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AIRsight™ Infrared/Raman Microscope

Application News

Unstained Analysis and Evaluation of Bone Quality Characteristics of Rat Femur Cross Section by AIRsight Infrared/Raman Microscope

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User Benefits

- The distribution of hydroxyapatite and collagen can be confirmed without staining bone samples.
- The optimum analysis technique for high-sensitivity detection of analysis target components can be selected from infrared and Raman spectroscopy.
- The component ratio and degree of maturity of components related to bone quality characteristics can be evaluated by a detailed analysis of the infrared/Raman spectra.

■ Introduction

In prevention of osteoporosis, in addition to bone density, which simply indicates the mass of bone minerals per unit volume of bone tissue, the quality of the bone tissue (bone quality characteristics) is also considered important. Bone tissue consists mainly of hydroxyapatite, an inorganic mineral, and collagen, an organic substance. Bone quality characteristics are determined by evaluating properties such as the ratio of inorganic and organic components (mineral-to-matrix ratio) and the crystallinity of the hydroxyapatite (mineral maturity). Both micro-infrared spectroscopy and micro-Raman spectroscopy can measure the distribution of the chemical components of bone without staining the bone samples, and bone quality characteristics can also be acquired by a detailed spectral analysis. While both methods are based on vibrational spectroscopy, it can be said that micro-infrared spectroscopy and micro-Raman spectroscopy exist in a complementary relationship, as some types of information can only be obtained by one of these methods.

For this article, an unstained analysis of a cross section of a rat thighbone (rat femur) was carried out using a Shimadzu AIRsight infrared/Raman microscope. Since AIRsight realizes both micro-infrared spectroscopy and micro-Raman spectroscopy in a single instrument, various components can be targeted for analysis if AIRsight is used. In addition, it is also possible to acquire bone quality characteristics information by selecting the more optimal analysis method.

■ Infrared/Raman Microscopy Measurement of Rat Femur

Infrared microscopy reflection measurement and Raman microscopy measurement of a cross section of a rat femur were carried out. After extraction of the femur, freeze-sectioning was done in the direction perpendicular to the longitudinal axis using a HistoCore AUTOCUT R microtome (manufactured by Leica Microsystems GmbH). The sectioned specimen can be measured while maintaining the parallelism of the measurement surface by setting the specimen on the sample stage attached to the microtome jig, as shown in Fig. 1. In order to analyze both the inorganic and organic components of the bone, decalcification treatment to remove the hydroxyapatite component to facilitate processing was not applied in this experiment.

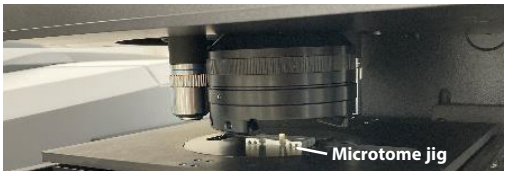

Fig. 1 Condition of Cross-Sectional Observation of Rat Femur

Fig. 2 shows an observation image of cross section of the rat femur. It can be understood that the entire specimen is in focus, in spite of the wide field of view, because the measurement surface and the sample stage are parallel.

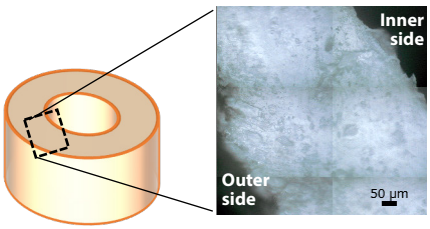

Fig. 2 Observation Image of Cross Section of Rat Femur

Table 1 shows the mapping measurement conditions of the infrared spectroscopy measurement and Raman spectroscopy measurement.

Table 1 Measurement Conditions

Instruments	: IRTracer™-100, AIRsight
Infrared spectroscopy measurement	
Resolution	: 8 cm^{-1}
Accumulation	: 200 times
Apodization function	: SqrTriangle
Aperture size	: 50 µm × 50 µm
Step width	: 50 µm
Mapping region	: 650 µm × 300 µm
Detector	: T2SL
Raman spectroscopy measurement	
Accumulation	: 5 times
Exposure time	: 10.0 s
Objective lens	: 50x
Excitation wavelength	: 785 nm
Laser diameter	: 5 µm
Step width	: 50 µm
Mapping region	: 650 µm × 300 µm
Detector	: CCD

■ Attribution of Femur by Infrared/Raman Spectra

Fig. 3 shows the spectra and the main attributions of the rat femur by infrared spectroscopy (FTIR) and Raman spectroscopy.

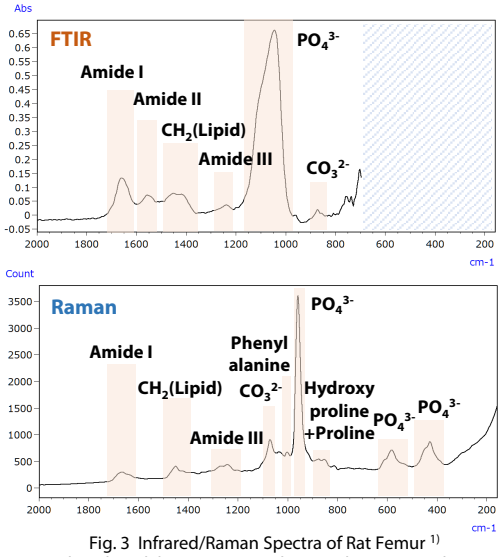

Fig. 3 Infrared/Raman Spectra of Rat Femur [1)]
(Phe: Phenylalanine, Hyp: Hydroxyproline, Pro: Proline)

Since this is an undecalcified specimen, simultaneous confirmation of the peaks originating from both the collagens (Amide I/II/III) and hydroxyapatite (PO_4^{3-}) was possible. Among the conditions observed here, the peaks that could only be confirmed by either the infrared or Raman spectrum were observed, and even the intensities of the same attributions were different, and the peak shapes were also different.

■ Separation and Observation of Distribution of Components by Multivariate Analysis

Multivariate curve resolution (MCR) is a multivariate analysis technique that makes it possible to acquire the spectra (pure spectra) and concentration information for each component from spectra that include multiple components. Multiple pure spectra which vary independently are selected from the spectrum of a mixture containing multiple components, and an optimization calculation is carried out by the alternating least squares method.

• Component distribution analysis Collagen and hydroxyapatite

A Raman mapping measurement of the femur was carried out, after which the collagen and hydroxyapatite in the acquired mapping data were separated by the MCR method, and a chemical image of the relative concentration distribution was prepared. Fig. 4 shows the qualitative analysis results of the spectra of each component, and Fig. 5 shows the chemical images. The colors blue and red show relatively low and high concentrations, respectively.

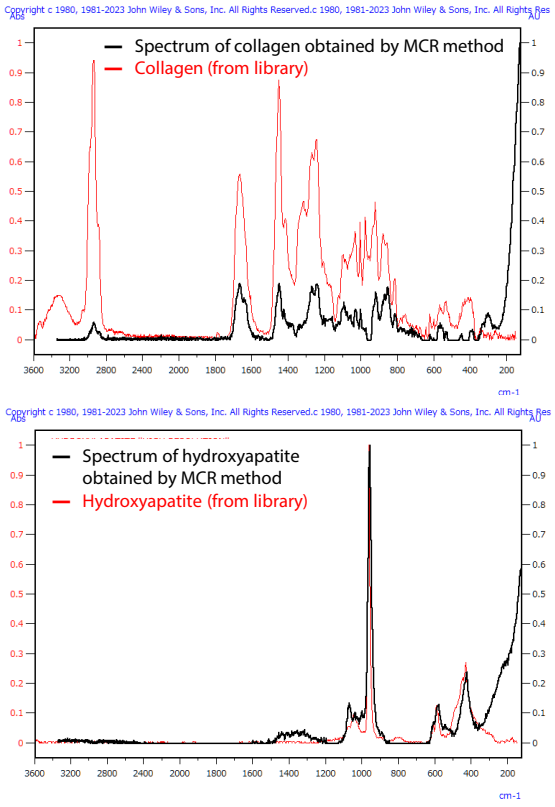

Fig. 4 Results of Search of Component Spectra Separated by MCR Method

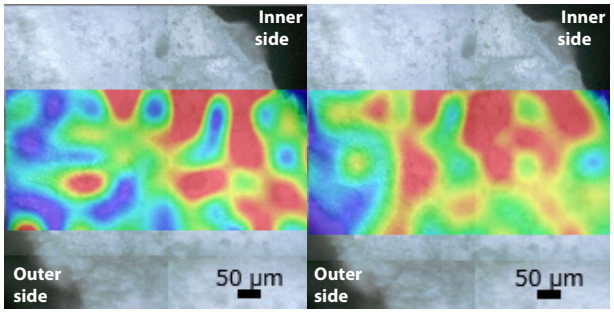

Fig. 5 Chemical Images Prepared by MCR Method
(Left: Collagen, Right: Hydroxyapatite)

From Fig. 4, it was found that collagen and hydroxyapatite can be separated by the MCR method. The chemical images in Fig. 5 revealed that hydroxyapatite is distributed across the entire bone cross section, but in contrast, the distribution of collagen shows a comparatively large content on the inner side of the bone.

■ Distribution of Peaks Originating from Each Component and Bone Quality Characteristics

With both infrared and Raman spectroscopy, variations can sometimes be seen in the intensity of the entire spectrum due to the surface state of the specimen. In this experiment, the relative component distribution was confirmed by using the ratio of the peaks originating from each component. In all the component distributions, blue indicates a relatively low concentration and conversely, red shows a high concentration.

• Bone quality characteristic ① Mineral-to-matrix ratio

The ratio of PO_4^{3-} and amide I originating from the component ratio of hydroxyapatite and collagen is called the Mineral : Matrix ratio, and it has been reported that this ratio increases with the elapsed time of bone formation [2]. Fig. 6 shows the peak area ratio of PO_4^{3-} and amide I of the infrared spectrum. Although a similar peak area ratio can also be obtained from the Raman spectrum, the infrared spectrum data were adopted here because both peaks can be clearly confirmed.

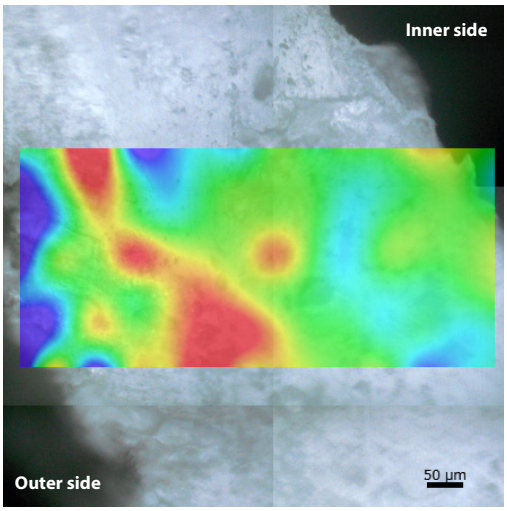

Fig. 6 Peak Area Ratio of Infrared Spectra (PO_4^{3-}/Amide I)

Looking at Fig. 6, the strong distribution in the direction following the contour of the bone cross section near the outer side of the bone indicates that bone formation has proceeded to a greater degree in this part.

• Bone quality characteristic ② Carbonate-to-phosphate ratio

The ratio of CO_3^{2-} (carbonate) to PO_4^{3-} (phosphate) in hydroxyapatite changes the solubility of the bone, and has been reported to affect the metabolism (bone remodeling) of bones [3]. Fig. 7 shows the peak area ratio of CO_3^{2-} and PO_4^{3-} according to the Raman spectrum. The peak originating from CO_3^{2-} can be measured by both infrared spectroscopy and Raman spectroscopy, but here the data from the Raman spectrum were used, as the peaks can be confirmed more clearly.

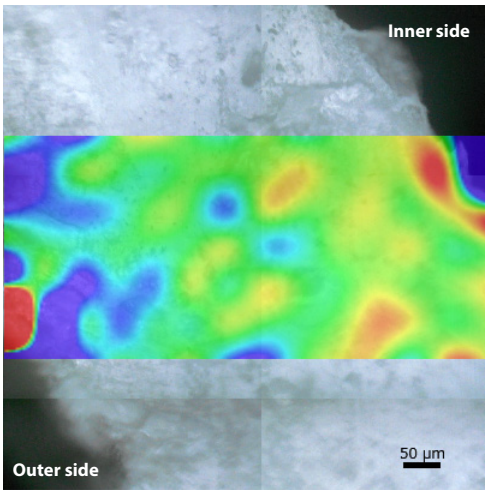

Fig. 7 Peak Area Ratio of Raman Spectrum (CO_3^{2-}/PO_4^{3-})

Since the components are distributed to the edge area in this measurement, a condition in which metabolism (bone remodeling) has been activated in this region could be confirmed.

• **Bone quality characteristic ③ Mineral maturity**

Mineral maturity is an index related to the crystallinity of hydroxyapatite. Although multiple methods for evaluation of mineral maturity are available, the method based on the ratio of the 1030 cm^{-1} and 1110 cm^{-1} peaks in the infrared spectrum of a rat femur was used in this experiment. The peak wavenumber (frequency) of PO_4^{3-} in the infrared spectrum differs depending on the crystal condition, as 1030 cm^{-1} is the peak originating from hydroxyapatite with a defect-free crystal structure, while 1110 cm^{-1} originates from hydroxyapatite with poor crystallinity. The shapes of these peaks overlap in the infrared spectrum, but it is possible to separate the overlapping peaks while maintaining quantitativity by applying secondary derivative processing to the infrared spectrum (Fig. 8).

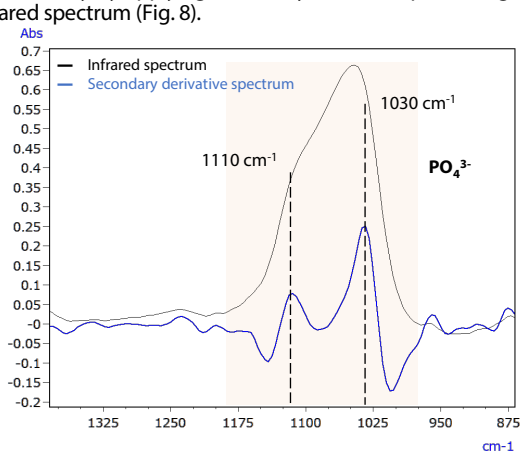

Fig. 8 Infrared Spectrum of Rat Femur and Secondary Derivative Spectrum
(In the secondary derivative spectrum, positive and negative are inverted.)

Fig. 9 shows the peak height ratio of the secondary derivative spectrum at each measurement point of the infrared mapping. Although positions with high mineral maturity are scattered throughout the bone cross section, these points are distributed in a belt-line form slightly closer to the center than the contour line of the bone.

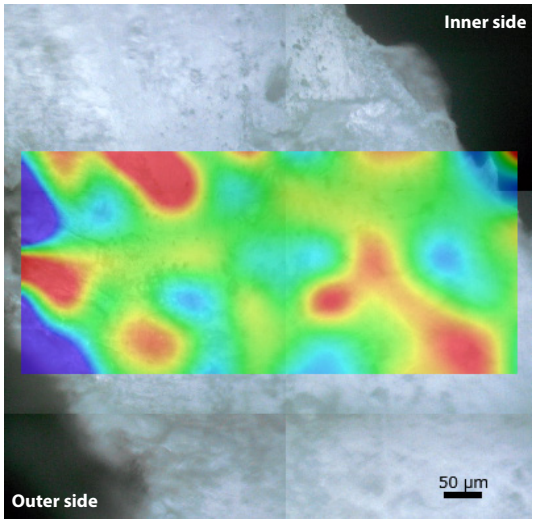

Fig. 9 Peak Ratio (1030 cm^{-1}/1110 cm^{-1}) of
Secondary Derivative Spectrum of Infrared Spectrum

• **Bone quality characteristic ④ Component ratio of phenylalanine and hydroxyapatite**

In some cases, peaks cannot be confirmed by infrared spectroscopy (i.e., infrared inactive components), but components of this type can be measured by Raman spectroscopy. Examples include the amino acids phenylalanine, hydroxyproline, and proline in Fig. 3.

In this experiment, the component ratio of phenylalanine and hydroxyapatite was evaluated by using the Raman mapping data of the femur specimen. Fig. 10 shows the chemical image of the relative ratio of phenylalanine and PO_4^{3-} obtained from the Raman spectrum. Although it was found that phenylalanine is strongly distributed to the outer side of the bone, this is considered to be an effect of the periosteum (bone membrane covering).

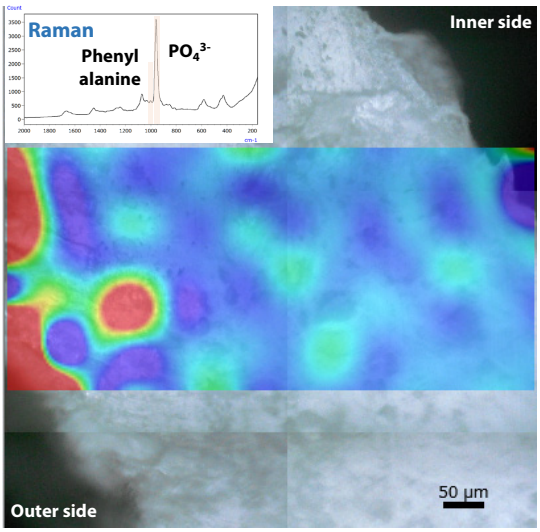

Fig. 10 Peak Area Ratio by Raman Spectrum
(Phenylalanine/PO_4^{3-})

■ Conclusion

An unstained analysis of the cross section of a rat femur and an evaluation of the distribution of various indices related to bone quality characteristics were carried out using the AIRsight infrared/Raman microscope. Since AIRsight makes it possible to conduct analyses by both infrared spectroscopy and Raman spectroscopy with a single instrument, a larger number of components can be analyzed simultaneously without removing the specimen from the sample stage. In addition, the component spectra of multiple components and chemical images of their concentration distributions could be acquired from the mixture spectrum by using the multivariate analysis method. It was also possible to evaluate crystallinity by secondary derivative processing of superimposed peaks. It may be noted that all of the analysis processing mentioned in this article can also be carried out with the dedicated software AMsolution for infrared/Raman microscopes.

<Acknowledgement>

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