European Journal of Pharmaceutics and Biopharmaceutics 116 (2017) 76–84

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

Studies for improved understanding of lipid distributions in human skin by combining stimulated and spontaneous Raman microscopy

A. Klossek [a], S. Thierbach [a], F. Rancan [b], A. Vogt [b], U. Blume-Peytavi [b], E. Rühl [a,*]

[a] Physikalische Chemie, Institut für Chemie und Biochemie, Freie Universität Berlin, Takustr. 3, 14195 Berlin, Germany
[b] Klinisches Forschungszentrum für Haut-und Haarforschung, Charité Universitätsmedizin, 10117 Berlin, Germany

ARTICLE INFO

Article history:
Received 22 May 2016
Revised 7 October 2016
Accepted in revised form 7 November 2016
Available online 15 November 2016

Keywords:
Skin
Stimulated Raman spectroscopy
Microscopy
Lipids
Proteins

ABSTRACT

Advanced Raman techniques, such as stimulated Raman spectroscopy (SRS), have become a valuable tool for investigations of distributions of substances in biological samples. However, these techniques lack spectral information and are therefore highly affected by cross-sensitivities, which are due to blended Raman bands. One typical example is the symmetric CH_2 stretching vibration of lipids, which is blended with the more intense Raman band of proteins. We report in this work an approach to reduce such cross-sensitivities by a factor of 8 in human skin samples. This is accomplished by careful spectral deconvolutions revealing the neat spectra of skin lipids. Extensive Raman studies combining the complementary advantages of fast mapping and scanning, i.e. SRS, as well as spectral information provided by spontaneous Raman spectroscopy, were performed on the same skin regions. In addition, an approach for correcting artifacts is reported, which are due to transmission and reflection geometries in Raman microscopy as well as scattering of radiation from rough and highly structured skin samples. As a result, these developments offer improved results obtained from label-free spectromicroscopy provided by Raman techniques. These yield substance specific information from spectral regimes in which blended bands dominate. This improvement is illustrated by studies on the asymmetric CH_2 stretching vibration of lipids, which was previously difficult to identify due to the strong background signal from proteins. The advantage of the correction procedures is demonstrated by higher spatial resolution permitting to perform more detailed investigations on lipids and their composition in skin.

1. Introduction

Advanced Raman techniques, such as Stimulated Raman-spectroscopy (SRS) [1–3] and Coherent anti-Stokes Raman scattering (CARS) [4–6], have gained in recent year importance for fast imaging of biological samples. These are label-free techniques which offer chemical contrast and sensitivity without any kind of dye or marker, as is required for e.g. fluorescence microscopy [7]. Maps of substance distributions can be recorded in an acceptable amount of time, which is due to the enhanced Raman efficiency of the SRS- and CARS-processes compared to spontaneous Raman scattering. One of the first and most investigated examples for demonstrating the potential of these techniques was the skin from humans and animals (cf. e.g. [1,5]). The spatial distribution of the skin lipids and proteins is of specific interest, since these represent two of the primary components. Moreover, lipids and proteins exhibit a distinct spatial distribution, which emphasizes the need of advanced imaging- and mapping-techniques. It is well-accepted that the stratum corneum, the main barrier of skin, consists of corneocytes which are embedded in a lipid matrix. A common model to describe the stratum corneum is the bricks-and-mortar model, where the corneocytes are the bricks and the surrounding lipids represent the mortar [8]. The skin layer underneath the stratum corneum is the viable epidermis, which exhibits a significantly lower local lipid concentration. Studies on human skin indicated that even the stratum corneum exhibits a gradient in lipid distribution forming a barrier to transepidermal water loss [9,10]. In contrast, the protein distribution in top skin layers appears to be more uniform [11].

Various spontaneous Raman studies of skin have established a widely accepted understanding of the CH_n-Raman bands ($1 \leqslant n \leqslant 3$) reaching from 2800 cm^{-1} to 3100 cm^{-1} [12]. Three main peaks can be distinguished. Two of them are attributed to lipids, exhibiting symmetric CH_2-stretching vibrations ($\nu_s CH_2$) near 2850 cm^{-1} and the asymmetric vibrations ($\nu_{as} CH_2$) around 2880 cm^{-1} [13,14]. The third and dominating band is localized

* Corresponding author.
E-mail address: ruehl@zedat.fu-berlin.de (E. Rühl).

http://dx.doi.org/10.1016/j.ejpb.2016.11.001
0939-6411/

around 2930 cm^{-1} and attributed to symmetric CH_3 stretching vibrations (ν_sCH_3) of proteins [14,15].

Maps of the lipid distribution derived from spectroscopic studies are limited to studies of ν_sCH_2, as performed at 2845 cm^{-1} (see e.g. [1,16]. Spectral deviations from the lipid bands reaching up to ±10 cm^{-1} have been observed, which depend on various factors, such as relative humidity [15], species, gender [14], and localization in skin [17]. However, selection of ν_sCH_2 is a remarkable choice, especially since deconvolutions of the CH_n bands indicate that $\nu_{as}CH_2$ around 2880 cm^{-1} is more intense [12]. This choice is primarily justified by the overlapping protein band, which causes a strong background at this wavenumber, so that the weaker one near 2850 cm^{-1} is selected. Nevertheless, investigations of the lipid distribution by SRS or CARS around 2850 cm^{-1} are still affected by a significant contribution from proteins. As a result, studies using the asymmetric CH_2 stretch vibration have not been reported to the best of our knowledge. As a result, advanced Raman techniques, such as SRS or CARS, lack distinct spectral information near blended vibrations. Such limitations are not just related to the limited spectral resolution reaching from a few cm^{-1} for lasers with pulse widths of about 7 ps [18] to tens of cm^{-1} that is typical for 100 fs-laser pulses [19]. In addition, there is only slow tuning of the optical parametric oscillator (OPO), which is often used to match the wavelengths in multi-photon processes used for SRS or CARS studies, requiring typically a few seconds per nanometer. Such time scales limit the advantage of enhanced efficiency compared to spontaneous Raman scattering.

In this work we outline a method to correct for blended Raman bands leading to backgrounds or cross-sensitivities in biological samples. We use as an example lipids within human skin, which are investigated at 2850 cm^{-1} and 2880 cm^{-1} by means of spontaneous and stimulated Raman scattering. We combine the complementary advantages of wide spectral information in spontaneous Raman spectroscopy with the increased efficiency in SRS leading to faster data acquisition times. We report on maps of proteins and lipids demonstrating the advantages of the reported data analysis as compared to maps derived without such corrections. Enhanced spatial resolution of different species as well as the use of the previously not used $\nu_{as}CH_2$ vibrations around 2880 cm^{-1} for probing skin lipids is reported. In addition, we discuss further differences between the results obtained from spontaneous and stimulated Raman techniques, by analyzing the same skin regions. These differences are found to be due to the detection geometry, i.e. transmission and reflection, as well as rough skin samples representing another source of scattered light. Consideration of these contributions is reported for avoiding misinterpretations of the collected data.

2. Experimental

2.1. Raman spectromicroscopy

Stimulated Raman spectromicroscopy (SRS) studies were performed using an IX83 Olympus microscope, equipped with a 50x NIR objective (LCPLN50XIR, Olympus). The samples were excited by a PicoEmerald laser system (APE, Berlin), with a pulse width of 6 ps and a repetition rate of 80 MHz. This laser emits two pulses, where one was kept at fixed wavelength (Stokes pulse, 1064.2 nm). The wavelength of the preceding pump pulse was adjusted according to the desired Raman transition using the signal wave of the optical parametric oscillator (OPO). Specifically, we used for probing proteins the transitions at 2934 cm^{-1}, which corresponds to the wavelength of the pump pulse of 811.2 nm. Lipids were probed at 2880 cm^{-1} ($\nu_{as}CH_2$) and 2850 cm^{-1} (ν_sCH_2), requiring that the pump pulse was adjusted to 814.5 nm and 816.5 nm, respectively. Measuring the SRS process was realized in Stimulated Raman-Loss (SRL) detection technique [1]. The Stokes beam was modulated by an electrooptical modulator (EOM) to 20 MHz. Modulations of the transmitted pump beam were detected by a fast photo diode (DET36A, Thorlabs) and a lock-in amplifier (HF2TA + HF2LI Zurich Instruments). In order to avoid radiation damage of the samples the laser power was limited to 10 mW and 20 mW for pump- and Stokes-beam, respectively. Stimulated Raman maps were performed by scanning the microscope stage with a dwell time at one spot of 160 ms. Possible unwanted backgrounds from cross-phase modulation or other effects [20] are reduced by subtracting background maps performed at 2500 cm^{-1}, corresponding to a pump beam wavelength of 840.6 nm, where no Raman signal is emitted from skin samples.

Spontaneous Raman measurements in the spectral region from 2690 cm^{-1} to 3100 cm^{-1} were performed in reflection geometry setup using a modified optical microscope (BX41, Olympus) equipped with 100x standard objective (MPLN, Olympus). The Raman excitation was carried out by a 532 nm CW laser (Millennia, 5 W, Spectra Physics) and detected by a fiber-coupled 80 cm Jobin Yvon spectrometer (Dilor triple XY 800, Horiba) using three 1800 l/mm gratings in the subtractive mode. The spectrometer is equipped with a Synapse Si CCD-detector (Horiba), which is cooled to −66 °C. The laser power was limited to about 1.5 mW, so that thermal damage of the samples was minimized. Raman spectra were taken each point two times at an integration time of 60 s. The spectral resolution of the 1024 data point spectra are limited by the entrance slit of 0.2 mm to ~2.5 cm^{-1}.

The samples were oriented so that the laser focus is directed to the skin first. Therefore, the measurements are not compromised by the glass slide serving as a substrate for the skin samples.

2.2. Skin samples

Human skin has been embedded in the medium Tissue-Tek® (O. C.T.) to slice cryo-cross-sections at −20 °C with a thickness of typically 6 µm. From these samples, regions of healthy and uniform skin have been selected for Raman studies. They were stored at room temperature for some days during SRL and spontaneous Raman measurements to avoid damage due to repeated freeze-thaw cycles.

3. Results and discussion

Human skin samples from different donors and thicknesses have been investigated in order to determine the distribution of proteins and lipid within the viable epidermis and the stratum corneum, which can be used for investigating changes which are induced by topical drug delivery. These samples were investigated by spontaneous and stimulated Raman spectroscopy. As a result of these studies, we identified Raman spectra representing two limiting cases regarding the concentration of lipids, as will be outlined in the following. Raman spectra of these regions were averaged in order to derive representative results of the entire stratum corneum and the viable epidermis, respectively, as shown in Fig. 1 (a) and (b). The depicted spectra were measured by stimulated (left hand side) and spontaneous Raman (right hand side), yielding comparable results. Black curves in Fig. 1(a) and (b) are characterized by intense peaks from lipid-related ν_sCH_2 vibrations around 2850 cm^{-1} and $\nu_{as}CH_2$ vibrations around 2880 cm^{-1} (marked by vertical dashed lines). These Raman spectra represent the upper layers of the stratum corneum. The red curves shown in Fig. 1(a) and (b) are typical for the viable epidermis and contain only low intensity in the spectral region of lipids, whereas the contribution of proteins is dominant (ν_sCH_3 around 2930 cm^{-1}). It can be