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In vivo measurement of the water content in the dermis by confocal Raman spectroscopy

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Background/purpose: Dermal water plays an important role in the physical properties of the skin. Recently, researchers have attempted to directly measure the dermal water content *in vivo* using magnetic resonance imaging, near infrared spectroscopy, and Raman spectroscopy. However, these methods have limitations. Although confocal Raman spectroscopy has been developed to measure the water content in the skin, no reports have suggested that this instrument can measure the dermal water content. This report describes a method for measuring the dermal water content *in vivo* using confocal Raman spectroscopy.

Methods: We used a confocal Raman spectrometer and adjusted the laser exposure time and depth increments according to the skin depth. Age-related changes in the dermal water content of the forearm were examined in 30 young and 30 elderly male subjects. Diurnal changes in the dermal water content of the forearm were examined in 12 elderly male subjects.

Results: Adjusting the exposure time and depth increment dramatically improved the signal-to-noise ratios of the Raman spectra. Elderly dermis had significantly higher water content than young dermis. Moreover, the dermal water content displayed a diurnal change.

Conclusion: This study demonstrates that the dermal water content can be measured *in vivo* using confocal Raman spectroscopy.

Key words: water content – dermis – confocal Raman spectroscopy – *in vivo*

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WATER PLAYS important roles in maintaining the condition of the skin. In the stratum corneum, water interacts with a natural moisturizing factor and keratin to give elasticity to the stratum corneum (1). In the dermis, the dermal fluid correlates with the skin elasticity (2). In addition, the elderly have higher dermal water content than the young (3–5). These findings suggest that a non-invasive method to monitor the water content in the skin would be very helpful for understanding the roles that water plays in the skin.

Many studies have directly measured the water content in the skin *in vivo*. Magnetic resonance imaging (MRI) is useful for mapping the water distribution. MRI can detect the protons in the water, so proton density and other parameters have been used to measure the water content (6, 7), T2 (8, 9), and MT activity (10, 11). MRI has a depth resolution of about 50–100 μm (6–11). Although MRI directly measures the water content in the skin, the measurement parameters are relative values. To directly measure the water content in the skin, spectroscopic measurements are also performed.

Attenuated total reflectance infrared spectroscopy (ATR-IR) detects OH vibrations in water, therefore the water content in the skin can be determined from the absorbance of the OH band, such as 2100 cm^{-1} (12) or 3300 cm^{-1} (13, 14). However, ATR-IR can only measure the superficial water content in the skin.

Near infrared spectroscopy (NIR) also detects OH vibrations in water, therefore the water content in the skin can be determined from the absorbance of the OH band, such as 1450 nm (15) or 1900 nm (16, 17). The water content also can be estimated using a partial least square regression based on the NIR spectra (18, 19).

Raman spectroscopy also detects OH vibrations in water, hence the water content can be determined from the absorbance of the OH band, such as 3250 cm^{-1} (20). However, the water content at an arbitrary skin depth is difficult to measure using NIR and Raman spectroscopy.

Recently, Caspers et al. (21) developed confocal Raman spectroscopy for evaluating the water content and the natural moisturizing factors of the skin. Confocal Raman spectroscopy can directly measure the water content from the skin surface down to the upper epidermis (22, 23) with high depth resolution (5 μm). Moreover, it produces a more absolute measurement value than other methods. Using confocal Raman spectroscopy, the water content is calculated from the ratio of integrated Raman signals for water and protein. The proportionality constant is estimated from the Raman spectra of various solutions of proteins in the water (21). However, no *in vivo* study has used confocal Raman spectroscopy to evaluate the dermal water content. Theoretically, the dermal water content can be measured using confocal Raman spectroscopy. The object of this report is to measure the dermal water content *in vivo* using confocal Raman spectroscopy.

Methods

Instrumentation

Raman spectra depth profiles from the skin surface to the dermis were obtained using confocal Raman spectrometer (Model 3510; River Diagnostics BV, Rotterdam, the Netherlands). The spectra in the region 2600–4000 cm^{-1} were obtained using a 671 nm laser. The water content (mass%) was calculated from the intensity ratio of the Raman bands of water (3350–3550 cm^{-1}) and protein (2910–2965 cm^{-1}) (21). The water content at depth increments between the measured points were interpolated using the software Model 3510 (Skin tool box; River Diagnostics BV).

Subjects

For this study, we designed three experiments to determine: (1) the laser exposure time, (2) age-related changes in the water content in the forearm skin, and (3) diurnal changes in the water content in the forearm skin. One healthy Japanese male subject was enrolled for the experiment to determine the laser exposure time. For the age-related changes experiment, 60 healthy Japanese male subjects were divided into two groups: a young group consisting of 30 subjects 20–24 years of age (mean age: 21) and an elderly group consisting of 30 subjects 60–68 years of age (mean age: 64). For the diurnal change experiment, 12 healthy Japanese male subjects 60–68 years of age (mean age: 64) were enrolled. Informed consent was obtained from all subjects.

Measurements

The experiments in this report were approved by the ethics committee at Kanebo cosmetics. All measurements were conducted after the subject had rested for 20 min in the measurement room, and the measurement sites were not washed. While the measurements were being taken, the temperature was maintained at 22 °C and the relative humidity at 50%.

(1) Determining the laser exposure time:
Several Raman spectra were obtained from the same region of the forearm.
(2) Age-related changes in the water content in the forearm skin:
Three Raman spectra were obtained from the forearm, and the water content for each depth point was averaged.
(3) Diurnal changes in the water content in forearm skin:
Measurements were taken twice a day between 08:30 and 10:30 hours in the morning and 04:30 and 6:30 hours in the afternoon. Three Raman spectra of the forearm were obtained and the water content for each depth point was averaged.

Statistical analysis

The dermal water content at the depths between 70 and 196 μm (endpoint of measurement) were averaged for statistical analysis because the dermis was about 70 μm below the skin surface (24–26). Statistical analysis was performed using EXSAS 7.5.2.2 (Arm Systex, Osaka, Japan), which is based on SAS (SAS Institute Japan, Tokyo, Japan). The two age groups were compared using the two-tailed Student's t-test. The paired t-test was used for analyzing diurnal changes. A probability of $P < 0.05$ was considered significant.

Results

Determining the laser exposure time

The confocal Raman spectrometer (Model 3510; River Diagnostics BV) can obtain Raman spectra from the skin surface to the upper epidermis using a 1-s laser exposure time (Fig. 1a). However, Raman spectra of the deeper layers of the skin, such as the dermis, cannot be obtained using a 1-s laser exposure time because the deeper the measurement depth, the weaker the Raman scattering. Therefore, to measure the water content in the

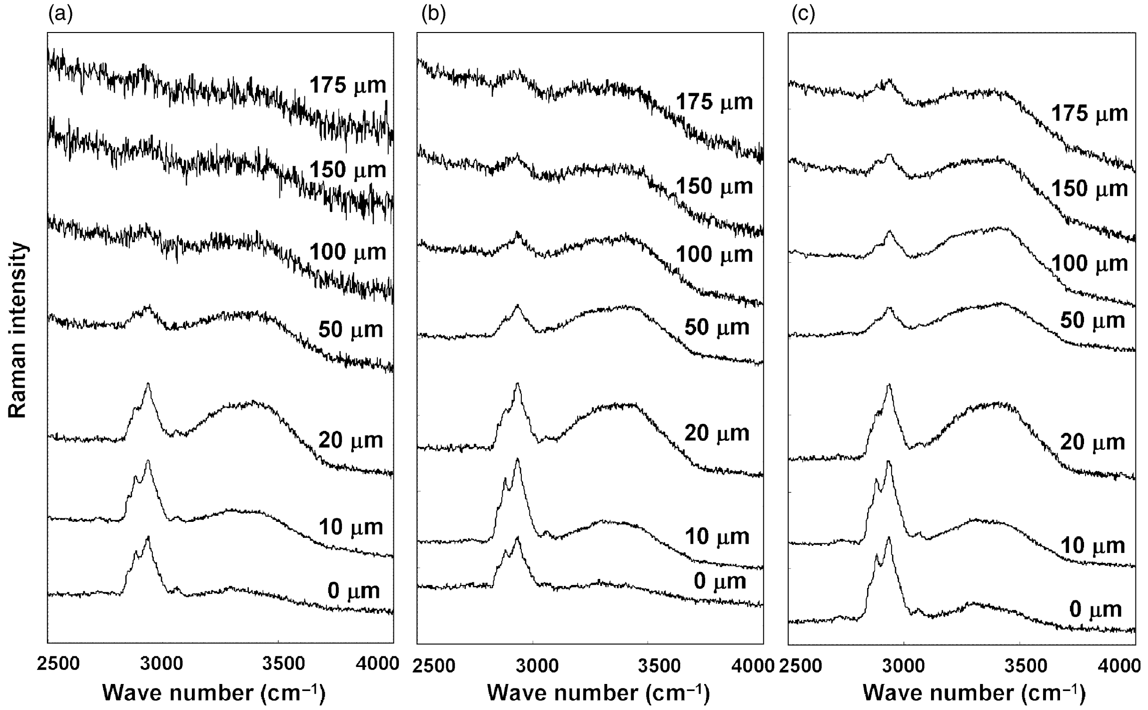

Fig. 1. Raman spectra were improved by adjusting the laser exposure time. (a) All spectra were obtained using 1-s exposures. (b) The spectra were obtained as follows: from the skin surface to 40 mm: exposure time of 1 s; below 40 mm: exposure time of 5 s. (c) The spectra were obtained as follows: from the skin surface to 40 mm: exposure time of 1 s; from 40 to 100 mm: exposure time of 5 s; below 100 mm: exposure time of 30 s.

dermis, the laser exposure time must be adjusted according to the skin depth.

At an exposure time of 1 s, the Raman spectra signal-to-noise ratio was lower in the deeper portions than at a skin depth of 50 μm (Fig. 1a). Consequently, the laser exposure time was increased from 1 to 5 s for measurements at skin depths below 40 μm. This improved the signal-to-noise ratio at depths between 40 and 100 μm, but the signal-to-noise ratio was still low at depths below 100 μm (Fig. 1b). Finally, the laser exposure time was increased from 5 to 30 s for measurements below 100 μm. These adjustments of the laser exposure time drastically improved the signal-to-noise ratio for all of the Raman spectra (Fig. 1c).

Therefore, for the succeeding experiments, we set the following laser exposure times and depth increments: from the skin surface to 40 μm: 1-s exposure and 2 μm increment; 40–100 μm: 5-s exposure and 5 μm increment; and 100–200 μm: 30-s exposure and 25 μm increment.

Age-related changes in the water content in forearm skin

The profiles for the water content in elderly and young forearm skin differed (Fig. 2a). Elderly forearm skin had a much higher water content than young forearm skin. Moreover, elderly forearm skin had a significantly higher dermal water content (70 μm below the skin surface) than young forearm skin (elderly: 73.3 ± 1.6%; young: 69.3 ± 1.7%; $P < 0.001$; Fig. 2b).

Diurnal changes in the water content in forearm skin

The profiles for the water content in forearm skin differed in the morning and the afternoon (Fig. 3a). The dermal water content was entirely higher in the afternoon than in the morning. The water content in the uppermost dermis (50–100 μm) was particularly higher in the afternoon than in the morning. Moreover, the dermal water content was significantly higher in the afternoon than in the morning (morning: 74.4 ± 1.9%; afternoon: 76.1 ± 2.1%; $P < 0.001$; Fig. 3b).

Discussion

First, we examined whether confocal Raman spectroscopy could measure the water content in the dermis *in vivo*. In this experiment, confocal Raman spectroscopy indicated that the dermal water content was significantly higher in the elderly subjects than in the young subjects. This agrees with reported results for the gravimetric method (3, 4) and *ex vivo* Raman spectro-

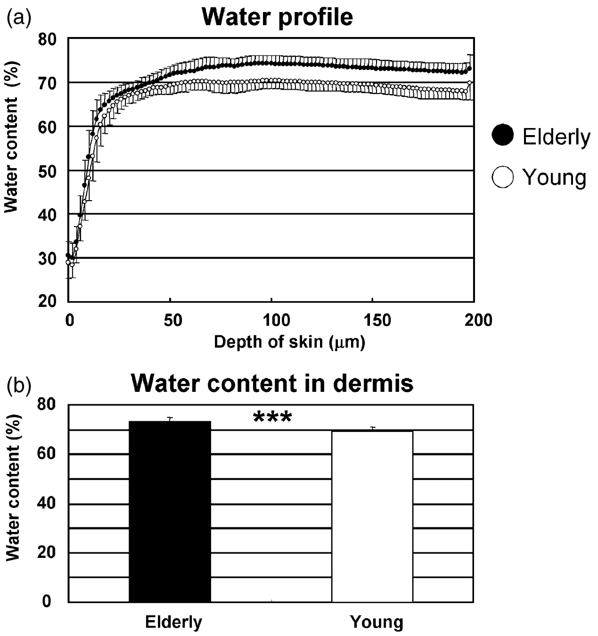

*Fig. 2. Elderly forearm skin had significantly higher dermal water content than young forearm skin. (a) Depth profiles of the water content in elderly and young forearm skin. (b) Comparison of the dermal water content in elderly and young forearm skin. Values are the mean value with standard deviation. A probability of P < 0.05 was significant: ***P < 0.001.*

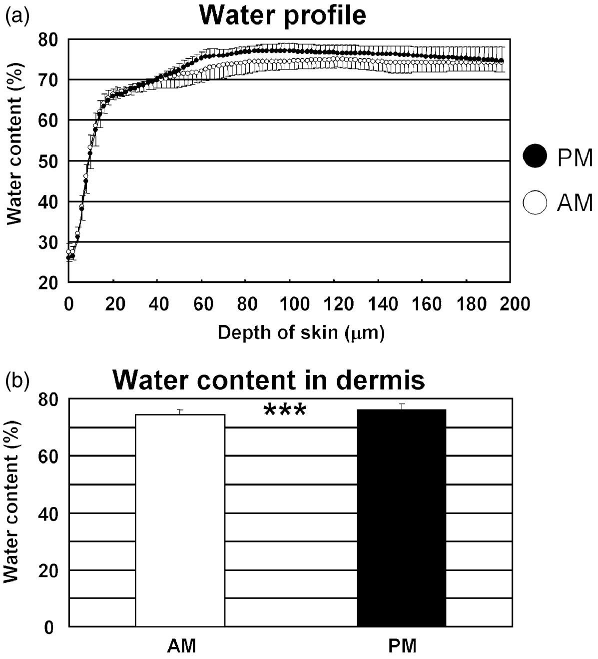

*Fig. 3. The dermal water content in forearm skin was significantly higher in the afternoon than in the morning. (a) Depth profiles of the water content in forearm skin in the morning and in the afternoon. (b) Comparison of the dermal water content in the morning and afternoon. Values are the mean value with standard deviation. A probability of P < 0.05 was significant: ***P < 0.001.*

scopy (5). Therefore, *in vivo* measurement of the dermal water content using confocal Raman spectroscopy was considered highly reliable.

On the other hand, studies using *ex vivo* Raman spectroscopy (5) and MRI (6) demonstrated that the mobile water content in the dermis, which is not bound in the substrates of the skin, was higher in elderly subjects than in young subjects. This suggests that the higher mobile water content may be due to the higher total dermal water content and/or to fewer substrates in the skin, such as collagen and glycosaminoglycans. In the present study, the nature of the water was not determined because we focused on measuring the total dermal water content, and our instrument could not obtain the tetrahedron water cluster band (180 cm^{-1}). In the future, it may be possible to determine the nature of the water in the skin by analyzing the OH band (3100–3800 cm^{-1}), which was used in the present study.

Gniadecka et al. (27) used high-frequency ultrasound to show that the echogenicity (the number of low echogenic pixels in an image) of forearm skin after rising from bed gradually decreases during the day. The echogenicity detected by high-frequency ultrasound correlated with the dermal water content ($r = 0.47$) measured by MRI (28). Although these results suggest that the dermal water content may change diurnally, the diurnal change in the dermal water content cannot be confirmed using high-frequency ultrasound. We used confocal Raman spectroscopy to determine whether the dermal water content changed diurnally and found that the water content in the uppermost dermis (70–100 μm) tended to be higher in the afternoon than in the morning and that the dermal water content was significantly higher in the afternoon than in the morning. These results confirm that diurnal changes in the dermal water content can be detected using confocal Raman spectroscopy.

In this study, we used wide depth increments between the measurement points in the dermis in order to obtain rough water profiles of the dermis. Therefore, the depth resolution differed little from that of MRI. The benefit of using confocal Raman spectroscopy is that the depth increment between measurement points can be freely adjusted. The depth resolution of the confocal Raman spectroscopy used in this study was 5 μm. With confocal Raman spectroscopy, the smaller the region of interest, the higher the depth resolution.

In conclusion, this study demonstrates that the dermal water content can be measured *in vivo* using confocal Raman spectroscopy.

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