# Atomic force microscopic studies on the structure of bovine femoral cortical bone at the collagen fibril-mineral level

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The structure of cortical bone at the collagen-mineral level was investigated by means of atomic force microscopy. Surfaces of the specimens treated with collagenase and ethylenediaminetetraacetic acid (EDTA) were examined. Images of blob-like objects observed in intact specimen became clearly outlined after collagenase treatment; the sizes of the blob decreased, suggesting that each blob had been fragmented by the collagenase treatment. Following EDTA treatment of an intact specimen, an image of thread-like objects appeared; the thread was partly constructed by trains of blobs and the other parts of the threads had a periodic pattern along its longer axis. The period was almost equal to the collagen D-period of the Hodge–Petruska model, indicating that the threads are collagen fibrils and that the blobs are related to the mineral phase in bone. It was concluded that minerals were deposited on and along collagen fibrils. A decorated collagen fibril model for the spatial relationship between mineral and collagen fibril was proposed. According to our model, the mineral inside the collagen fibril is about one forth of the extrafibrillar mineral.
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## Introduction

Despite many years of study by many talented investigators, the details of the ultrastructure of mineralized tissue are still poorly understood. Controversy remains over (1) the shape of bone mineral particles [1–5], (2) the locus of the particles with respect to collagen fibrils [6, 7], and (3) the portions of minerals inside and outside the fibril [8–11]. These three issues are much interrelated. In order to try to clarify these issues, Tao *et al*. investigated the ultrastructure of bone by means of atomic force microscopy (AFM) [12]. It has been proved informative to examine AFM images obtained from materials previously well investigated by more traditional techniques such as transmission electron microscopy (TEM) and X-ray diffraction. In high magnification atomic force micrographs, Tao *et al*. found blob-like objects of various sizes ranging from 50 to 500 nm in diameter [12]. This range of blob sizes is similar to that in scanning electron microscopic images reported by Turner and Jenkins [2]. The rigidity of the blobs was estimated to be 0.7–0.9 GPa for the interior of the blobs and more than 10 GPa for the inter-blob regions. Information on these sizes was considered to be closely related to the three unsolved issues listed above. The mechanism by which the blob was constructed, however, was not revealed.

The aim of this investigation was to obtain conclusive information about the structure of bone at the collagen fibril-mineral level. We considered that knowledge of the structure of the blob-like objects observed by Turner and Jenkins [2] and Tao *et al*. [12] in bone would contribute to the understanding of bone morphology. We used AFM to observe bone surfaces prepared by two types of treatment: treatment with collagenase, which was expected to eliminate collagen fibrils from the surface of the specimen, and treatment with ethylenediaminetetraacetic acid (EDTA) for the elimination of minerals from the surface. From the obtained images, we examined the spatial relationship between mineral particles and collagen fibrils in cortical bone.

## Experimental

The bone samples used in this study were obtained from the mid-diaphysis of 20-month-old bovine femora. Optical microscopic examination showed that all of the samples were generally plexiform bone but were partly remodeled into the Haversian system. Surfaces perpendicular and parallel to the bone axis were examined. Specimens parallel to the bone axis were cut from a radial rectangular plate of 100 mm (length) × 15 mm

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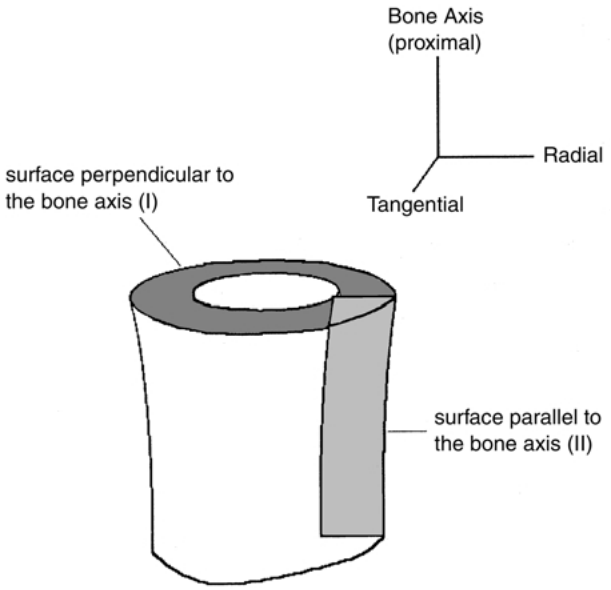


*Figure 1* Surfaces examined by AFM. The surface perpendicular to the bone axis was referred to as I and that parallel to the bone axis as II.

(width) × 0.5 mm (thickness), and those perpendicular to the bone axis were prepared from the plate cut in round slices as shown in Fig. 1. Twenty specimens were prepared for AFM observation; ten of these specimens had surfaces parallel to the bone axis and the other 10 specimens had surfaces perpendicular to the bone axis. Specimens were shaped using emery paper under tap water, and final polishing was carefully done using apatite paste. The average size of the specimens was $10 \times 10 \times 0.5\,mm^3$. The surface of the specimen thus polished was assessed using an optical microscope if there were scratches on the surface caused by the polishing. As well as the treatment for intact specimens, two types of treatment were also performed on the surface: collagenase treatment and EDTA treatment. Collagenase was purchased from Sigma Chemicals. Treatment was performed in 100 µg/ml of collagenase aqueous solution controlled at pH 7.0 with a buffer at 37 °C for five days and 12 days. The solution was changed every three days. EDTA treatment was performed in 0.5 M EDTA aqueous solution at pH 8.0 at 37 °C. Treatment was conducted for 4, 8, and 12 h. After the treatment, bone specimens were washed in distilled water for 3 h. Before the AFM measurement, each specimen was washed in an ultrasonic bath. The specimen codes and treatments are shown in Table I. The

TABLE I Bovine femoral bone specimens for AFM measurements

| Specimen code | Surface | Treatment |
|---|---|---|
| NI | ⊥ (perpendicular to the bone axis) | — |
| NII | // (parallel to the bone axis) | — |
| CI5D | ⊥ | collagenase, 5 days |
| CII5D | | collagenase, 5 days |
| CI12D | ⊥ | collagenase, 12 days |
| CII12D | | collagenase, 12 days |
| EDI4H | ⊥ | EDTA, 4 h |
| EDII4H | | EDTA, 4 h |
| EDI8H | ⊥ | EDTA, 8 h |
| EDII8H | | EDTA, 8 h |

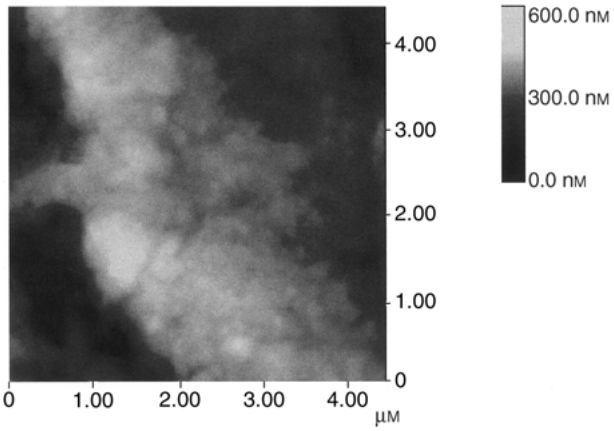


*Figure 2* AFM image of the surface of untreated bone perpendicular to the bone axis (NI).

AFM measurements were performed using a Nanoscope III AFM from Digital Instruments Inc. Images were obtained by the constant force method in air. Care was taken to ensure that the specimens were always in a wet state.

## Results

Images of EDI4H, EDI8H, and EDII8H were not obtained. These specimens were too pliant for AFM images to be obtained by contact mode observation. Another measurement method, such as tapping mode observation, is considered to be useful for these specimens, and such experiments are now in progress.

### Image of a surface perpendicular to the bone axis

The surface perpendicular to the bone axis corresponds to that examined by Tao *et al*. [12] using AFM. Fig. 2 shows an AFM image for NI. The image shows part of lamellar structure. The cave-like lower (dark) area is considered to be a lacuna or a canaliculus for an osteocyte, judging from the size of images. The lamellar structure was found to be composed of blob-like objects. The average diameter of the blobs is about 0.14 µm. Larger blobs in the picture seem to have been made from two or three smaller blobs of average size. Judging from the range of

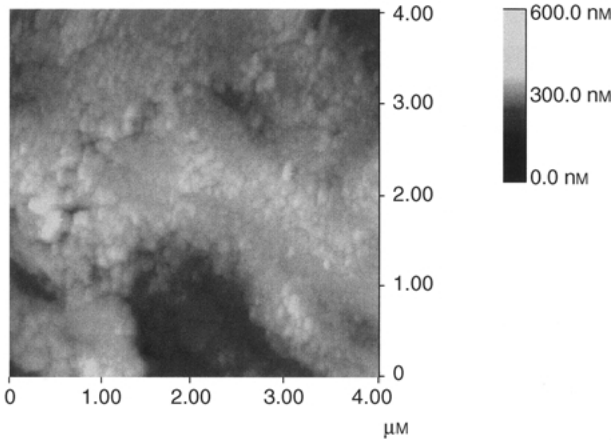


*Figure 3* AFM image of the surface of a collagenase-treated specimen (CI12D). The surface is perpendicular to the bone axis. Collagenase digestion was performed for 12 days.

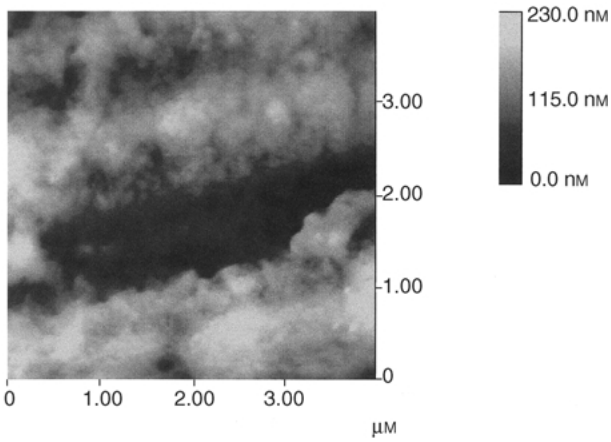


*Figure 4* AFM image of the surface of untreated bone parallel to the bone axis (NII).

sizes observed here, the blob-like objects are thought to be the same as those observed by Tao *et al*. [12].

Images of the CI5D specimens, which had been treated with collagenase for 5 days, were not different from those of intact specimens. Fig. 3 shows an image of the surface of CI12D, treated with collagenase for 12 days. The lamellar structure is partly observed in the image. The dark area is also thought to be a lacuna or a canaliculus for an osteocyte. Images of blob-like objects in intact specimen became clearly outlined after the collagenase treatment; the sizes of the blobs became smaller, suggesting that the blobs had been fragmented by the treatment. In the case of intact bone, the blob-like objects seemed to be connected to each other. In the image in Fig. 3, the blobs appear individually and there seems to be some space between adjacent blob-like objects. A careful inspection of the pictures shows that the blobs seem to be arranged along lines.

## Image of a surface parallel to the bone axis

Fig. 4 shows an AFM image of an intact surface that is parallel to the bone axis (NII). Blob-like objects were observed on the surface as they were in the image shown in Fig. 2 (NI). The average size of the blobs was 0.12 μm, which is similar to the average size of the blobs observed on the NI surface, which is perpendicular to the bone axis. From a simple geometrical consideration, the blob-like objects observed on the surface parallel to the bone axis are regarded as the side-view image of the blobs in the image shown in Fig. 2. Fig. 5 shows an AFM image of the surface treated by collagenase for 12 days (CII12D). Just like the AFM image of CI12D shown in Fig. 2, the blobs observed in the image of collagenase-treated surface of CII12D were reduced in size as compared with that in the intact specimen.

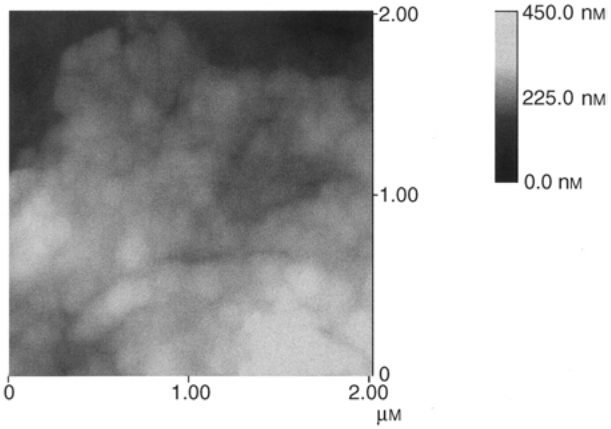


*Figure 5* AFM image of the surface of a collagenase-treated specimen (CII12D). The surface is parallel to the bone axis. Collagenase digestion was performed for 12 days.

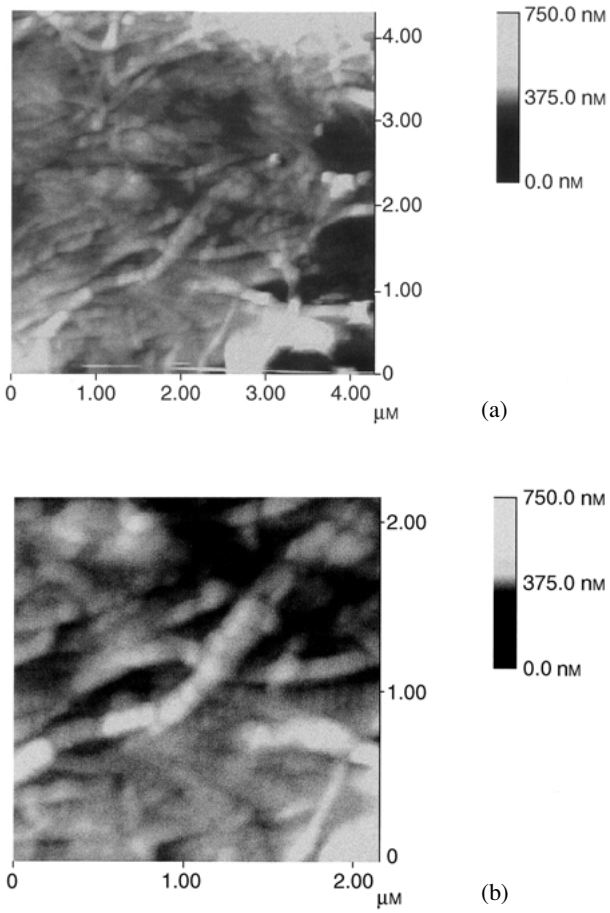


*Figure 6* (a) AFM image of the surface of an EDTA-treated specimen (EDII4H). The surface is parallel to the bone axis. (b) Magnified image of the central area of Fig. 6(a).

Fig. 6(a) shows an AFM image of EDII4H, the EDTA-treated surface. Following EDTA treatment, thread-like images that were made of trains of the blob-like objects appeared. This image is similar to an AFM image of a demineralized turkey leg tendon presented by Lees *et al*. [9]. The orientation of the threads is almost uniaxial, along the bone-axis direction. The magnified image in Fig. 6(b) shows that each thread has a periodic pattern along its longer axis. Along the threads, there are two types of periodic patterns; a period along the threads with well-contrasted image and that with insufficiently contrasted image. The periods of the two types of image were different. The average value of the period for well-contrasted image was 0.12 μm and that for less-contrasted image was 67 nm.

# Discussion

## Size of blobs and collagen fibrils

The average diameter of blobs observed in intact specimens (both NI and NII) was 0.12–0.14 μm. The size and shape of the blobs changed following surface

treatments with collagenase and EDTA. After the collagenase treatment, the blobs in the perpendicular and parallel surface images were reduced in size, as if they had been fragmented. Treatment of a bone surface with collagenase is known to eliminate collagen fibers from the bone surface but leave the minerals almost unchanged. The blob-like objects that remained after collagenase treatment are therefore thought to be composed of minerals. EDTA treatment, on the other hand, is expected to eliminate minerals in bone and collagen fibers almost unchanged. The period of 67 nm observed for the EDTA-treated specimen (EDII4H, Fig. 6) was very similar to the bone collagen D-period of the Hodge–Petruska model [13, 14], indicating that the threads are collagen fibrils. The width of the threads was about 0.11 μm, which accords well with that of the model [15] for collagen fibril in bone. The well-contrasted part with a period of about 0.12 μm on the thread in Fig. 6(a) and (b) is something precipitated on the collagen fibril thread. The width of the object was also about 0.14 μm. The average diameter of the blobs in intact specimens was 0.14 μm. These same values indicate that the structure of the collagen fibril with well-contrasted objects on it is the same as that observed as blobs in NI and NII. Some of the objects and/or the blobs disappeared following EDTA treatment. These objects were therefore thought to be mineral particles. Following EDTA treatment for 4 h, destruction of minerals was insufficient, and some mineral units remained on the collagen fibril, which were observed as the well-contrasted objects. Though we do not have any idea why the blob had the period of about 0.12 μm along the collagen fibril, the period may originate from the D-period of collagen. The blob size and the period relating to the collagen fibril determined from AFM images are listed in Table II.

## Mineral-collagen arrangement

Comparison of blob and collagen fibril sizes listed in Table II suggested that the spatial relationship between collagen fibrils and mineral particles is as follows (see Fig. 7).

1. Each collagen fibril with a diameter of 0.11 μm has a crust of about 0.01–0.02 μm in thickness made of minerals (extrafibrillar minerals).

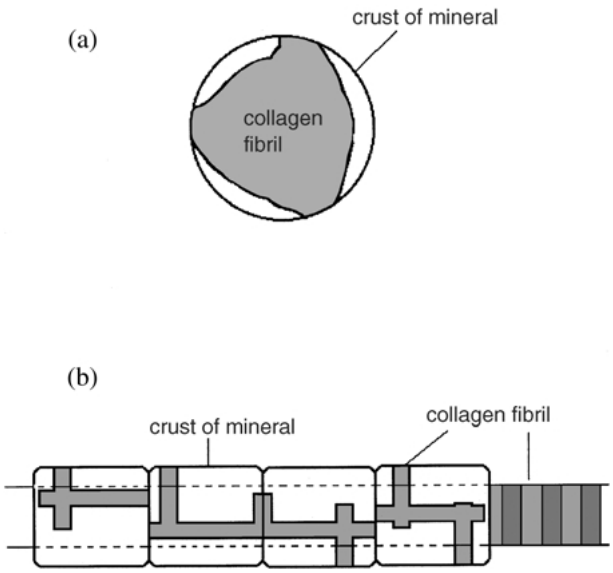


*Figure 7* Schematic drawings of the mineral-collagen fibril arrangement in bone, (a) cross-section of a blob and (b) side-view of a train of blobs. Collagen fibril contains mineral particles inside.

2. The crust is usually divided into a few pieces by intercalation of collagen microfibrils.
3. Collagen fibril inside the mineral crust still contains mineral particles (intrafibrillar minerals).

Point (2) was deduced from images for CI12D and CII12D. According to Tao *et al*. [12], the modulus inside the blob was much smaller than that among the blobs. Though it is difficult to estimate the modulus value of an object by contact-mode measurement, the model presented above can explain the modulus distribution observed by Tao *et al*. [12]; in NI, the surface corresponding to Tao *et al*. [12], inside the blob is a collagen-rich area and outside the fibrillar cylinder there are mineral-rich parts, where the modulus of the former is much smaller than that of the latter.

Minerals in the collagen fibril (collagen-rich part) are so-called intrafibrillar minerals and those outside the fibril are so-called extrafibrillar minerals. We can determine the ratio of intrafibrillar minerals to extrafibrillar minerals using the model presented here. Assuming that 80% of the crust is mineral and 20% is collagen fibril (values chosen on the basis of a comparison of Figs 2 and 3), the ratio of minerals inside the fibril was estimated as follows:

TABLE II The size and aspect ratio of the blob, collagen fiber diameter, and periods along the fiber. All figures are described as in the form of average ± standard error

| Specimen code | Blob size[a](μm) | Aspect ratio[b] | Collagen fiber diameter (μm) | Period along the fiber (μm) | |
|---|---|---|---|---|---|
| NI | 0.14 ± 0.01 | 1.0 ± 0.1 | | | |
| NII | 0.12 ± 0.01 | 0.97 ± 0.02 | | | |
| CI5D | 0.141 ± 0.003 | 1.12 ± 0.02 | | | |
| CII5D | 0.143 ± 0.003 | 1.00 ± 0.02 | | | |
| CI12D | 0.076 ± 0.002 | 0.63 ± 0.01 | | | |
| CII12D | 0.097 ± 0.003 | 0.83 ± 0.03 | | | |
| EDII4H | | | 0.11 ± 0.01 | 0.067 ± 0.002[c] | 0.12 ± 0.01[d] |

[a]Diameter value of the horizontal direction of each image.
[b]Horizontal diameter/vertical diameter.
[c]Less-contrasted image in Fig. 6(b).
[d]Well-contrasted image in Fig. 6(b).

Volume of crust $V_c = \pi[(0.14/2)^2 - (0.11/2)^2]h$
Volume of mineral in the crust $m_c = 0.8V_c$
Volume of fibril intercalating in the crust $f_c = 0.2V_c$
Volume of mineral in the collagen fibril

$$m_f = [f_c + \pi(0.11/2)^2 h]x$$

Volume fraction of mineral in bone

$$0.4 = (m_c + m_f)/\pi(0.14/2)^2 h$$

Here, $x$ is the fraction of mineral inside the fibril to the fibril volume, and $h$ is the length of the blob along the collagen fibril. Blob diameter of 0.14 μm and collagen fibril diameter of 0.11 μm were used. As for the volume fraction of mineral, the value of 0.4 was used in this estimation. We obtained $x = 0.14$,

$$m_c : m_f = 0.8V_c : [0.2V_c + \pi(0.11/2)^2 h]x \sim 4.7 : 1.4$$

About 77% of the mineral is estimated to be outside the fibril and 23% of the mineral is estimated to be inside the fibril. The ratio of intrafibrillar mineral to the total amount of mineral in bone has been reported to be 70–80% by Katz and Li [8] and 70% by Sasaki and Sudoh [11]. Katz and Li [8] and Sasaki and Sudoh [11] determined these values by the X-ray diffraction method. Mineral particles of the size observed by AFM as crusts of collagen fibrils, however, are not detectable by the X-ray diffraction. Lees *et al*. [9], using an electron micrographic method, proposed that 70–75% of the mineral is extrafibrillar and 30–25% is within the fibril. Pidaparti *et al*. [10] stated on the basis of the macroscopic observation by Bonar *et al*. [16] that only 25% of the mineral is inside the collagen fibril in bone. These values are similar to the percentages estimated on the basis of the model presented in this paper.

## Conclusions

Based on a comparison of AFM images of intact, collagenase-treated and EDTA-treated surfaces of bovine femoral cortical bone specimens, a model for the arrangement of extrafibrillar mineral around collagen fibrils was proposed. The fraction of extrafibrillar mineral to the total amount of mineral in cortical bone was estimated using this model. It was estimated that 77% of the mineral is outside the collagen fibril. This value is similar to those reported in the literature, indicating that the model presented here is valid.

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