Anisotropic aspects of solubility behavior in the demineralization of cortical bone revealed by XRD analysis

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Abstract

Dissolution of cortical bone mineral under demineralization in 0.1 M HCl and 0.1 M EDTA solutions is studied by X-ray diffraction (XRD). The bone specimens (in the form of planar oriented pieces) were cut from a diaphysial fragment of a mature mammal bone so that a cross-section surface and a longitudinal section surface could be analyzed individually. This permitted to compare the dissolution behavior of bone apatite of different morphologies: crystals having the *c*-axis of the hexagonal unit-cell generally parallel to the long axis of the bone (major morphology) and those having the *c*-axis almost perpendicular to the bone axis (minor morphology). For these two types of morphology, the crystallite sizes in two mutually perpendicular directions (namely, [002] and [310]) were estimated by Scherrer formula in the initial and the stepwise-demineralized specimens. The data obtained reveal that the crystals belonging to the minor morphology dissolve faster than the crystals of the major morphological type, despite the fact that the crystallites of the minor morphology seem to be only a little smaller than those of the major morphology; the apatite crystallites irrespective of the morphology type are elongated in the *c*-axis direction. We hypothesize that the revealed difference in solubility may be caused by diverse chemical modifications of apatite of these two morphological types, since the solubility of apatite is strictly regulated by anionic and cationic substitutions in the lattice. The anisotropy effect in solubility of bone mineral seems to be functionally predetermined and this should be a crucial factor in the resorption and remodeling behavior of a bone. Some challenges arising at XRD examination of partially decalcified cortical bone blocks are discussed, as well as the limitations of estimation of bone crystallite size by XRD line-broadening analysis.

Keywords Apatite · X-ray diffraction · Cortical bone · Crystal orientation · Morphology · Demineralization · Crystallite size

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1 Introduction

Bone mineral has an apatite-like structure with hexagonal symmetry [1–3]. Crystals, as deduced by direct [4, 5] and indirect methods [6–9], can be rod-shaped, prism-shaped, or elongated platelets with dimensions of 20 to 50 nm along the *c*-axis perpendicular to the basal plane of the hexagonal unit cell. As stated by many authors [10–15], bioapatite crystals in a bone possess not only shape anisotropy but also a natural texture. In a compact bone (diaphysis of long bones), the *c*-axis of crystals is mostly parallel to the bone long axis. Furthermore, there is evidence [8, 10, 14, 15] that there are at least two types of apatite morphology in cortical bone: one (main part) with the *c*-axis almost parallel to the long axis of the bone and the other (small part) in which the *c*-axis is oriented almost perpendicular to the bone long axis (Fig. 1). The dual-oriented nature or two-morphology model of bone mineral particles has been described based on data of X-ray pole figure analysis [15]. This paper also reported the estimated major-to-minor morphology ratio to be about 7:3.

Several authors have shown that bioapatite crystal lengths determined microscopically are longer than those obtained by X-ray diffraction (XRD). This can be attributed to the fact that ‘crystallite size’ is not synonymous with ‘particle size’, while XRD is sensitive to the crystallite size [16]. A mineral particle of bone apatite might consist of one or more than one crystallites or coherent domains. Usually, XRD data provide the average values and provide no information regarding the size distribution of the crystallites in a sample [7, 8]. However, it is stated that normal sound bone has a mixture of small (recently formed) crystals and larger, more mature crystals [17]. Moreover, there is experimental evidence about certain optimal (rather broad) size distribution in a healthy bone. It appears that both increase and decrease in mean crystal size affect the mechanical properties of the bone and are usually caused by pathology [17].

Demineralization of cortical bone has been the subject of many studies [18–24]. Partial demineralization is extensively used in cortical bone grafts for enhancement of their osteoinductive properties [23]. Moreover, in vitro demineralization of biological hard tissues attracts interest because in a living organism the bone mineral dissolves during lifetime (normal physiological remodeling). Bone resorption is usually carried out by osteoclasts; this active cellular process runs due to release of carbonic acid [25]. If the rate of bone resorption

Fig. 1 Schematic view (not to scale) of bioapatite crystals of different morphologies in a bone diaphysal fragment. A: crystal of the major morphology and corresponding unit cell orientation, B and D: crystals of the minor morphology and corresponding unit cell orientations; C: crystal axis perpendicular to the (002) plane; E: collagen fibrils (prevalent orientation)

exceeds the rate of new bone formation, the loss of bone mass is occurring accompanied by weakening and fragility of bone. Generally, it takes place due to pathology or diseases (e.g., osteoporosis) or under such negative external factors as prolonged weightlessness and immobilization of a biological object.

Unfortunately, up till now, there have only been a few studies devoted to in vitro methods to decrease mineral content of bone using decalcifying chemical agents that alter the bone mineral density and some biomechanical properties to mimic osteoporosis in humans [26, 27]. Removal of the mineral from bone can be accomplished using a variety of chemical agents that chelate (ethylenediamine tetraacetic acid, EDTA) or solubilize (hydrochloric acid, HCl) the mineral phase [18, 24, 28].

A few studies have applied XRD to evaluate the effects of acid concentration and immersion time on the demineralization of cortical bone [19, 21, 22]. The publications state that the decrease of the apatite's XRD peaks intensity and the broadening of their width is caused by crystal lattice degradation and the removal of the mineral from the bone matrix.

The objective of our study is to observe the evolution of bone apatite structural parameters under in vitro demineralization in HCl and EDTA solutions taking into account the effects of preferred orientation and ordered arrangement of the bioapatite crystals in cortical bone in accordance with the two-morphology model. For this purpose, we use oriented bone specimens (planar pieces) cut out from a diaphysal fragment of a mature mammal bone so that a cross-section surface and a longitudinal section surface can be analyzed individually (Fig. 1). One of the main obstacles for XRD analysis of partially demineralized planar bone specimens is the formation of the so-called 'advancing reaction front' separating the demineralized portion from the still mineralized part of cortical bone [23]. In our study, we consider some limitations arising in XRD examination of partially decalcified cortical bone blocks.

2 Materials and methods

2.1 Sample preparation

Bone specimens were prepared from the femur of a mature cow, which was obtained from a local butcher immediately after slaughter. The diaphysis fragment of the bone (about 7 cm length and 5 cm diameter) was boiled in distilled water, mechanically cleaned and dried in air. The specimens were cut from nearby locations in order to minimize variations in mineral content and characteristics. In preparing the tissue for cutting, the periosteum and endosteum were removed to obtain a bone fragment that may be characterized as cortical bone. The planar oriented specimens (ca. $15 \times 10 \times 3$ mm) were cut from the diaphysis so that surfaces to be analyzed were (a) perpendicular to the longer bone axis (cross-sectioned specimens) and (b) parallel to the longer bone axis (longitudinally sectioned specimens). Observable surfaces of the specimens were smoothed using sandpaper under constant water irrigation.

2.2 Characterization of bone microstructure

For anatomical characterization of the investigated bone fragment, we performed scanning electron microscopy (SEM) examination using an electron microscope REMMA102 (SELMi, Ukraine). This examination showed that the specimen could be characterized as osteonal bone (Fig. 2).

Fig. 2 Microstructure of the bone specimen (the examined surface is a cross-section perpendicular to bone long axis). 1 - Haversian systems; 2 - intermediate lamellae; 3 - cementing substance surrounding Haversian systems (consisting of mineralized matrix with few collagen fibers); 4 - Haversian system in reconstruction stage; 5 - interstitial system

2.3 Demineralization process

The chemical agents were 0.1 M HCl and 0.1 M EDTA solutions. Demineralization was performed at room temperature in 2 l solutions under constant stirring, following the procedure described in [21]. The solution-to-sample volume ratio was almost 500 in order to avoid saturation, which can affect the demineralization process. At pre-determined immersion times, the specimens were withdrawn from the corresponding bath, washed in distilled water and dried in air. X-ray diffraction spectra were recorded for a succession of several demineralization stages of the same specimen.

2.4 Structural characterization of bone mineral

X-ray diffraction examination was carried out using a Siemens diffractometer D 5000 with Ni-filtered Cu-K α radiation and conventional Bragg-Brentano geometry [29]. The current and voltage on the X-ray tube were 30 mA and 40 kV, respectively. The receiving slit size was 0.1 mm. The irradiated area on the specimen surface was about $3 \times 14 \text{ mm}^2$. A micrometer screw was used to carefully mount the specimens so that their flat surfaces could meet tangentially with the focusing circle of the diffractometer. The mounting accuracy was about 10 μm . The specimens were scanned in continuous mode over a range of 2θ angles (θ being the Bragg angle) from 10° to 60° to obtain a general diffraction pattern and then in stepwise mode (step size of 0.005° held at 1.0 s/step) over appropriate small ranges of 2θ angles to have accurate (002) and (310) line shapes. All procedures of the profile processing, i.e., background separation, $K\alpha_2$ -stripping, selection of profile shape function and determination of integral

breadth, were carried out with a commercial X-ray diffraction software calculating errors at each stage. No smoothing of the diffraction line was applied to avoid eventual distortion of the line shape. All diffraction profiles were approximated by the Lorentzian profile function. The total error in peak fitting and calculation of the integrated intensity did not exceed 15%. Crystalline phase identification was performed with system software using JCPDS cards as standards. The crystallite (coherent scattering region) sizes (D) were estimated from the physical broadening of the (002) and (310) lines by applying the Scherrer formula [29, 30]:

$$D = \frac{K \cdot \lambda}{\beta \cdot \cos \theta},$$

where β is the line breadth of the pure diffraction profile resulting from small crystallite sizes, λ is the X-ray wavelength for $\text{CuK}\alpha$ and K is a constant roughly equal to unity and related to crystallite shape (shape factor). Although the Scherrer equation to some extent is limited by the uncertainties in K and β , the equation is widely used for the ease to calculate [9]. Powdered polycrystalline NaCl was used as the reference specimen free of size and distortion broadening. The (200) line width of NaCl after $\text{K}\alpha_2$ -stripping was recalculated for 2θ angles corresponding to hydroxyapatite (002) and (310) lines and was used for correction of instrumental broadening.

3 Results and discussion

The data obtained (Fig. 3) reveal that all peaks of the diffraction patterns of the untreated specimens correspond to the hydroxyapatite lattice (JCPDS 9-432). Also, the natural texture of bone mineral is manifested by prominent differences of XRD patterns of cross-sectioned and longitudinally sectioned bone specimens. It should be mentioned that with Bragg–Brentano geometry the diffraction pattern is formed by crystallographic planes oriented parallel to the examined specimen's surface. Therefore, in accordance with the two-morphology model of bone apatite [15], the ($h00$) or ($hk0$) peaks from longitudinally sectioned specimens and the ($00l$) peaks from cross-sectioned specimens belong to apatite crystals of the major morphology with the c -axis almost parallel to the bone long axis. Similarly, the ($00l$) peaks from longitudinally sectioned specimens and the ($h00$) or ($hk0$) peaks from cross-sectioned specimens are related to apatite crystals of the minor morphology with the c -axis oriented almost perpendicular to the bone long axis. It is important that in the latter case the ($h00$) and ($hk0$) reflections are formed by the total pool of the crystals of the minor morphology independently from their radial orientations (see Fig. 1), while the ($00l$) reflections are formed only by the crystals with the c -axis oriented perpendicular to the surface of the longitudinally sectioned specimen. Therefore, the data from the ($00l$) reflections correspond to only a small fraction of crystals of the minor morphology. This applies to the integrated intensity reflecting the number of crystals (total area of reflecting crystal planes) and, as we believe, does not apply to the line broadening, which correlates with the size of the crystallites. However, as far as we know, the correlation of the longitudinal size of bone apatite crystallites of the minor morphology and radial orientation of their c -axis was never studied.

For the untreated cross-sectioned bone specimen (Fig. 3), the (002) and (004) reflections show anomalously large intensity, confirming that the favored orientation of apatite crystal along the long axis of bone is in the $[00l]$ direction. For the longitudinally sectioned bone

Fig. 3 X-ray diffraction patterns of cross-sectioned and longitudinally sectioned bone specimen after immersion in 0.1 M HCl and EDTA solutions for the specified time. For comparison, diffractograms have been stacked

specimen, the (002) and (004) reflections are very weak, which indicates that the fraction of crystals with the *c*-axis almost perpendicular to the bone axis is much less numerous than the major fraction oriented along the bone long axis. Similarly, the reflections from planes that are perpendicular to the basal plane of the hexagonal cell (namely, (210) and (310)) are stronger for longitudinally sectioned bone specimens and weaker for cross-sectioned bone specimens.

For both (longitudinally and cross-sectioned) bone specimens the (00 *l*) reflections are much sharper than the (*h*00) or (*hk*0) ones. It means that the apatite crystallites are elongated along the *c*-axis for both types of morphology in bone.

It should be mentioned that X-ray line broadening is assumed to be caused not only by the small crystallites size (domain size) but also by lattice strain (within the domain) [7, 8]. A reliable separation of size and strain contributions to broadening requires at least two diffraction lines corresponding to different orders of reflection from the same planes [8, 29]. Unfortunately, the available pair of lines (namely, (002) and (004)) may provide the size and strain parameters only for one crystallographic direction, exactly perpendicular to hexagonal basal plane of apatite [8, 31]. The single-line methods, applied for crystallite size and microstrain determination, yield far less reliable results [7]. Since we intend to compare the alteration of crystallites sizes in at least two dimensions during stepwise demineralization, we need to ignore the contribution of the lattice microstrain to the lines broadening. Alternatively, a rough estimation of bone mineral solubility behavior can be done using the total inverse breadth of a diffraction peak as a size/strain parameter, known as ‘crystallinity’ [7, 32, 33]. Nevertheless, it seems that the size of the crystallites might be a more traditional and acceptable parameter, bearing in mind the mentioned assumption about neglecting the contribution of microstrain. Therefore, we will consider the variations in the (002) line breadth as the variations of the crystallite length, while the (310) line breadth reflects the size in the direction

normal to the crystallite length (width or thickness). Table 1 lists the integral intensity of the (002) and (310) diffraction peaks and crystallite sizes (in two mutually perpendicular directions) for two types of morphology taking into account the above considerations.

According to Table 1, in the initial bone specimen, the crystals of strongly pronounced (major) morphology are only a little bigger than the crystals of the less pronounced (minor) one, despite the fact that the intensity of the lines of the major morphology is much stronger. During demineralization the intensities of XRD peaks decrease and their widths become more broadened demonstrating progressive degradation of the crystal lattice and removal of the mineral from the bone matrix. However, the dissolution rate of crystallites of different morphologies seems to be quite different. The crystals of the minor morphology dissolve faster in both HCl and EDTA solutions (Table 1 and Fig. 4).

It is evident from Table 1 that the rapid decrease of the intensity $I_{(002)}$ of the line, which corresponds to the major morphology, during demineralization is not accompanied by the equivalent decrease of the crystallites size. Moreover, $D_{(002)}$ at first stage of demineralization demonstrates some growing trend. This can be explained by the decrease of content of small crystallites with remarkable imperfection of the lattice because of their faster dissolution. Thus, their contribution to the statistically integrated line intensity is reduced, whereas the average size is increased. Not only is this effect due to the mineral loss but in addition X-ray absorption in the progressively advancing decalcified layer reduces the intensity of diffraction lines with demineralization time [21]. Therefore, the represented values of the line intensity have only indicative meaning and cannot be used as a quantitative parameter of bone mineral loss with the demineralization process.

It should be noted that when bone demineralizes for 48 h in 0.1 M EDTA, the (002) line of the minor morphology completely disappears, while the (310) line is still clearly distinguishable and allows to estimate the values of $I_{(310)}$ and $D_{(310)}$. This is due to the above-mentioned fact that the crystals of the minor morphology may have random orientation of their *c*-axis in the cross-sectional plane of bone fragment (Fig. 1). The (002) line of the longitudinally sectioned bone specimen is formed by only a small part of the crystals possessing appropriate direction of the *c*-axis, whereas the (310) line of the cross-sectioned specimen is formed by all crystals of the minor morphology irrespective of their *c*-axis orientation on the transversal plane of bone. For the same reason, the inverse proportion of the line intensities ($I_{(002)}$ to $I_{(310)}$ ratio) is observed for the minor morphology in comparison with the major morphology.

Table 1 Integral intensities of the (002) and (310) diffraction peaks ($I_{(002)}$ and $I_{(310)}$, arbitrary units) and crystallite sizes ($D_{(002)}$ and $D_{(310)}$, nm) in accordance with the two-morphology model of bone mineral for bone samples demineralized for 6 and 12 h using 0.1 M HCl and for 24 and 48 h using 0.1 M EDTS

		Untreated specimen	6 h, HCl	12 h, HCl	24 h, EDTA	48 h, EDTA
Major morphology	$I_{(002)}$	117.5	58.5	36.3 (A)	82.8	80.1
	$I_{(310)}$	81.8	81.4	66.5 (A)	81.9	78.5
Minor morphology	$I_{(002)}$	25	B	C	~ 20.0 (A)	C
	$I_{(310)}$	45	B	C	~ 45 (A)	~ 65.7 (A)
Major morphology	$D_{(002)}$	27	28	~ 20 (A)	28	27
	$D_{(310)}$	8.5	7.7	~ 6 (A)	7.5	6.5
Minor morphology	$D_{(002)}$	22	B	C	~ 19 (A)	C
	$D_{(310)}$	7.5	B	C	~ 5.6 (A)	~ 4.4 (A)

Note: A – diffraction peak is not suitable for adequate line-broadening analysis because of smearing and distortion, measurement error exceeding 30%; B – diffraction peak is very diffuse and hardly pronounced; C – diffraction peak not found

Fig. 4 Idealized model illustrating the transformation of crystallites for the two morphologies under demineralization of cortical bone. The sizes of crystallites (in nm) are represented with the data from Table 1

Figure 4 represents schematically the transformation of bioapatite crystals of different morphologies in a bone diaphysis fragment under demineralization based on the data from Table 1. Here the multiplicity of orientation of the crystals of the minor morphology on the transversal plane of bone is not taken into account.

After a 6 h-treatment in the 0.1 M HCl (or 24 h in the 0.1 M EDTA) solution, the diffraction patterns exhibit a halo at angles $2\theta \approx 20^\circ$ (Fig. 3), indicating an amorphous phase. This amorphous phase is probably not a product of apatite decomposition but the result of the interaction of the bone organic components with the demineralizing solution. To confirm this, we performed the experiments on demineralization of a bone specimen after previous pyrolytic decomposition of organic components (by annealing at 450°C for 1 h). At this temperature, the organic component of the bone was completely thermally decomposed and the halo did not arise on the diffractograms. In diffraction patterns of the longitudinally sectioned bone specimen (Fig. 3), the halo is asymmetric and shifted towards small 2θ angles. This is likely due to preferred orientation of collagen molecules and reflects cortical bone anisotropy related to collagen orientation. This may also indicate that structural properties of collagen fibrils are influenced by demineralization procedures.

It is interesting to note that in case of the major morphology, the line intensity $I_{(002)}$ decreases during demineralization faster than $I_{(310)}$. It cannot be explained by influence of the halo, because this effect is more evident in case of HCl demineralization and only scarcely pronounced in case of EDTA demineralization, while the halo for both cases is similar in shape and intensity. It probably relates to the different chemical properties of the different apatite's faces in the acidic conditions. Several studies have demonstrated that the dissolution rates along the principal crystallographic directions of apatite are rather different. It was found [34] that $\{001\}$ facets of apatite crystals in enamel are more resistant to acidic attack than the $\{100\}$. Another work [35] reported that the dissolution rates along the three principal directions of a natural apatite depend on pH; the following relationship was found:

[001] > [110] > [100] for pH = 0 – 1 but the order was reversed for pH > 3. This may explain our result: the faster destruction of the apatite crystal at a perpendicular direction to the long size (thinning) may lead to a decrease of the summarized area of the basal reflecting plans ({001} faces) and thus to reducing of corresponding line intensity. Thus, we suggest that not a proportional decrease of (002) and (310) intensities is caused by the alteration of the crystals shape (habitus) under process of demineralization.

On the other hand, the cross-sectional size of crystals $D_{(310)}$, as seen from Table 1, does not decrease as much as one would expect from the previous arguments. This parameter is statistically averaged and so the effect of the crystallite size reducing due to acidic attack can be suppressed because of total disappearance of smallest crystallites resulting in an increase of the mean size.

Some limitations of XRD examination of partially demineralized cortical bone block need to be considered in more detail. The first one is the formation of the decalcified layer on the bone surface due to the advance of the reaction front into the tissue. The layer grows with increasing immersion time of bone graft in the decalcifying agent. In our earlier publication [21] it was shown that this phenomenon induces (1st) the shift in the position of the diffraction lines due to the effect of apparent specimen displacement and (2nd) reduction in the X-ray intensity due to the absorption in the decalcified layer. Thus, the diffraction line position and intensity cannot be determined accurately. Nevertheless, the errors in the determination of the crystallite size in the bone block are not significantly increased by the decalcified surface layer formed, as was proven by additional measurements carried out to examine the effect of specimen displacement on the shape and angular position of the NaCl (200) line [21]. Certainly, we are unable to evaluate precisely the crystallite size in the depth layers of a bone sample associated exactly with the reaction front because the interface between the decalcified and the mineralized bone was found to be rather thin (about 20 μm [23]). However, it is clear that the contribution from the crystals of this interface layer to statistically averaged diffraction line width is sufficient to show the acid induced an alteration in crystallite size.

It is important to note that crystallite sizes determined from the broadening of XRD lines are not absolute but only ‘effective values’. The numerical values for the same bone fragment can vary depending on many instrumental factors and data processing conditions. Some authors have attempted to take into account the lattice microstrain contribution to the line broadening, while many authors (like we in this study) ignored this factor. That is why the data available from the references [7–9, 36] have a rather wide range. Certainly, the variety of values depends also on the biological species where the bone has been collected [37].

Moreover, line-broadening measurements in wide-angle XRD provide the information averaged across the investigated region because the X-ray probed area of the bone tissue contains many diverse structural elements (e.g., osteons, interstitial lamellae, etc.) [8, 36, 38]. Thus, the crystallite size from XRD line-broadening analysis guarantees only semi-quantitative estimation and may indicate just a general trend. Nevertheless, this parameter is very important and widely used for characterization of the microstructure of bone mineral and its evolution in real or simulated conditions [7, 17].

Noteworthy is that the considered limitations here cannot affect the main findings of this study about different dissolution rates for the crystallites of the different morphologies.

Finally, we would like to emphasize that the task of the present work was not to study the kinetics of bone demineralization. By applying the different chemical agents and immersion time, we aimed to compare the stability of crystals of different morphological types under an acidic environment (simulating the resorption process at bone remodeling). Our results

indicate that apatite crystals of the major morphology are more resistant to dissolution than the crystals of the minor morphology. This distinction in solubility may be due to different chemical modifications of apatite of the two morphological types. It is well known that the solubility behavior of apatite is strictly regulated by anionic and cationic substitutions in the lattice [2, 3, 39]. Unfortunately, there are currently no possibilities to evaluate directly the chemical differences of apatite crystals oriented along and across the long axis of cortical bone. Our preliminary attempts to identify the chemical dissimilarity of apatite crystals belonging to different morphologies by means of assessment of their lattice parameters (a and c) did not provide us with clear and unambiguous results. Moreover, a precise determination of the lattice parameters is beyond the scope of the present work. On the other hand, in vitro dissolution of apatite in bone may also be dependent on local fluid convection, the surface area in contact with fluids, and thus can be influenced by spatial arrangement of the crystals into a collagen matrix. Perhaps, these two assumed causes may have a combined effect. To clarify this point further, investigation should be performed.

The variable solubility and thus implied chemical distinction of dual-oriented apatite crystals may play an important role in the maintenance of functional properties of living bone exposed to different negative external factors like microgravitation or immobilization of a biological object.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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