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NIR RAMAN SCATTERING FOR THE STUDY OF BIOCHEMICAL FEATURES OF THE HUMAN SKIN EPIDERMIS AND A SKIN SURFACE MICRO-MAPPING *IN VITRO*

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The Raman spectroscopy is characterized as a high potential, accurate technique for the non–invasive research of various biological tissues and also human skin under *in vivo* and in vitro conditions. In this paper, we present the investigation of Raman spectroscopy of normal–appearing human skin epidermis *in vitro*. NIR Raman spectroscopy could give some insight on human skin structure and mechanisms governing the behavior of this layer. Moreover, in–depth measurements allow determining relative concentration modifications of the substantial constituents: water, ceramides, cholesterol, fatty acids, proteins, urea, etc. in the thickness of the human skin epidermis. The spectra were obtained using Renishaw inVia Reflex spectrometer in the Raman shift range: 300 cm^{-1}–1450 cm^{-1} and excitation by near infrared NIR λ = 785.0 nm diode laser to avoid a possible fluorescence of the skin tissues and determine the respective advantages of such wavelength. The article presented spectra of the epidermis from which obtained biochemical information regarding its composition. Raman bands assigned to proteins present in the spectrum of the dermis upper layer and appear at 852 cm^{-1}, 1090 cm^{-1}, 1120 cm^{-1}, 1440 cm^{-1}, 1447 cm^{-1}. Prominent contributions of lipids, observed as a small–intensity Raman shifts at: 1060 cm^{-1}–1127 cm^{-1} due to chain C–C stretching, and some small peaks at: 1290 cm^{-1}–1305 cm^{-1} due to CH_2 torsion, vibrations at 1440 cm^{-1}–1447 cm^{-1} due to C-H rupturing. Skin surface point measurements and surface micro-mapping were also performed on the human fingertip. Such information is important for the development of *in vivo* and *in vitro* diagnosis of skin diseases and the improvement of transdermal drug administration.

Key words: human skin tissue, epidermis, Raman scattering, micro-mapping, NIR spectroscopy.

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INTRODUCTION

Raman spectroscopy is one of the most effective and widely used methods for the research of biological molecules (Patterson 1995, Gremlich & Yan 2011). It is known that the Raman spectroscopy uses light excited vibrational–rotational energy states of molecules to obtain information about the composition, structure, interaction of molecules and their complexes. The main advantages of the method are selectivity speed of measurement and suitability for the study of different surfaces. In addition, the number of agents required to study Raman spectroscopy limited by the transverse dimensions of the focused laser beam only. The narrow spectral linewidth of the laser excitation and the use of multichannel recording system allow measuring spectra in the range: 1 cm^{-1} to 3800 cm^{-1} or in a broader range. High power density laser radiation provides information about spectrum, even at low concentrations of analyte. Apply of Raman spectroscopy with biological objects, allows obtaining information on transitions between vibrational energy levels, the molecular vibrations of molecules, and also the negative impact on the object reduced to a minimum (Lieber et al. 2008, Matousek & Stone 2009, Darvin et al. 2004, Chance 1991, Das et al. 1997). The probability of inelastic Raman scattering is exceedingly low, and as a consequence, long integration times are required to acquire sufficient scattering signals for a single spectrum. For example, a traditional Fourier–transform Raman system requires up to 25–40 minutes of integration time to acquire one spectrum. Optical spectroscopy techniques are now widely used in the study of biomolecules and their functioning within live cells. (Tuchin 2000).

Human skin can be hypothetically divided into three main layers: the stratum corneum, the epidermis and the dermis. The stratum corneum has an important function of preserving the skin's moisture. It includes non-nuclear epithelial cells with plenty of keratin, called corneocytes coated with lipid bilayers. Ceramide is one type of lipid and the major component of bilayers. Approximately 80% of the dry weight of the horny layer is keratin. Human skin epidermis consists of living epidermal cells (Gawkrodger, 2002). Their constant growth involved in the production of new horn cells in the stratum corneum. Humidity in the epidermis is maintained by means of natural processes humoral regulation and lipids in the stratum corneum. The main components of the dermis are collagen and elastin, which are produced by cells and fibroblasts, which provide strength and elasticity. Another feature of the structure of the skin is that the boundary of the epidermis and the dermis has an undulating profile (Anderson & Parrish 1982, Lademann et al. 2008). Different morphological and functional conditions of the skin related to the histological, biochemical, physical and chemical changes may be characterized on the basis of information obtained via the Raman micro–mapping of the human skin surface and can be applied to obtain information regarding the molecular composition of the skin down to several hundred microns below the skin surface. (Barry et al. 1992, Gniadecka et al. 2004, Nijssen et al. 2007). Most prior studies involving the skin have been limited to either *ex vivo* samples or a few *in vivo* skin measurements, all requiring relatively long integration times (Svanberg 1997, Fishkin et al. 1997). NIR Raman Spectroscopy can minimize the time that is needed for skin analysis, surface mapping and eliminated the skin luminescence that is one of the disturbing factors in this kind of analysis. The human skin is a boundary between the body and environment, as an important object of study, which can give a huge amount of useful information about the human skin health.

Raman spectroscopy is the most rapidly developing area among all the available optical methods applied to the non–invasive investigation of human skin *in vivo* and *in vitro* (Puppels 1999, Caspers et al. 1998). Applying this technology can obtain sufficient information on the structure of epidermis and dermis, the number, and density of blood vessels, the concentration and spatial distribution of chromophores, fluorophores and the nature of the metabolic processes in the skin. Typical applications include *in vivo* quantitative analysis of erythema and skin pigmentation, skin color variations of the definition, monitoring the effectiveness of dermatological treatment,

the determination of skin photo–aging, cancer diagnosis and study of skin biophysics (Lademann et al. 2008, Rawlings & Matts 2005, Matousek et al. 2002).

Laser imaging (Raman–mapping) is becoming increasingly important in clinical diagnostics, due to the small depth of the penetration of the light into skin tissue of a few micrometers only. Raman spectroscopy has certain characteristics that make it particularly suitable for the skin studies *in vitro* and *in vivo*. Since epidermis much thinner than dermis (≈1400 μ), it can be easily studied using microscopy (Pence I., Mahadevan–Jansen 2016). The important and at the same time elusive goals of analytical sciences in the field of bio–medical research is to provide a safe non–invasive method for analyzing the chemical composition of the layers of living tissue or other turbid media. Therefore the aim of this preliminary study was to analyze the normal skin samples by NIR Raman spectroscopy, because such information is crucial, for example, in the diagnosis of skin diseases as an alternative diagnostic tool to established techniques such as histopathology, as a means of determining the state of health of a living cell.

MATERIAL AND METHODS

Skin samples preparation

Skin samples were collected from two volunteers (one man and one woman), the average age of volunteers 30 years). In each case dorsal (back) hand section from the index fingertips was employed and the sample was measured in unprocessed form. Tissue samples were measured at room temperature 22C° (+/- 1C°), relative humidity 25%.

Raman spectroscopy and micro–Raman mapping of human tissue

Renishaw InVia Reflex spectrometer with an external mounting NIR (air–cooled) diode laser operating at λ =785 nm as a source was used. For the decomposition of the light scattered by the sample, a 1200 lines/mm diffraction grating was used throughout this research. Skin samples analyzed through inVia Spectrometer and focused on a Renishaw air–cooled RenCam CCD array detector with insertion/retraction speed >20 mm s^{-1}, repeatability <0.5μm. For the measurements, either a 50x/0.50 long distance Near–IR optimized optics N PLAN objective (DM 3000, Leica) was employed, each providing a spatial resolution of <2 μm at the sample. For some measurements, the pinhole was set at 100 μm. Movement of the sample was made using a motorized XYZ sample stage with a pitch of 100 nm. In this study for the micro-Raman mapping, the laser is point focused, and the skin sample under test is translated past the laser focus, or the focus raster scanned across the object. The radiant power of the laser was maintained at or below 200 mW at the laser head and the power of the laser beam reached the sample was ~20 mW (+/– 0.5 mW) measured by Novall PD300-3W-V1 OPHIR equipment for laser measurements.

To minimize the heating of the tissue structures under the laser beam, the sample was placed just below the focus; the visible spot size was about ~30 μm. The transmitted part of the beam is incident on the sample at ~90 degrees. Before all measurements performed X–calibration and the system were spectrally calibrated to the 520.116 cm^{-1} spectral line of silicon reference sample. To evaluate the reproducibility of the Raman measurements, conducted separate measurements. Repeated spectra were taken from the same sites in triplicate from 2 different normal human skin samples.

Data analysis

Data collection was accomplished with Renishaw WiRE 3.3 software, but spectrometer scans and processing were controlled by a personal computer using Thermo scientific Grams 9.0 program. Additionally, processing of the spectral data, including decomposition of complex band–shapes analyses was accomplished using WiRE 3.3 Raman software for curve fitting. All obtained spectra were compared with S.T. Japan spectral databases and data from scientific articles.

RESULTS AND DISCUSSION

Applying Raman spectroscopy it's possible to determine minor details in the chemical composition of the skin layers and differentiate on the minimal differences in the spectra (Angel 2000, Lucassen et al. 2002, Tu 1982). The choice of the laser operating in the 785 nm range is also based on the fact that a large wavelength provides deeper penetration into the tissue thickness and at the same time is less dangerous to living cells (Lucassen et al. 2002).

These Raman spectra (Fig.1, Fig.2) are raw spectra with the small residues of the self–fluorescence of the skin at 1220 cm^{-1}–1320 cm^{-1} region, surface background subtracted only. It can be seen that at 1220 cm^{-1}–1320 cm^{-1} region the spectra has larger noise due to a high fluorescence background masking the underlying Raman signal (Fig.1, Fig.2). The fluorescence originates predominantly from the melanin component of skin located at the very surface regions of the probe sample but the fluorescence is a normal phenomenon and characteristic for some biological objects. Usually, the large spurious background fluorescence complicates the analysis of Raman spectra when working with wavelengths in the visible range and Raman shifts till 2500cm^{-1}, so it is more indicative for the spectra of up to 2000cm^{-1}.

Variability in the Raman frequency shifts by Raman shifts for any 3 consecutive spectra from the same skin site was negligible, confirming the relative stability of spectral peak positions. However, in terms of the Raman signal intensities (y–axis), the variances for the triplicate measurement sets showed a systematic change that was wave number–dependent, with relatively smoother spectra at lower Raman frequencies and increasing fluctuations at higher frequencies. It was decided to shift measurements to the region: 300 cm^{-1}–1500 cm^{-1} because of the increased fluorescence of the samples at 1800 cm^{-1}–2500 cm^{-1} region (data not shown).

The region with the highest peak vibrations and the maximum biochemical information is contained within the so–called fingerprint region of the spectrum of the skin surface was at the region: 450 cm^{-1}–1500 cm^{-1} and therefore this region was initially analyzed (Fig.1, Fig.2.). This region is an important source of information about the components of biological molecules such as nucleic acids, peptides, lipids, amino

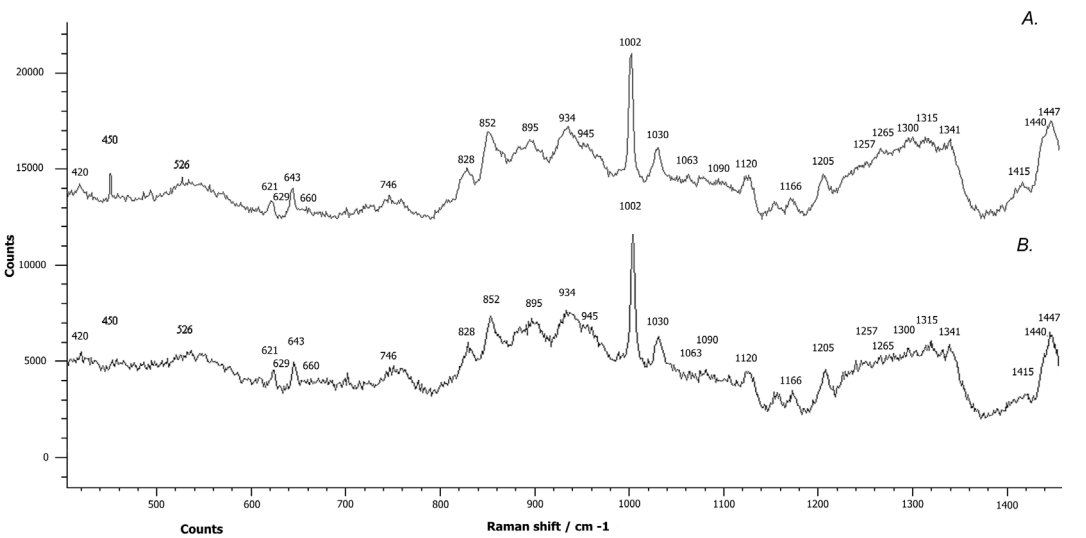

Fig.1. The estimates of pure Raman spectra: A. - the man's skin right–hand index fingertips epidermis, B. - the women's skin right–hand index fingertips epidermis, measured directly from the sample surface (*in vitro*).

acids and carbohydrates. Collagen is also visible in this spectrum: proline and hydroxyproline make up about one–fourth of the amino acids in collagen, a higher proportion than in most other proteins. The Raman spectrum (Fig.1) show a series of well–resolved bands at 828 cm^{-1}, 830 cm^{-1}, 852 cm^{-1} and these bands originate from the amino acid side chain vibrations of proline and hydroxyproline as well as from a C–C stretching vibration of the collagen backbone (Boukamp et al. 1988, Tfayli et al. 2007, Bonnier et al. 2013). Raman bands assigned to proteins present in the spectrum of the dermis upper layer and appear at 852 cm^{-1}, 1090 cm^{-1}, 1120 cm^{-1}, 1440 cm^{-1}, 1447 cm^{-1} and some small–intensity peaks at 1257 cm^{-1} due to C=O deformation and C–N stretching, while features at 1002 cm^{-1} and 1035 cm^{-1} are due to symmetric ring breathing and C=O in–plane bending (phenylalanine). These bands are also evident in the mean spectra of the other skin layers, but the collagen specific high–intensity peaks are absent and they are thus distinctive signatures of the dermal layer of the skin (De Gelder et al. 2007, Jess et al. 2006, Notingher & Hench 2006, Notingher et al. 2003).

It is known that the concentration of lipids is significantly reduced from the stratum corneum to the epithelium (Pappas 2009, Rawlings & Matts 2005). The obtained results show prominent contributions of lipids, observed as a small–intensity Raman shifts at this region: 1060 cm^{-1}–1127 cm^{-1} due to chain C–C stretching, and some small peaks at this region: 1290 cm^{-1}–1305 cm^{-1} due to CH_2 torsion, and also vibrations at 1440 cm^{-1}–1447 cm^{-1} due to C-H rupturing (Fig.1). All Raman spectral bands can be compared to strong features in the spectra of ceramide, phosphatidylcholine or sphingomyelin, but the most abundant classes of lipids present in the stratum corneum are cholesterol, fatty acids and ceramide, in which these skin compounds play the main role as the barrier for the skin. The absence of the intense Raman features at region: 720 cm^{-1}–730 cm^{-1}, highlights that sphingomyelin is absent from the stratum corneum. Similarly, the absence of a strong peak at 717 cm^{-1} in the spectrum of the skin seems to indicate that no contribution from the phospholipids can be observed (Chalmers & Griffiths 2001, Caspers et al. 1998). Raman peaks at 934 cm^{-1} and 945 cm^{-1} correspond to protein assignments: ν(C–C)

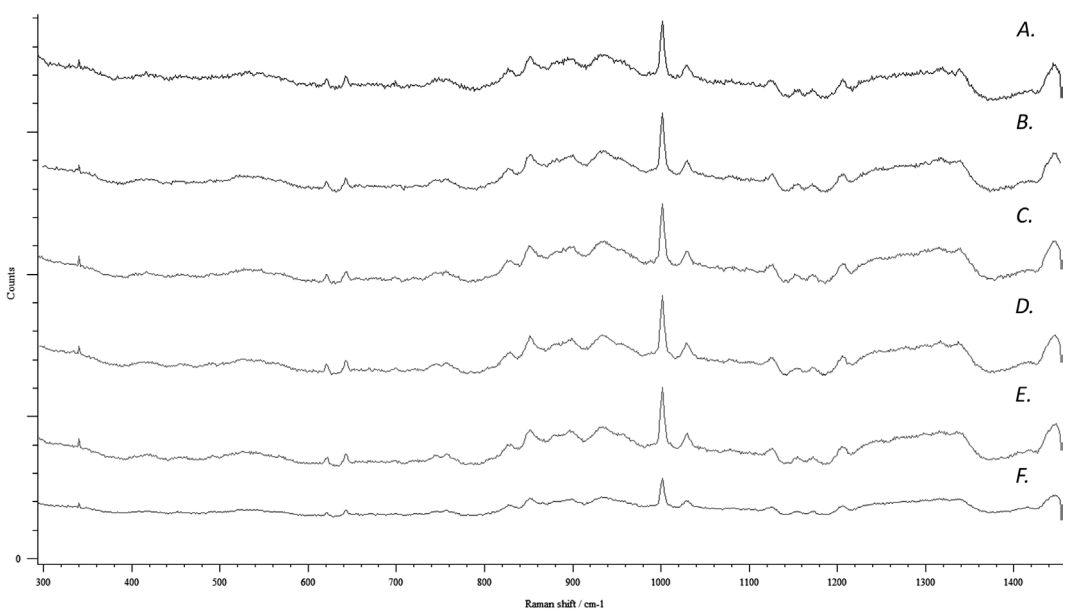

Fig. 2. Average Raman spectra of the unprocessed right–hand index fingertips skin epidermis of the 2 volunteers (in vitro). A,B,C- the man's skin samples D,E,F- the women's skin samples.

proline and v(C–C) valine at 1002 cm^{-1} to v(C–C) phenyl ring, at 1270 cm^{-1} to v(C–N), δ (N–H) Amide III at 1442 cm^{-1} to $\delta(CH_2)$, $\delta(CH_3)$ and also as lipid assignments as a $\delta(CH_2)$ scissoring (Fig.1) (Caspers et al. 2000, Caspers et al. 2001). However, in recent studies, the stratum corneum (the upper layer of the epidermis) is commonly described as mostly composed of ceramides, fatty acids and cholesterol although other lipids may be present in small quantities. Earlier studies indicated that phospholipids in the stratum corneum account for about 5% of the total lipids from samples taken from the legs or abdomen but smaller proportions in other location such as the face (Lampe et al.1983, Melnik et al. 1989, Harding et al. 2000). Fig.1 and Fig.2 illustrate that these regions: at 1210 cm^{-1}–1300 cm^{-1} and at 1400 cm^{-1}–1440 cm^{-1}, are regions of strong lipid contributions, however, spectral shifts and these characteristic differences are more likely due to differing contributions of lipids to the spectra relative to those proteins (Downing 1992, Mizutani et al. 2009).

Was noted that Raman mapping is a non-intrusive analytical technique well suited for fast analysis of skin epidermis samples to help identify and visualizing the biochemical composition or Raman signal overall intensity at the point. For the visualization of obtained single Raman spectra, was created micro–Raman signal intensity distribution map from the components that belong to the upper layer of the human skin epidermis and in the Raman shift region at 300 cm^{-1}–1500 cm^{-1} (Fig.3.). In this 2D micro–Raman map, it was shown, that the distinguishable field of the varying Raman signal strength is depending on the distribution of components of the composition of the human skin epidermis.
This image shows the distribution of Raman signal intensity at the point: higher intensity (red color), smaller intensity (green and blue color) as shown in the (Fig.3). During the mapping of the skin samples, the intensity of the Raman signal ranged between 2988.6 (dark blue) and 9717.8 (red). The main part of the skin surface has shown intensity in the range from ~3500 to ~5500 and from ~6500 to ~8000. The smallest investigated area with the highest signal intensity is colored in red and approximately within the range more than 9000. Sometimes the Raman mapping of rough transparent samples was thus far very challenging and time-consuming, due to the inability all the time to keep the sample in focus. However, applying focus tracking the Raman mapping allows avoiding this problem. Large

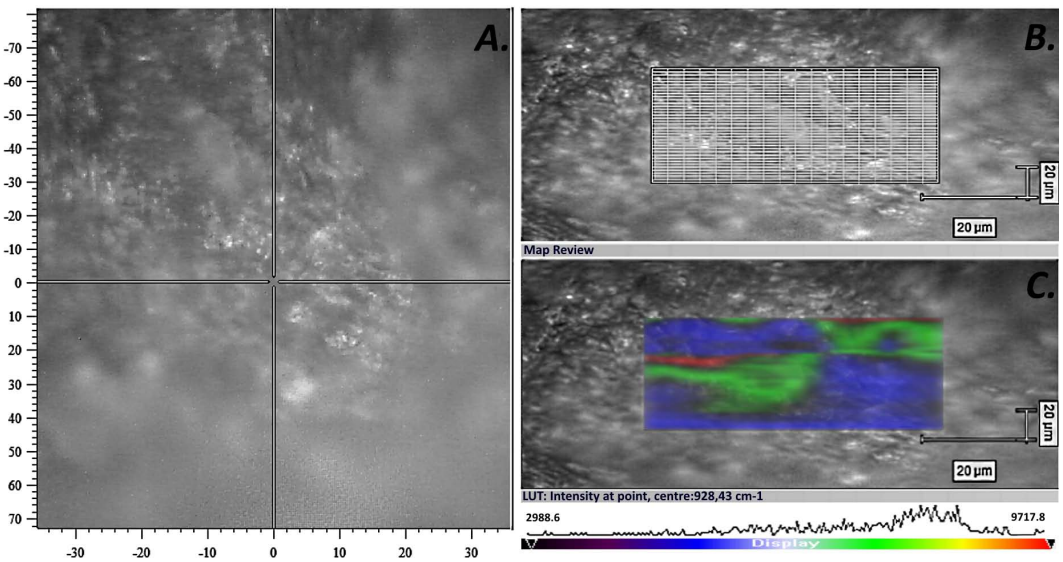

Fig. 3. Spatial 2D image of the human epidermis surface: A) an optical image; B) mapping scheme; C) micro–Raman signal intensity map.

area topographic coordinates can be precisely correlated with the large area Raman imaging data. This allows true surface Raman imaging on heavily inclined or rough surfaces, with the true surface held in constant focus, while maintaining the highest image quality.

During this study, it was noted, that the use of Raman spectroscopy enables certain characteristics which make Raman scattering particularly suitable for skin studies *in vitro*. Raman spectra of human skin *in vitro* show a significant decline in the absolute signal intensity with increasing distance between the laser focus and the skin surface and therefore require more time for the accumulation of the signal. This feature is mainly due to diffuse scattering of light, which is a much more potent effect on the skin than the absorption effect.

CONCLUSIONS

In the nowadays, the interest in diagnostics of different skin diseases are increasing and research in this field are very important. Recent studies with applying of Raman spectroscopy in skin analysis show that the use of non-invasive spectroscopy in diagnosis and monitoring has great potential, but unfortunately not fully disclosed. However, for the application of this type of diagnosis requires precise standardization of the methods and samples.

It was noted that significant spectral differences by gender corresponding to certain components in the Raman spectra of human skin taken from the very surface layer of the index fingertips epidermis of two volunteers at the nearest age and race group were not observed and bright new peaks expressed in Raman's finger–print area comparing skin samples does not appear. We have studied biochemical features of unprocessed human skin epidermis from young women and man, in each case dorsal (back) hand section from the index fingertips, using real-time *in vitro* NIR Raman spectroscopy with an integration time of fewer than 10 seconds per point, in the so–called Raman shift ranges fingerprint region of the spectrum at the region: 450 cm^{-1}–1500 cm^{-1}. Multi-point Raman micro-mapping also performed. The analysis shows that Raman spectroscopy can distinguish various biochemical compounds such as fatty acids, proteins, lipids, nucleic acids, peptides, carbohydrates and others from the surface layer of normal skin epidermis. Interest in such studies is caused by the possibility of creating biologically–safe diagnostic techniques for bio–tissues, the potential for the imaging study of surfaces with a spatial resolution comparable to the length of the source of emitting, and also the possibility of multi–functional diagnostics of the objects in a sparing mode.

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