Influence of water content on Raman spectroscopy characterization of skin sample

SOOGEUN KIM,1,2 KYUNG MIN BYUN,1,2,3 AND SOO YEOL LEE1,2,4
1Department of Biomedical Engineering, Kyung Hee University, Yongin 17114, South Korea
2Targeted Precision Treatment Research Center, Kyung Hee University, Seoul 02447, South Korea
3kmbyun@khu.ac.kr
4sylee01@khu.ac.kr

Abstract: We report that the Raman spectrum obtained from porcine skin varies significantly with the change of skin water content. At different water contents from 40 to 55 wt.%, the Raman spectra results using confocal Raman spectroscopy show that the spectral variation of porcine skin is highly affected by skin water content. Experimental data are consistent with the Monte Carlo calculation and it is proved that the intensity of the Raman spectrum depends on the angle distribution and collection efficiency of backscattered light from the sample surface for a varied water content. It is suggested that water content for a given skin sample should be controlled carefully to minimize errors and deviations in the Raman peak analyses.

© 2017 Optical Society of America

OCIS codes: (170.1870) Dermatology; (170.5660) Raman spectroscopy; (170.6510) Spectroscopy, tissue diagnostics; (290.5860) Scattering, Raman.

References and links
1. Introduction

Over the past decades, applications of Raman spectroscopy have been actively investigated for disease diagnosis in various tissues and organs such as cervix [1–3], skin [4–7], and gastrointestinal tract [8–11]. Among them, skin provides the easiest access to researches with Raman spectroscopy; however, its complex and turbid nature make it one of the most challenging clinical targets [12]. Since Raman scattering signal is as weak as ~10⁻⁶ of the intensity of Rayleigh scattering [11] and is often overwhelmed by fluorescence background signal, skin disease diagnosis based on Raman spectroscopy has suffered from low accuracy and poor signal to noise ratio. To overcome these inherent disadvantages, a variety of techniques that can achieve a high diagnostic accuracy have been suggested. For example, Zhao et al. reported that the diagnostic specificity for fixed sensitivity of 0.99–0.90 was improved from 0.17–0.65 to 0.20–0.75 with wave-number selection based on a leave-one-out cross-selection procedure [13]. Lim et al. suggested that a combination of three fiber-optic-based optical spectroscopy modalities (diffuse optical spectroscopy, laser-induced fluorescence spectroscopy, and Raman spectroscopy) is necessary for accurate and noninvasive diagnosis of both melanoma and non-melanoma skin cancers in vivo [14]. Although diagnostic accuracy in Raman spectroscopy is strongly dependent on the instrumental systems and the data processing algorithms, it can be also affected by the experimental condition of the skin sample. It is because a variation of optical property by skin condition change may induce a significant contrast in Raman scattering signals even under
the same experimental condition. In terms of water content, one of the key parameters for skin conditions, earlier studies reported that optical properties of skin tissue such as absorption and scattering coefficients are influenced by a degree of the water content [15,16]. Interestingly, it has been known that the water content of human skin changes with body parts and measurement time. Nakagawa et al. found that water content in forearm skin of healthy male increased up to 5.7 wt.% in the afternoon compared to the value in the morning [17]. Hence, due to the time- and point-varying optical properties of target skin, which is accompanied by its water content change, it is likely that the same skin sample may produce different results in Raman scattering spectrum profile.

Despite a lot of Raman spectroscopy studies on improving the instruments and data processing techniques in skin disease diagnosis [4–7,13,14], the water content effect on the Raman signal measure has been relatively less taken into account [18]. In this study, we intend to present an influence of water content on the Raman spectroscopy experimentally using confocal Raman microscope with a 785 nm excitation laser. Especially, it is demonstrated that the Raman spectrum profile obtained from a skin tissue changes with its water content and the Monte Carlo simulations are well consistent with the experimental measurement.

2. Materials and methods

2.1 Sample preparation

Abdominal porcine skin obtained from a local abattoir immediately after postmortem was used as the sample because of its similarity in structural characteristics to human skin [19,20]. The porcine skin samples were stored in a saline solution at 4°C to minimize dehydration and structural change until experiments. All experimental results were made at room temperature within 12 h from the collection.

The water content of porcine skin samples in weight percent \( w_t \) (wt.%) was estimated using the following equation:

\[
   w_t = \frac{M_{wet} - M_{dry}}{M_{wet}} \times 100.
\]

where \( M_{wet} \) is the mass of either native or partially dehydrated samples and \( M_{dry} \) is the mass of dehydrated samples which are air-dried at room temperature for 24 h. During the experiment, the water content of native samples was reduced by blowing with a cold dryer to ensure uniform drying conditions. Based on Eq. (1), the water content of ten native samples was estimated to be \( \sim 55 \text{ wt.\%} \). Five samples of them was used for optical property measurement and the others was used for Raman measurement.

2.2 Measurement of optical properties

The optical properties of absorption and reduced scattering coefficients of porcine skin were obtained over the water content ranging from 40 to 55 wt.% at a step of 5 wt.%. For each water content condition, five samples were tested and each sample was measured five times at different positions. The size of each sample is about 10 mm \( \times \) 10 mm \( \times \) 0.56 mm. First, the total reflectance and transmittance at each water content were measured using a double-integrating sphere (AvaSphere-30, Avantes, port diameter = 6 mm) [21]. And then, from the measured data, the optical properties were calculated using an inverse adding-doubling program [22,23]. Specifically, the program repeatedly calculated the total reflectance and transmittance with trial values of optical properties until the calculated total reflectance and transmittance matched the measured values.

In double-integrating sphere measurements, the sample thickness is recommended to be \( \sim 1/10 \) of the port diameter or smaller to reduce an edge loss [23]. The thickness of native skin samples was \( \sim 560 \mu \text{m} \) by digital vernier calipers (CD-20CP, Mitutoyo Corp.) and the value
was decreased with dehydration to ~540, 510, and 490 μm at the water content of 50, 45, and 40 wt.%, respectively. In addition, the edge loss compensation function of an inverse adding-doubling program was applied in computing the optical properties.

2.3 Raman spectroscopy

Five abdominal porcine skin samples were used for Raman spectral measurements. The samples were collected from the same abdominal skin of a porcine (20 mm × 20 mm × 1 mm). The size of each sample is about 5 mm × 5 mm × 1 mm. Each native sample (55 wt.%) was dehydrated to 50, 45, and 40 wt.%. For each water content condition, each sample was measured five times at different positions. As a result, the average Raman spectrum at each water content condition was calculated from the twenty-five measurements obtained from the five samples. The Raman spectra of the samples were measured at the sample surface using a confocal Raman microscope system equipped with a 785 nm laser module (I0785MM0350MF, Innovative Photonic Solutions) as shown in Fig. 1. The laser power and spot diameter on the sample surface were 50 mW and 120 μm, respectively. The Raman scattering signals were collected in a 10 × objective lens (LDJ 10, Shibuya Optical Co., LTD., NA 0.3) and detected by a Czerny-Turner spectrograph (SR-303i-A, Andor Technology) with a low dark current deep-depletion CCD (iVac, Andor Technology). All Raman spectra of the samples at varying water content condition were acquired in the range of 600 to 3600 cm\(^{-1}\) with a spectral resolution of 0.8 cm\(^{-1}\). The acquisition time of 60 s was required to accomplish a good signal to noise ratio (SNR) in Raman spectra. The minimum SNR was ~5.3.

After the Raman measurements, the instrument and CCD noise were removed from the measured Raman spectra and then, the noise-corrected Raman spectra were smoothed by the Savitzky-Golay digital filter with smoothing width of 9 and degree of 3 [11], followed by baselining using improved modified multi-polynomial fitting [24].

2.4 Monte Carlo simulation

A Monte Carlo model of steady-state light transport in tissue [25] and the corresponding convolution program [26] were used to calculate diffuse reflectance on the porcine skin.
surface in the cylindrical coordinate system. For simplicity, the layered structure of porcine skin was approximated into a single layer and its equivalent optical properties were measured using the porcine skin samples of thickness 560 μm as explained in section 2.2. Through the Monte Carlo simulation based on probability distribution analysis, impulse responses of photons within the single layer porcine skin were first calculated using the infinitely narrow photon source. The photons of 10⁹ were used to yield an acceptable results. And then, the responses to photon source of finite size were calculated using the convolution program based on Green function. The laser spot radius of 60 μm in Raman experiments was used for this simulations. The detail simulation code is presented in Ref [27].

3. Results and discussion

Figure 2 shows the Raman spectra of porcine skin samples measured at varying water content. Each Raman spectrum represents the average (solid and dashed lines) and standard deviation (filled areas). All Raman spectra contain the peaks that are consistent with normal skin samples, for example, polysaccharide (855 cm⁻¹), C-C stretching in α-helix (940 cm⁻¹), phenylalanine ring breathing (1005 cm⁻¹), Amide III in proteins (1270 cm⁻¹), CH₂ deformation (1450 cm⁻¹), Amide I in proteins (1660 cm⁻¹), CH₃ stretching (2880 cm⁻¹), OH stretching (3260 cm⁻¹) [4,7,18,28]. It is interesting to note that the peak intensity of the Raman spectra varies with the water content of the sample, even though the laser power on the sample surface is kept constant during the experiment. From all the Raman peaks obtained, it is observed that a significant decrease of the peak intensity takes place as the water content increases from 40 to 55 wt.%. For a confocal Raman microscope, the intensity of Raman scattered light depends on the concentration of the Raman-active molecules, the volume of the sample illuminated by the Raman excitation laser, the intensity of the Raman excitation laser, and the scattering properties of the sample [11]. In our experimental conditions, the possibility of concentration change of the Raman-active molecules is very little because the sample was identical and the external form of the sample showed little change with a varied water content. Therefore, it seems that the intensity change of Raman scattering is associated with the change of the scattering properties of the sample in different water content condition.

![Fig. 2. Raman spectra of porcine skin in (a) fingerprint and (b) high wave-number measured at varying water content.](image-url)
Fig. 3. (a) Absorption ($\mu_a$) and reduced scattering ($\mu_s'$) coefficients and (b) diffuse reflectance in different water content samples. The error bars is for standard deviation.

Figure 3(a) shows the calculated absorption ($\mu_a$) and reduced scattering ($\mu_s'$) coefficients of porcine skin samples at a wavelength of 785 nm. It is found that $\mu_s'$ increases up to twice as the water content varies from 40 to 55 wt.%. The observed increase of $\mu_s'$ may be attributed to the refractive index mismatch between collagen fibrils and interstitial space as a result of the increased water concentration within the interstitial space [29]. On the other hand, $\mu_a$ is not highly influenced by the change of water content conditions, which is probably due to the absence of blood perfusion in the in vitro sample. Based on the variation of $\mu_a$ and $\mu_s'$, it is presented that the amount of backscattered light from the sample surface is governed by the variation of $\mu_s'$. Previous studies on the biological tissues demonstrated that if $\mu_s'$ is much larger than $\mu_a$ and $\mu_a$ remains constant, an increment of $\mu_s'$ leads to a notable increase of backscattered photons from the tissue surface [30,31].

Figure 3(b) shows the diffuse reflectance, defined as the ratio of laser power of the backscattered light to that of the incident light, using a single-integrating sphere system [21]. The specular reflectance for the normal incident light is simply subtracted from the measured raw data. It should be emphasized that an increase of backscattered light is found with a growing water content, resulting in an increase of $\mu_s'$. This observation is not consistent with the variation of Raman peak intensity in Fig. 2 because the peak intensity decreases as the water content increases. Such conflicting experimental results can be understood by suggesting that the collection efficiency of an objective lens for backscattered light varies with the water content of the sample. Practically, when confocal Raman microscope is employed, intensity of the measured Raman spectra depends not on the amount of backscattered light from the sample surface but on the backscattered light of which reflectance angle is within the acceptance angle of an objective lens ($\theta_a$, the half-angle of the acceptance cone of an objective lens). In an air environment, the acceptance angle is determined by the numerical aperture (NA) of an objective lens, i.e., $\theta_a = \sin^{-1}NA$, which is equal to 0.3 rad in this study.

To estimate the reflectance angle distribution of the backscattered light from the sample surface, the Monte Carlo simulation is carried out at a wavelength of 785 nm for each water content condition from 40 to 55 wt.%. The water content-dependent $\mu_a$ and $\mu_s'$ data in Fig. 3(a) and the laser power of 50 mW and spot radius of 60 $\mu$m in Raman experiments are used for the reflectance angle calculations. Figure 4(b) exhibits the calculation results of the normalized diffuse reflectance profiles as a function of reflectance angle and radial position for different water content conditions. The zero values for reflectance angle and radial position indicate the normal direction to the sample surface and the distance from the center of laser spot on the sample surface, respectively.
In Fig. 4(b), notable change in diffuse reflectance profile is found according to the water content condition. For a low water content, backscattered light from the sample surface mostly occurs within the range of the laser spot radius of 60 μm. A significant portion of the backscattered light appears to be included in the acceptance angle of the objective lens. On the contrary, the backscattered light by the 55 wt.% condition spreads out over the angular and radial range. These simulation results suggest that the portion of the backscattered light collected by the objective lens is eventually decreased as the water content of the porcine skin sample increases. For 40, 45, 50, and 55 wt.% conditions, the portion of the backscattered
light considered to be collected by the objective lens used in the Raman experiments is calculated using the results of Fig. 4(b) to be reduced as 85, 66, 54, and 35%, respectively. Considering these values, the measured diffuse reflectance in Fig. 3(b) is replotted in Fig. 5 as the calculated diffuse reflectance, showing that the diffuse reflectance considered to be collected by the objective lens is decreased as the water content of the sample increases. An overall trend in Fig. 5 is exactly consistent with the decrease of the intensity of the Raman spectra with an increasing water content from 40 to 55 wt.% as shown in Fig. 2.

For an accurate diagnosis of skin cancers such as melanoma, basal cell carcinoma (BCC), and squamous cell carcinoma (SCC) using Raman spectroscopy, the spectral alterations reflecting changes in composition and structure of proteins and lipids have been carefully investigated [4–6]. To discriminate between normal and cancerous skin, Gniadecka et al. compared the ratio of Raman peak intensities between CH \(_2\) deformation and Amide I in proteins (I\(_{1450\text{ cm}^{-1}}\) / I\(_{1660\text{ cm}^{-1}}\)) [4]. They found that when is compared with normal skin, the ratio value of melanoma is about two times higher, whereas that of BCC is lower. Lieber et al. reported that the peak at Amide I in proteins (1660 cm\(^{-1}\)) is higher in BCC and SCC than in normal skin [5].

It is interesting to find in Fig. 6 that the intensity ratio between CH\(_2\) deformation and Amide I in proteins (I\(_{1450\text{ cm}^{-1}}\) / I\(_{1660\text{ cm}^{-1}}\)) varies due to the change in water content of the porcine skin sample independent of skin cancers. The variation of the intensity ratio is possible because the protein vibrational modes are being disrupted by dehydration [18]. Although the peak intensity at Amide I in proteins (1660 cm\(^{-1}\)) increases with a decreasing water content as shown in Fig. 2, it appears to be not due to skin cancers such as BCC and SCC as explained above. As a result, in this study, we confirm that the spectral alterations due to the variation of skin water content may affect the diagnostic accuracy for skin cancers. In practical Raman experiments, it is suggested that water content for a given skin sample should be kept constant and controlled carefully to minimize errors and deviations in the Raman peak analyses.
Fig. 6. The ratio of Raman peak intensities between the CH\textsubscript{2} deformation and Amide I in proteins (I\textsubscript{1450 cm\textsuperscript{-1}} / I\textsubscript{1660 cm\textsuperscript{-1}}) in different water content samples. The error bars is for standard deviation. The p-value calculated using the ANOVA of mean differences is less than 0.001.

4. Conclusion

In this study, we presented that the Raman spectrum and its peak intensity ratio changed significantly as the water content of porcine skin varied in the range between 40 and 55 wt.%. More specifically, as the water content increased, the spreading of the backscattered light over a larger angular range, rather than the increase in its intensity, became the dominant factor in the variation observed in the intensity of the Raman spectra. Since the spectral alterations of the Raman scattering signals are critical for disease diagnosis, our experimental and numerical results implies that the water content dependency of the skin Raman spectrum should be considered for an accurate diagnosis of skin diseases.

Funding

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean Government (MSIP) (2015R1A5A1037656 and 2015M2A2A7A03043177).