depth 1 and depth 2. In this way changes in molecular composition at successive distances to the skin surface were analyzed, until the spectrum measured furthest below the skin surface had been used in the analysis.

Semiquantitative analysis The qualitative analysis described above only analyzes the changes in the concentrations of molecular compounds between two locations in the skin. In order to obtain a semiquantitative determination of the concentrations of NMF and sweat constituents as a function of distance to the skin surface, the fitting procedure described above was slightly altered (illustrated in **Fig 3*b***). Instead of using an *in vivo* skin spectrum, an *in vitro* spectrum of a washed/delipidized stratum corneum sample was included in the set of fit spectra. Therefore, each individual *in vivo* skin spectrum was modeled with a fixed set of fit spectra, consisting of the model set and the spectrum of washed/delipidized stratum corneum. The fit coefficients for the model spectra thus obtained were normalized with respect to the fit coefficient of the spectrum of the washed/delipidized stratum corneum sample. In this way a measure is obtained for the concentrations of NMF and sweat constituents relative to the keratin concentration (keratin being present only in the stratum corneum sample and being its major component). This semiquantitative measure was used to monitor changes in the concentrations of NMF and sweat constituents as a function of distance below the skin surface.

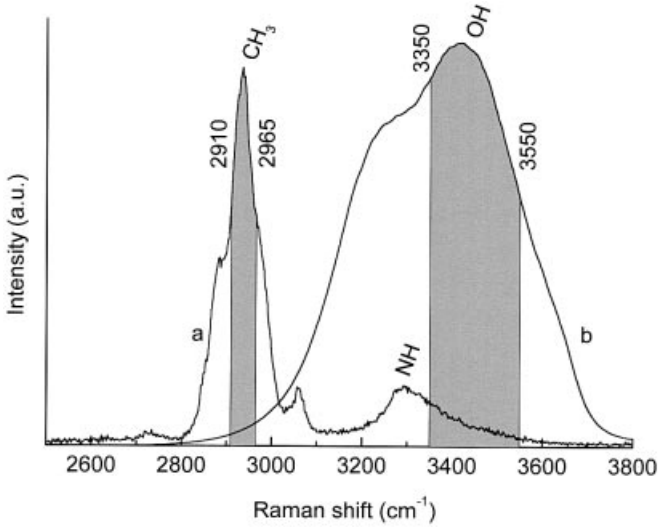

Figure 2. Raman spectra of water and dry stratum corneum. (*A*) Raman spectrum of freeze-dried stratum corneum. (*B*) Raman spectrum of water. The gray areas highlight the spectral intervals that are used in the calculation of water content in the skin.

In both the qualitative and the semiquantitative analysis the fit procedures used the spectral interval 400–1500 cm^{-1}. The reason for excluding the amide I band around 1650 cm^{-1} was that its bandwidth and position were found to vary. These changes, which are probably due to changes in protein secondary structure and protein–water interactions, cannot be modeled by the set of spectra used in the fit procedure. Nevertheless the least-squares algorithm would try to fit these spectral changes, using the available set of spectra. This was found to worsen the fit result in the 400–1500 cm^{-1} spectral interval and to significantly influence the fit coefficients of urocanic acid, which possesses an intense band at 1650 cm^{-1}.

The model set contained 15 spectra. In some spectral intervals a degree of spectral overlap exists between spectra of the model set. Therefore, it was verified that the model set is an independent set of spectra, which is a prerequisite for the fits to yield reliable results. Two tests were performed. First we attempted to fit each individual spectrum of the model set by all other spectra of the model set. This was not possible. For each of the model spectra a very poor fit was obtained, judged by the large Raman features in the fit residuals (i.e., the features of the spectrum being fitted that cannot be accounted for by the set of fit spectra). Second, for each of the compounds in the model set an independently measured spectrum was fitted with the complete model set. It was verified that only the spectrum of that compound, as included in the model set, contributed to the fit (i.e., had a fit contribution greater than 99%). Together the results of these tests show that the model set forms indeed a sufficiently independent set of spectra for the purposes of this study.

Estimation of errors in the fit results An independent set of fit spectra will yield unique fit results. The presence of noise in the spectra may affect the fit results, however. Therefore an estimate

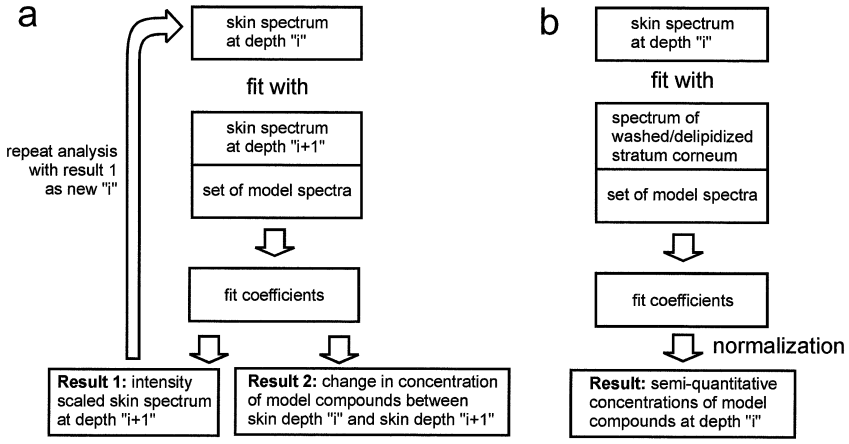

Figure 3. Schematic representation of the fit methods. (*a*) Flow chart of the method used to qualitatively analyze the changes in Raman spectra as a function of distance to the skin surface. (*b*) Flow chart of the method used for semiquantitative determination of the concentration of skin constituents as a function of distance to the skin surface.

was made of the influence of noise on the semiquantitative fit results. For this purpose noise was added to the *in vivo* skin spectra to artificially decrease the signal-to-noise ratio by a factor of 2, and a new set of fit coefficients was calculated according to the semiquantitative fit procedure described above. For each of the *in vivo* skin spectra this procedure was repeated 100 times. The standard deviation thus obtained for each of the fit coefficients was used as an estimate for the error in the fit coefficients.

RESULTS

The experimental results presented in this section reflect a total of 30 experiments in which multiple Raman depth scans were made through the stratum corneum of the thenar and the volar aspect of the arm of six healthy volunteers (three male, three female; between 23 and 37 y of age).

Water concentration profiling **Figure 4** shows a series of *in vivo* Raman spectra obtained from the stratum corneum of the thenar (palm of the hand) at a range of depths below the skin surface. A first-order polynomial was subtracted from each spectrum. The spectra were normalized to the CH-stretching band ($I_{2910–2965}$), which originates from the stratum corneum proteins. The O-H stretching modes of water are visible as a broad spectral band centered around 3450 cm^{-1}. The distances to the skin surface are indicated in the figure. The spectra clearly illustrate that the ratio between the water signal and the protein signal varies strongly as a function of distance to the skin surface.

In vivo water concentration profiles are displayed in **Fig 5**. **Figure 5**(***a***) shows four *in vivo* depth profiles for the volar aspect of the forearm. **Figure 5**(***b***) shows four *in vivo* profiles for the thenar. Each profile is from a different location on the thenar or arm, revealing significant local variations in the shape of the water concentration profiles. Repeated recordings at one location, however, scanning either from or to the skin surface, resulted in highly reproducible profiles (results not shown). The left-hand ordinate in **Fig 5(*a*, *b*)** represents the Raman signal intensity ratio ($I_{water}/I_{protein}$). The error bars plotted on the top trace represent the 4% random error margin in the determination of this intensity ratio (see *Data Analysis*). The right-hand ordinate represents absolute water concentration in grams of water per 100 g of wet tissue, as calculated from expression 2. The errors in the absolute water concentration are indicated along the right-hand ordinate. These are systematic errors as calculated from expression 3.

NMF Similar to the measurements described above Raman depth scans were made in the 400–1850 cm^{-1} spectral interval. **Figure 6** shows a series of *in vivo* Raman spectra obtained at a range of depths in the stratum corneum of the thenar. As the absolute signal intensity of the spectra decreased with depth (increments of 10 μm), the intensities of the spectra were normalized as described in *Data Analysis*. Although the signal collection time for each spectrum was only 30 s, the quality of the spectra is high and the spectra show many clear spectral features. Several spectral variations can be discerned, two of which are marked by an asterisk to guide the eye.

Inspection of the differences between consecutive spectra revealed two regions where significant changes in stratum corneum molecular composition occur, namely between the skin surface and 10 μm below, and between 60 and 70 μm below the skin surface (see **Fig 7**). Only minor changes were observed between consecutive spectra in the depth range from 10 to 60 μm below the skin surface.

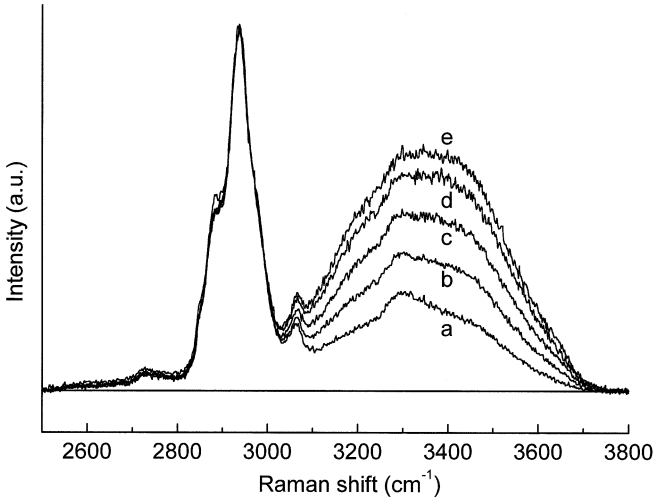

Figure 4. *In vivo* Raman spectra of the stratum corneum in the spectral interval 2500–3800 cm^{-1}. Illustration of the spectral changes due to differences in water content. The spectra were obtained at the thenar, at different depths below the skin surface. Distance to skin surface: (*a*) 0 μm; (*b*) 75 μm; (*c*) 80 μm; (*d*) 85 μm; (*e*) 90 μm. The spectra were normalized on the intensity of the protein signal (2910–2965 cm^{-1}). Signal collection time for each spectrum, 3 s; excitation wavelength, 730 nm; laser power on the skin, 100 mW.

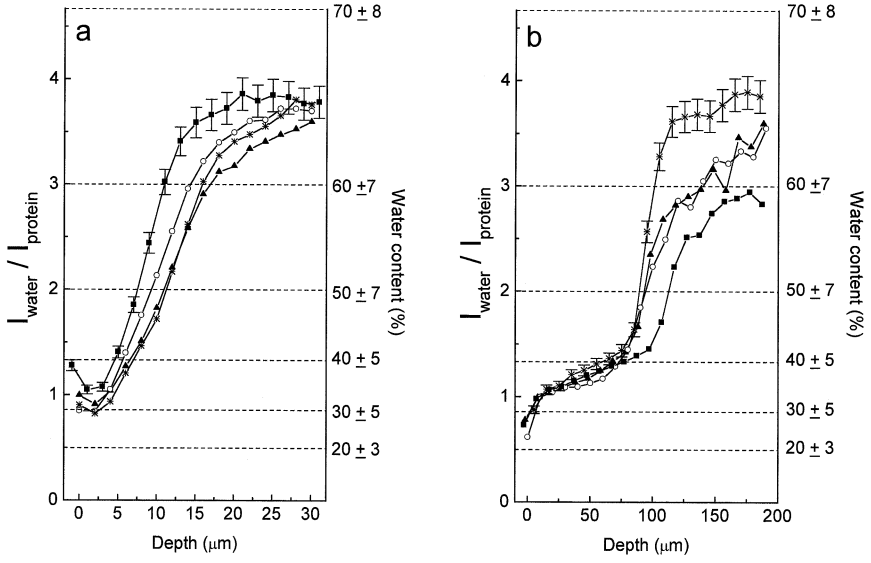

Figure 5. *In vivo* water concentration profiles of the stratum corneum. (*a*) Four water concentration profiles, calculated from Raman measurements on the volar aspect of the forearm. Different symbols are used for profiles obtained for different measurement locations. (*b*) Four water concentration profiles based on Raman measurements on the thenar. Different symbols mark different measurement locations. Signal collection time: 3 s per data point. The left-hand ordinate is the ratio between the Raman signal intensities of water and protein ($I_{3350–3550}/I_{2910–2965}$). The right-hand ordinate represents the absolute water content in mass-% (grams of water per 100 g of wet tissue), as calculated from expression 2. Note that this is a nonlinear scale. The error bars and error margins are explained in the text.

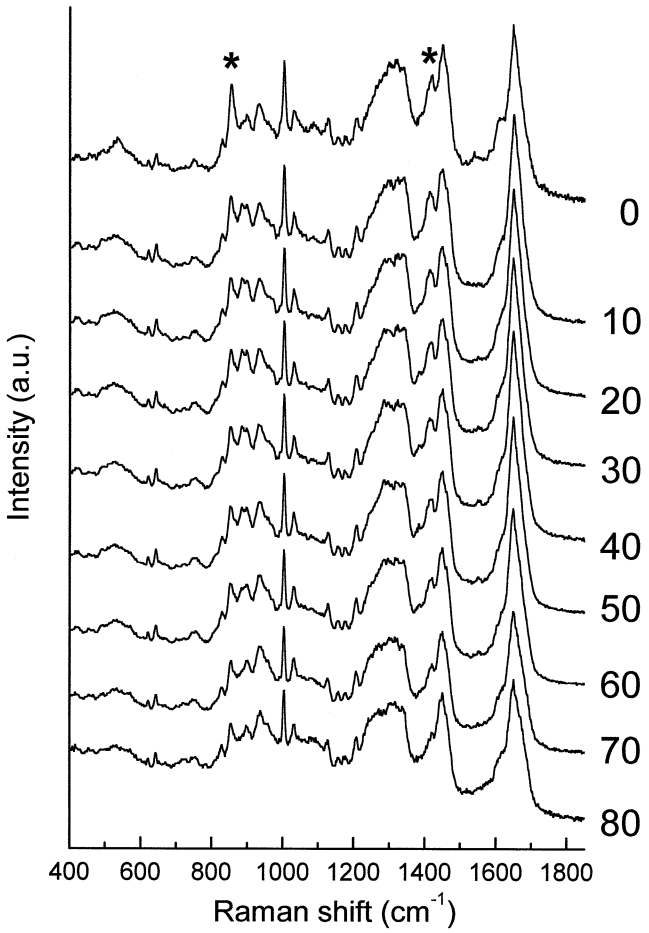

Figure 6. *In vivo* Raman spectra of the stratum corneum of the thenar in the 400–1850 cm^{-1} spectral interval. Raman spectra were recorded of the stratum corneum of the thenar at a range of depths, starting at the skin surface down to 80 μm below the skin surface. Numbers to the right of the spectra indicate this depth in μm. The spectra were intensity normalized (see *Data Analysis* for details on the normalization procedure) and offset along the ordinate for clarity of presentation. Signal collection time: 30 s per spectrum.

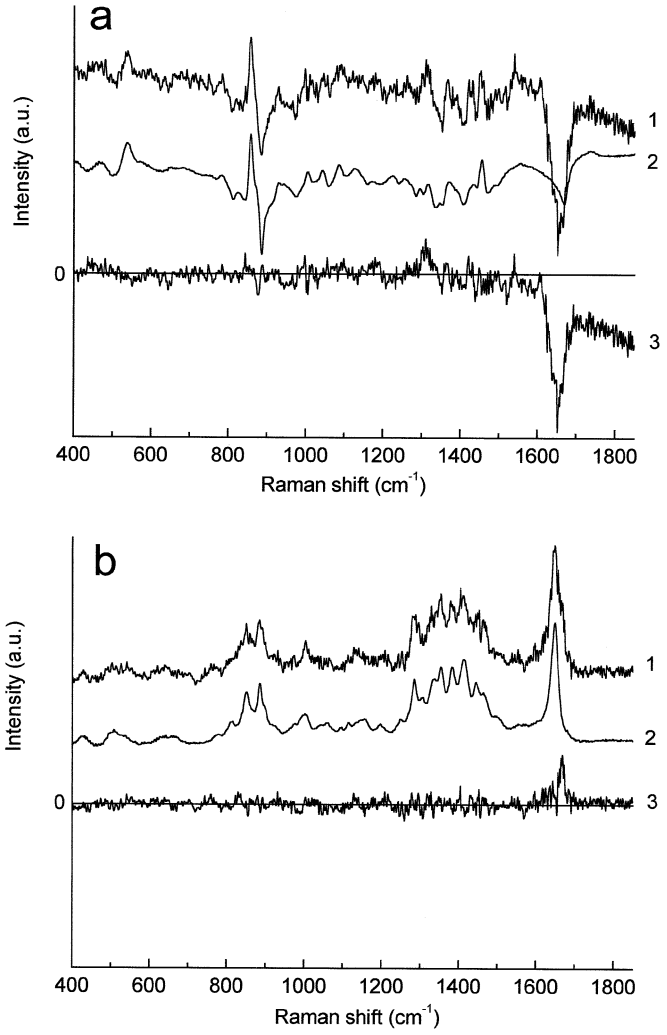

Figure 7. Analysis of the differences in Raman spectra obtained at different depths below the skin surface by means of the qualitative least-squares fit procedure with a set of model spectra of NMF and sweat constituents. (*a*) (1) Difference between the Raman spectra obtained at the skin surface and 10 μm below the skin surface (0 μm–10 μm); (2) fit result; (3) residual (curve 1–curve 2). (*b*) (1) Difference between the Raman spectra obtained at 60 μm below the skin surface and 70 μm below the skin surface (60 μm–70 μm); (2) fit result; (3) residual (curve 1–curve 2). The spectra are all plotted on the same scale. Details of the fit procedure are given in *Data Analysis*.

A qualitative analysis of the origin of these spectral changes was carried out by a multiple regression fitting procedure, using a set of model spectra, which consisted of spectra of solutions of NMF and sweat constituents, and spectra of ceramide and water. The results of this analysis are displayed in **Fig 7**. The spectral interval 400–1850 cm^{-1} is shown. In the fitting calculations only the 400–1500 cm^{-1} interval was used, as explained in *Data Analysis*. In this 400–1500 cm^{-1} spectral interval, no clear remaining spectral features are visible in the fit residuals, indicating that virtually all of the changes in molecular composition that are reflected in the Raman spectra have been accounted for.

Analysis of the spectral changes between the skin surface and 10 μm below the surface yielded large fit contributions from the sweat constituents lactate and urea, as well as fit contributions from NMF constituents. In contrast, analysis of the spectral differences between 60 and 70 μm below the skin surface showed only fit contributions from NMF constituents.

If a difference in NMF concentration is the origin of the spectral differences between 60 and 70 μm below the skin surface, then the relative contributions to the fit of each of the amino acid (derivative) spectra should reflect the composition of NMF. We therefore repeated the qualitative analysis for five independent *in vivo* Raman experiments and for each experiment we determined the relative fit contributions of the dominant NMF constituents, expressed in mole percent. **Figure 8** shows a comparison between this Raman analysis and published reports on the composition of NMF (*in vitro* determinations based on various methods) (Tabachnick and LaBadie, 1970; Pratzel and Fries, 1977; Koyama *et al*, 1984).

The qualitative analysis of the differences in the Raman spectra obtained from different distances to the skin surface enabled identification of the molecular compounds that vary in concentration. A different analysis is needed to quantify these variations in concentration as a function of distance to the skin surface.

For semiquantitative determination of molecular concentration profiles a fit procedure was applied in which each individual *in vivo* spectrum was modeled by a set of spectra representing all main skin constituents (see *Data Analysis*). A spectrum of washed/delipidized stratum corneum was used to represent the keratin fraction in skin. Fit residuals were only slightly larger than in the qualitative analysis described above (results not shown). In this way for each *in vivo* spectrum a complete set of fit coefficients was obtained (one for

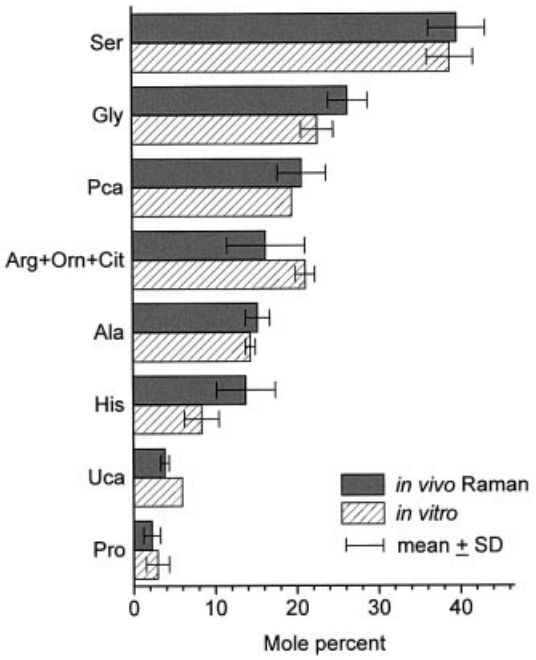

Figure 8. Comparison of the composition of human NMF as determined by *in vivo* Raman spectroscopy and results of previously published *in vitro* studies. The bar plot shows the molar fractions of the predominant NMF constituents as determined by *in vivo* Raman spectroscopy (*solid bars*) and by three *in vitro* methods (*hatched bars*). The fractions of Arg, Orn, and Cit have been combined (Arg is the precursor of Orn, which is the precursor of Cit). All concentrations have been normalized such that the total sum of Ser, Ala, Arg, Orn, Cit, Gly, Pro is 100 mole-%. The concentrations of PCA, UCA, and His were not included in this normalization, because none of the *in vitro* studies in this comparison provides values for both PCA and UCA (being the precursor of UCA, His was also excluded). The Raman results are based on qualitative analysis (as described in *Data Analysis*) of the differences in Raman spectra obtained at different distances from the skin surface. The mean molar fractions and standard deviations shown in the figure are based on the analysis of five independent sets of Raman data. *In vitro* methods: Tabachnick and LaBadie (1970), amino acid analysis of skin scrapings of the trunk and extremities; Pratzel and Fries (1977), amino acid analysis of stratum corneum that was removed from the buttocks by tape-stripping; Koyama *et al* (1984), amino acid analysis of water-soluble compounds extracted with a water-filled glass cup from various body regions.

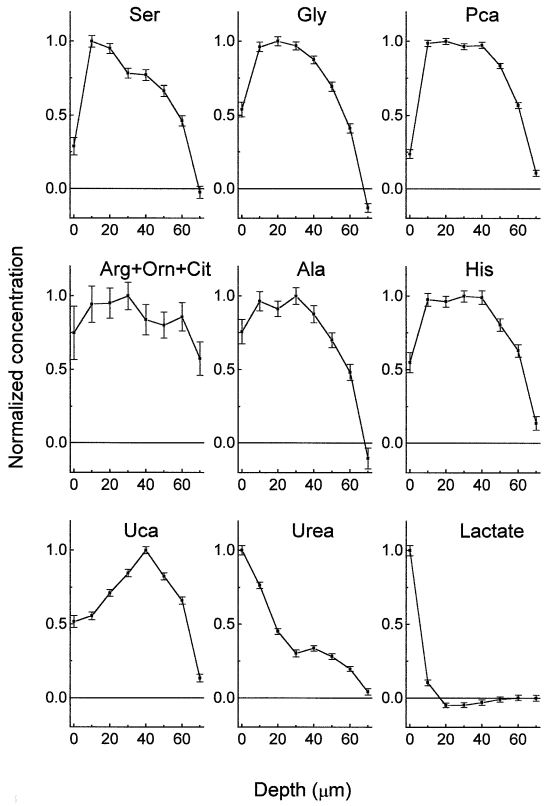

Figure 9. Semiquantitative *in vivo* concentration profiles of NMF and sweat constituents in the stratum corneum of the thenar as determined by Raman spectroscopy. The concentration profiles have been determined from the Raman spectra displayed in **Fig 6** by application of the fit procedure described in *Semiquantitative analysis* in *Data Analysis*. Each profile has been normalized to its maximum concentration. Error bars represent the uncertainty in the fit results as explained in *Estimation of errors in the fit results* in *Data Analysis*.

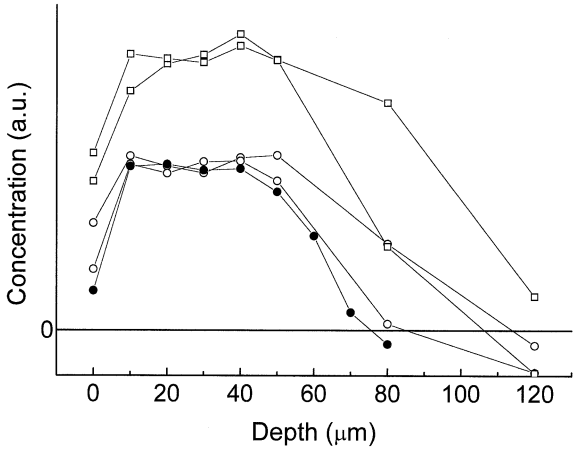

Figure 10. Semiquantitative concentration profiles of PCA as determined by Raman spectroscopy. Concentration profiles were measured in the stratum corneum of the thenar of two volunteers: subject A (*squares*), male, 30 y; subject B (*circles*), female, 30 y. Open and solid symbols refer to experiments on two different days.

each of the spectra of the model set). These fit coefficients were normalized to the fit coefficient of washed/delipidized stratum corneum. Under the assumption that the keratin concentration is relatively constant throughout the stratum corneum, the fit coefficients provide semiquantitative measures of the concentrations of NMF and sweat constituents as a function of distance to the skin surface. **Figure 9** shows the concentration profiles of the dominant NMF and sweat constituents, as determined by semiquantitative analysis of the measurements shown in **Fig 6**. The error bars represent the uncertainty (standard deviation) due to noise in the *in vivo* spectra (see *Data Analysis*).

Figure 10 shows the concentration profiles of PCA, one of the major NMF constituents, as determined from five series of Raman measurements. Spectra were obtained at different locations of the thenar for two subjects (one male, one female). A clear difference is observed in the relative concentrations of PCA between these subjects (i.e., the concentration of PCA relative to that of keratin).

DISCUSSION

The *in vivo* water concentration profiles of the stratum corneum of the arm as determined by Raman spectroscopy (**Fig 5*a***) are very similar to *in vitro* results obtained by X-ray microanalysis (Warner *et al*, 1988; von Zglinicki *et al*, 1993). These *in vitro* results show a continuous rise in water concentration in the stratum corneum from 15% to 25% at the skin surface to about 40% at the boundary between stratum corneum and stratum granulosum. This is followed by a steep rise to a constant level of about 70% in the

viable cells. Our results obtained with Raman spectroscopy show a somewhat higher water content of about 30% ± 5% at the skin surface, continuously increasing to about 65% ± 8% at a depth of about 15 μm. If we assume, in accordance with Warner *et al* (1988) and von Zglinicki *et al* (1993), that the boundary between the stratum corneum and the stratum granulosum is located near the high end of the steep water concentration gradient, it follows from **Fig 5(*a*)** that the stratum corneum thickness at the volar aspect of the arm is 10–15 μm. This is in agreement with known literature values (Holbrook and Odland, 1974). The stratum corneum is much thicker at the thenar than at the volar aspect of the arm. Also the water profiles obtained from the thenar (**Fig 5*b***) are qualitatively and quantitatively distinct from the water profiles of the arm (**Fig 5*a***). In contrast to the arm, where the water concentration increases throughout the stratum corneum, the stratum corneum of the thenar shows only little variation in water concentration. A steep water gradient exists at about 80–120 μm below the skin surface, however, with an increase in water content from 40% (± 5%) to about 60% (± 7%). The *in vitro* results of Warner and coworkers showed a similar water discontinuity at the stratum corneum–stratum granulosum boundary in the skin of the lower leg. If we again assume that this discontinuity demarcates the boundary between the flat stratum corneum cells and the water-rich cells of the viable epidermis, the Raman results imply a stratum corneum thickness at the thenar of 90–110 μm. The results of the *in vivo* Raman experiments on the arm (**Fig 5*a***) also suggest the presence of such a discontinuity. The present depth resolution of 5 μm of our setup precludes a definitive conclusion, however.

Absolute determination of water concentration by Raman spectroscopy suffers from a relatively large uncertainty caused by the relative error of 15% in the calibration constant *R*, which links Raman intensities to molecular concentrations (see *Data Analysis*). We are investigating ways to obtain a better absolute calibration. In many applications, however, the determination of relative differences in water concentration is of interest, rather than the absolute water concentration. These relative differences can be determined with a much higher accuracy than the absolute water content (see **Fig 5**), because in this case only the experimental (random) errors in the determination of Raman intensities in the CH-stretching region and in the water band play a role.

NMF is produced in the lower part of the stratum corneum and this would be expected to give rise to a noticeable increase in the concentration of free amino acids in this region. Therefore, it seemed likely that this would be reflected in the Raman spectra. As shown in **Fig 7(*b*)**, the differences between the Raman spectra obtained at 60 and 70 μm below the skin surface can be almost completely accounted for by differences in the concentration of NMF constituents. Moreover, the relative concentrations of the NMF constituents that were determined from the fitting procedure compare well with published reports on the NMF composition as determined by various *in vitro* methods (see **Fig 8**). This indicates that the changes in molecular composition observed at a depth of 60–70 μm are indeed due to a difference in the concentration of NMF constituents.

According to Rawlings *et al* (1994) a zone of stable filaggrin exists in the lowermost part of the stratum corneum where the epidermal water barrier is formed. The conversion of filaggrin to NMF would take place above this zone, i.e., closer to the skin surface. This is consistent with our observations. The concentration profiles (**Fig 9**) actually show that for most NMF constituents the concentration approaches zero at 70 μm below the skin surface. Between 70 and 50 μm the concentrations increase sharply and from 50 to 10 μm below the skin surface the concentrations remain constant. This suggests that, for this particular depth series, the conversion from filaggrin to NMF takes place between 50 and 70 μm below the skin surface. Sweat is excreted at the skin surface. This is reflected in the concentration profiles of the sweat constituents lactate and urea, which have their maximum at the skin surface and drop rapidly below the surface.

If we combine the water concentration profiles (as in **Fig 5*b***) and the NMF concentration profiles (as in **Fig 9**) the following overall picture emerges. In the stratum corneum of the thenar the zone of stable filaggrin is located between about 110 and 70 μm below the skin surface. Between 80 and 50 μm below the surface filaggrin is converted to NMF and between 50 and 10 μm no further changes take place. The concentration of NMF drops in the uppermost part of the stratum corneum.

At depths of about 80 μm or more below the skin surface the results of the quantitative analysis, which was used to determine concentration profiles, became less reliable as the residuals of the fits increased strongly. This indicates that the set of fit spectra, used to model the *in vivo* spectra, does not suffice to adequately represent the actual molecular composition of the stratum corneum at depths greater than about 80 μm. This is in accordance with the fact that at this depth the zone of stable filaggrin is reached.

The concentration profiles of PCA, as determined at different locations of the thenar (**Fig 10**), show that some variation exists in the depth at which filaggrin is converted to NMF. Throughout the larger part of the stratum corneum (between 10 and 50 μm), however, the concentration profiles for a single subject are remarkably similar, whereas a clear difference is observed between the two subjects. These results may indicate the possibility of detecting interpersonal variations in the NMF content of the stratum corneum.

CONCLUDING REMARKS AND OUTLOOK

In vivo confocal Raman microspectroscopy is a novel technique for noninvasive spatially resolved determination of molecular concentration profiles. We have demonstrated that water concentration profiles across the stratum corneum can be measured *in vivo*, both in thin and in thick stratum corneum (arm and thenar). Presently an automated depth scanning Raman instrument is being developed, which will enable the recording of a complete water concentration profile within a total signal collection time of 30–60 s or less. It was shown that variations in the concentration of NMF significantly contribute to the differences in Raman spectra obtained at different depths in the stratum corneum and that NMF concentration profiles determined *in vivo* by Raman spectroscopy are in agreement with published results of *in vitro* studies.

The fact that the technique is completely noninvasive makes it uniquely suitable for studies in which changes in molecular concentrations or molecular concentration profiles are induced, e.g., by topical application of chemicals or drugs, hydration/dehydration as a result of chemical or physical challenges, or as a result of disease processes and treatments. It may therefore be expected that future confocal Raman microspectroscopy studies will generate important data related to skin physiology and pathology.

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