THE EVALUATION OF PREFERENTIAL ALIGNMENT OF BIOLOGICAL APATITE (BAp) CRYSTALLITES IN BONE USING A TRANSMISSION X-RAY DIFFRACTION METHOD

Katsunari Sasaki, Takayoshi Nakano, Yukichi Umakoshi, Joseph D. Ferrara, and Toshihiko Sasaki

1Division of Innovative Technology and Science, Graduate School of Natural Science and Technology, Kanazawa University, Kanazawa 920-1192, Japan
2Division of Materials and Manufacturing Science, Graduate School of Engineering, Osaka University, Suita 565-0871, Japan
3Rigaku Americas Corporation, 9009 New Trails Drive, The Woodlands, Texas 77381-5209

ABSTRACT

A two-dimensional quantitative XRD measurement of the orientation of biological apatite (BAp) in an isolated trabecula of a mouse ulna was made using a transmission mode X-ray diffraction system with a 0.3 mm diameter parallel beam MoKα source. The same measurement was done with CuKα radiation. Copper Kα is the most commonly used radiation for powder diffraction because it provides good resolution of the resultant lines but is more cumbersome because it requires slicing the bone into thin sections since CuKα radiation is not very penetrating. The preferred orientation of the BAp c-axis from the orecranon to the caput ulnae was measured for 12-week mutant osteopetrotic (op/op) and littermate mice. It was found that the transmission optical method using MoKα radiation provides a simple and non-destructive method for measuring bone quality.

INTRODUCTION

X-ray diffraction is a well known method for the analysis of materials. The method is used to measure properties such as lattice parameters, crystallite, preferred orientation, residual stress, and pole figures. Structural materials such as steel, aluminum, and plastics are often strengthened by modification of the crystal properties of the material. For example, the materials may be processed to enhance preferred orientation or residual stress. X-ray diffraction techniques have changed dramatically since the development of two-dimensional detectors such as the image plate (IP), multiwire proportional counter (MWPC), and charge coupled device (CCD) [1]. The use of these detectors provides two-dimensional information and reduces measurement times [2]. Bone is usually evaluated using bone mineral density (BMD), but recent research suggests that BMD is not a sufficient predictor of whole bone mechanical properties [3,4]. Nakano et al. have measured preferred orientation of the BAp crystallites by X-ray diffraction techniques because the preferred alignment of the BAp c-axis in various bones varies depending on the shape, stress distribution in vivo, and the related mechanical function [5]. The analysis of the BAp orientation also explains the behavior of bone regeneration by the tissue engineering technique since the change in the BAp orientation does not agree with that in BMD during the regenerative process [6,7]. CuKα (λ = 1.5414 Å) radiation was used for all these X-ray measurements. The absorption of CuKα radiation by BAp is quite large. In these experiments only information about the surface of the bone was observed [8]. Thus, the use of CuKα is not practical for medical in vivo applications or even routine ex vivo applications.
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The purpose of this study is to show the advantage of the use of higher energy characteristic X-ray MoKα radiation (λ = 0.7107 Å) and transmission mode X-ray diffraction. The use of higher energy radiation reduces the absorption and improves the signal-to-noise ratio, and the transmission method means the bone may be analyzed intact rather than sectioned. We investigated the areal distribution of the BAp c-axis on a cross-section of the ulna bone of 12-week osteopetrotic (op/op) mice. Osteoclasts rarely appear because of a defect in the expression of the macrophage colony-stimulating factor and littermate mouse using a transmission X-ray diffraction method.

MATERIALS AND METHODS

Apatite sample and bone specimens

To perform the absorption experiment, NIST calcium hydroxyapatite powder (SRM 2910) was placed into a 1.5 mm diameter glass capillary. Two bone samples, op and control (normal), ulnae of 12-week-old mice were fixed and kept in 10% formalin neutral buffered solution 6 months to avoid degradation. Figure 1 shows a photograph of the bones used for the experiment. The sample op/op is from a mutant osteopetrotic mouse. The sample control is from a normal mouse. The bone length of op/op was 14.6 mm and the control sample was 16.9 mm.

X-ray micro CT

The internal structure of each bone and its density was verified with a Rigaku R_mCT, a micro X-ray CT scanner specially designed for small animal imaging. The operated X-ray power was 90 kV, 88 μA with an exposure time of 17 s.

X-ray diffraction analysis

All X-ray diffraction experiments were performed using a Rigaku R-AXIS RAPID II system. This is a two-pinhole transmission, two-dimensional camera system with cylindrical detection geometry. The RAPID II employs an image plate (storage phosphor) developed by Fuji Film as the X-ray detector. The geometry of the apparatus resembles a vertical-screenless Weissenberg camera and was mainly designed for single crystal X-ray structure analyses and charge density studies. Figure 2 shows a schematic drawing of the R-AXIS RAPID II X-ray optics. The detector is 460 mm in the horizontal direction and 256 mm in the vertical direction with a radius of 127.4 mm. This provides a 2θ range from -60° to +144° in the horizontal direction and a 2θ range of ±45° in the vertical direction. The pixel size is 100 μm. A Rigaku ultraX rotating anode X-ray generator with either a Cu (λ = 1.5418 Å) or Mo (0.7107 Å) target and a graphite monochromator was used in the study. A 0.3 mm double pinhole collimator was used to spatially condition the X-ray beam. The X-ray generator was operated at 50 kV and 90 mA. Both CuKα and MoKα radiation were used to study the X-ray absorption characteristics of the NIST calcium hydroxyapatite reference sample, but only MoKα radiation was used to study the bone samples. The exposure time for the NIST apatite reference sample was 10 min. The bone samples were exposed for 1 min.

The samples were oriented on a 3-circle Eulerian goniometer with a manual XYZ stage. The Eulerian angles were ω, χ, and φ. The φ-axis was closest to the sample and provided for complete rotation of the sample around that axis. The χ-axis was next and used to tilt the sample as shown in Figure 2. In general, the ω-axis is used as the scanning axis. However, in this study the sample was fixed for each exposure. The XYZ stage was used to select the desired position on the bone sample for study. The distribution of the X-ray absorption of the bone samples were measured using a
Rigaku X-ray scintillation counter operating in integrating mode. The bone sample was placed in the beam and the X-ray intensity of the transmitted beam was measured as function of the X direction. To measure along Anterior-Posterior direction, the $\varphi$-axis was rotated 90°.

X-ray diffraction data were recorded using the Rigaku RAPID Auto software. The data were processed as follows. For each bone sample, 10 measurements were taken three times. First 10 mm $\times$ 45° segments in both the equatorial and meridional directions of the images (Figure 3) were extracted and converted to $2\theta$ versus $I$ profiles. Next the (002) and (310) reflections were identified and the integrated intensity was calculated.
RESULTS AND DISCUSSION

X-ray micro CT

The results of the CT measurements are shown in Figure 4. The op/op bone sample shows a spongier structure with thinner walls for the same diameter bone as compared to the control (normal) indicating presence of disease in the op/op sample.

Absorption

X-ray absorption is described by the equation

$$I = I_0 e^{-\mu t}$$  \hspace{1cm} (1)

where $I$ is the intensity of the transmitted X-ray beam in counts per second, $I_0$ is the intensity of the incident X-ray beam in counts per second, $\mu$ is the absorption coefficient in cm$^{-1}$, and $t$ is the thickness of the sample in cm. In general, X-ray absorption decreases with decreasing wavelength. Assuming a nominal density of 3.19 g/cm$^2$, $\mu = 159.09$ cm$^{-1}$ for CuK$\alpha$ and $\mu = 19.12$ cm$^{-1}$ for MoK$\alpha$ calcium hydroxyapatite. Substituting these values into Eq. (1) with a thickness of 0.05 cm for the bone sample indicates 99.96% of the Cu X-rays would be absorbed but only 38.4% for the Mo X-rays. This suggests that MoK$\alpha$ radiation is a much better choice for this experiment.

An absorption profile of the two bones is shown in Figure 5. The absorption profile shows that the peak transmission is about 40% to 20%, which is well within the range of reasonable values for transmission diffractometry. The curves show higher bone density at the edges for the control sample and higher density in the center for the op/op sample, consistent with the micro CT described above.

Figure 4. Results of X-ray micro CT (left: op/op mouse; right: control mouse).

Figure 5. Distribution of X-ray absorption of MoK$\alpha$ radiation (upper: Lateral to Medial direction; lower: Anterior to Posterior direction).
Angular resolution and diffraction plane

The disadvantage of Mo\(K\alpha\) radiation is the reduced angular resolution of the reflections. Figure 6 shows a typical Mo\(K\alpha\) diffraction pattern of the NIST calcium hydroxyapatite. The present work compares the ratio of the integrated intensities of the (002) and (310) reflections in BAp. Figure 6 also displays the region \(10^\circ \leq 2\theta \leq 20^\circ\) and shows these reflections which are well resolved with Mo\(K\alpha\) radiation.

It is known that the crystallites in BAp orient preferentially and that the geometry that optimizes the ratio of the (002) and (310) reflection requires a tilt of the bone axis of 6\(^\circ\), the \(2\theta\) angle of the (002) reflection for Mo\(K\alpha\) radiation, but the preferred orientation of the \(c\)-axis to the longitudinal axis of the bone is very broad. This suggests that careful orientation of the bone is not necessary to get meaningful results. The longitudinal axis of the bone simply needs to be within a few degrees of being aligned to the vertical axis.

Measurement of distribution of bone quality

Finally, we measured a series of 10 diffraction patterns at equidistant points along the longitudinal axis of the bone samples. The measurement points are shown in Figure 1. The observed images at point numbers 1, 3, 5, 7, and 9 for both op/op and control bones are shown in Figure 7. Each diffraction pattern was analyzed as described earlier and the resultant (002)/(310) ratios are plotted in Figure 8 with standard deviations. Crystallites with the \(c\)-axis oriented horizontally contribute to the equatorial sections of the diffraction pattern, and crystallites with the \(c\)-axis oriented vertically contribute to the meridional sections of the diffraction pattern. The meridional regions of the diffraction show a strong dependence to the distribution of BAp within the bone. Furthermore, the data show a weak dependence of the equatorial regions with the distribution of BAp in the bone. Both the control and op/op bone samples display a maximum (002)/(310) ratio near the center of the bone. For the control sample, the ratio of (002)/(310) is 14.0, almost the same as observed by Nakano et al. [5], at the maximum values, corresponding to the center of the bone. The op/op bone sample displays a (002)/(310) ratio value of 5.9 which indicates less preferred orientation and lower bone quality, consistent with the micro CT observations.
CONCLUSION

We have shown that MoKα radiation is a better choice for data collection for the determination of preferred orientation of BAp in bone samples because of reduced absorption. We have also shown that the reduced angular resolution is inconsequential because the (002) and (310) reflections are adequately resolved with MoKα radiation. An important aspect of this work is the demonstration that it is not necessary to tilt the bone by the Bragg angle of the (002) reflection to correct for the pole of BAp. Furthermore, simply orienting the bone vertically (or nearly so) is sufficient to collect meaningful data. Finally, we have shown that there is a clear difference in the (002)/(310) ratio depending on the quality of the bone being measured.

Figure 7. X-ray diffraction patterns for sample position numbers 1, 3, 5, 7, and 9.

Figure 8. Distribution of the (002)/(310) intensity ratio distribution along the longitudinal direction.

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We have shown that MoKα radiation is a better choice for data collection for the determination of preferred orientation of BAp in bone samples because of reduced absorption. We have also shown that the reduced angular resolution is inconsequential because the (002) and (310) reflections are adequately resolved with MoKα radiation. An important aspect of this work is the demonstration that it is not necessary to tilt the bone by the Bragg angle of the (002) reflection to correct for the pole of BAp. Furthermore, simply orienting the bone vertically (or nearly so) is sufficient to collect meaningful data. Finally, we have shown that there is a clear difference in the (002)/(310) ratio depending on the quality of the bone being measured.
The method we propose has several advantages. First, it is not necessary to orient the bone to better than a few degrees to get meaningful results. Second, it is possible to obtain $c$-axis distribution along the longitudinal direction of the bone without sectioning the bone. Finally, this work has shown it is possible to measure the $c$-axis distribution easily with little sample preparation. This method can be used to study mechanism of bone growth through the $c$-axis distribution.

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