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Connective tissue research

Relationship between nanoscale mineral properties and calcein labeling in mineralizing bone surfaces

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Abstract

Bone's mineral properties, such as particle thickness and degree of alignment have been associated with bone quality. Bone formation, remodeling, aging of the tissue and mineral homeostasis influence mineral particle properties leading to specific patterns across bone. Scanning small angle X-ray scattering (sSAXS) with synchrotron radiation is a powerful tool, which allows us to study bone's nanoscale mineral properties in a position-resolved way. We used sSAXS, fluorescence light microscopy and backscattered electron (BSE) imaging to study bone's mineral properties at the tibial midshaft of in vivo-loaded mice. By combining these techniques, we could detect local changes in mineral properties. Regions labeled with calcein fluorochrome have lower mean mineral thickness and degree of mineral alignment. We also observed thinner and less aligned mineral particles near blood vessels. We conclude that mineral properties (i) are altered by fluorochrome labeling and (ii) depend on the proximity to blood vessels.

Keywords

Bone mineral, calcein, mice, synchrotron sSAXS

Introduction

Bone has a hierarchical structure defined at the nanometer- scale as a composite material constituted by a collagen-rich organic matrix in which mineral crystals with a thickness of only a few nanometers are embedded (1,2). The mineral phase is known to act as an ion reservoir and to confer stiffness and hardness to the bone (3). Bone's material quality is closely associated with the mineral crystal properties, such as size and arrangement (2).

Some studies indicate that mineral crystal size increases with aging (4). Newly formed tissue can be identified based on fluorochrome labeling, since these agents selectively bind to sites of new mineralized tissue formation, and are incorporated into the mineralization front. Although this technique has been routinely used for decades, it remains unclear why this selective binding occurs, although some studies suggest that this is due to the smaller crystal size formed in the initial phase of mineralization, in comparison with older mineralizing sites (5).

We previously studied the bone's adaptive response to in vivo tibial loading in female mice (6). These mice were given calcein to label new tissue formed in response to loading and growth. Our aim in the present study was to investigate mineral particle properties of this new tissue by using various high-resolution methods. Synchrotron sSAXS (1mm monochromatic X-ray beam) was combined with fluorescence and BSE imaging, to analyze mean mineral thickness and degree of alignment.

Methods

In vivo loading and sample preparation

Female 10, 26 and 78 week old C57Bl/6J mice underwent in vivo cyclic compressive loading of the left tibia

(1200 μe) as previously described (6), their right tibia was an internal control ($n=2/\text{age}$). Calcein was given via intraperitoneal injection, 12 and 3 d. before euthanasia. Both tibiae were fixed in 100% ethanol, embedded in polymethyl methacrylate and sectioned using a microtome (Leica SM2500S; knife: Leica VMH 400, Nussloch, Germany). One, 10 μm thick longitudinal section from each tibia was analyzed using BSE and sSAXS. Animal experiments were approved by the local legal animal welfare representative (LAGeSo Berlin, G0333/09).

sSAXS data acquisition, correction and analysis

Prior to sSAXS measurements, samples were imaged with fluorescence microscopy (Leica DMRB, Munich, Germany) to identify calcein labeled regions, which enabled us to distinguish between newly formed and mature bone (Figure 1a). A region of the tibial midshaft (125–300 μm x 30– 77.5 μm) was examined by sSAXS at the Nanofocus beamline (ID 13) of the European Synchrotron Radiation Facility (ESRF). A monochromatic high energy X-ray beam with a wavelength of 0.0812nm ($E=15\text{keV}$) and a diameter of 1mm was used to scan samples, with a step size of 1 μm within the plane perpendicular to the incident beam. The sSAXS patterns were captured with an ESRF FReLoN detector (pixel size: 51.7 μm x 51.5 μm , exposure time: 0.6 to 0.8 s). Up to 28 700 sSAXS intensity patterns were collected per sample. All 2D sSAXS patterns were corrected for dark current (CCD readout noise) and for incoming beam intensity fluctuations from ESRF source. 2D sSAXS patterns were radially and azimuthally integrated to obtain $I(q)$ and $I(X)$, respectively with custom-made software (Autofit, MPI-CI, Potsdam, Germany), with I being the scattering intensity, q the scattering length and X the azimuthal angle. From the resulting sSAXS intensity function $I(q)$ and based on Porod's law, the T parameter, the average mineral particle thickness in the scattering volume (Figure 1c), was derived (7). The function $I(X)$ was used to calculate ρ parameter, the degree of mineral particle alignment within the plane perpendicular to the primary beam. This parameter varies between 0 and 1 (7) (Figure 1b).

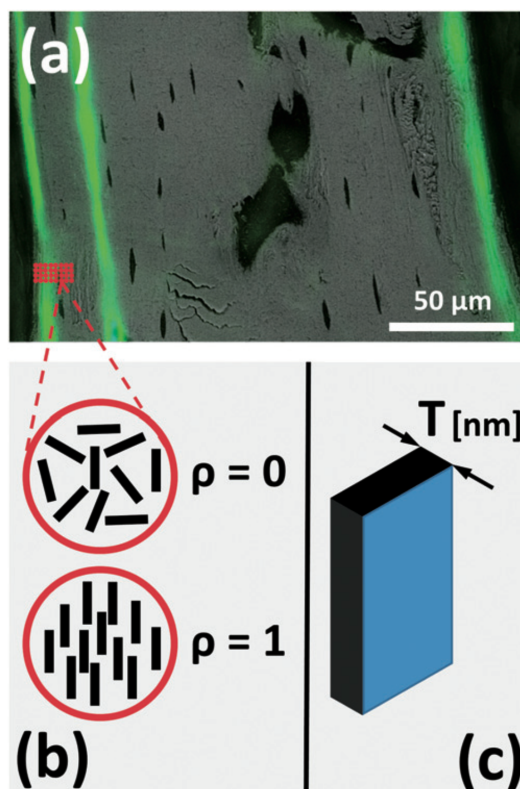


Figure 1. (a) Merged BSE and fluorescence image of tibial midshaft; (b) ρ parameter varies between 0 (randomly oriented mineral particles) and 1 (perfectly aligned mineral particles); (c) T parameter corresponds to mean mineral thickness.

BSE imaging

After sSAXS measurements and to visualize the degree of mineralization (mineral content) within the bone, the entire longitudinal section of each tibia was imaged with an environmental scanning electron microscope (FEI FEG- ESEM Quanta 600, Hillsboro, OR) in BSE mode (2) under low vacuum (0.8 Torr). An operating

voltage of 12.5 kV and working distance of 10 mm was used. Images collected using these multiple methods were overlaid onto each other to correlate the degree of mineralization and calcein label location (Figure 1a) with sSAXS maps of mean mineral thickness (Figure 2e) and degree of mineral alignment (Figure 2f) from the same regions.

Results

Areas labeled with calcein had lower T and ρ parameters

Calcein-labeled regions within the bone corresponded to regions of lower mean mineral thickness and degree of mineral alignment (Figure 2a–d). When plotting changes in T and ρ parameters along the first 30 μm from the bone surface, we detected a peak of low T and ρ at approximately 30, 15 and 8 μm in tibia from 10, 26 (Figure 2a and b) and 78 week old (Figure 2c and d) mice, respectively, which corresponded to the calcein label location. Correlating T and ρ maps with fluorescence microscopy images also indicated that this pattern corresponded to the location of the calcein label itself. One tibia from a 10 week old mouse with a diffuse calcein label did not adhere to this same pattern.

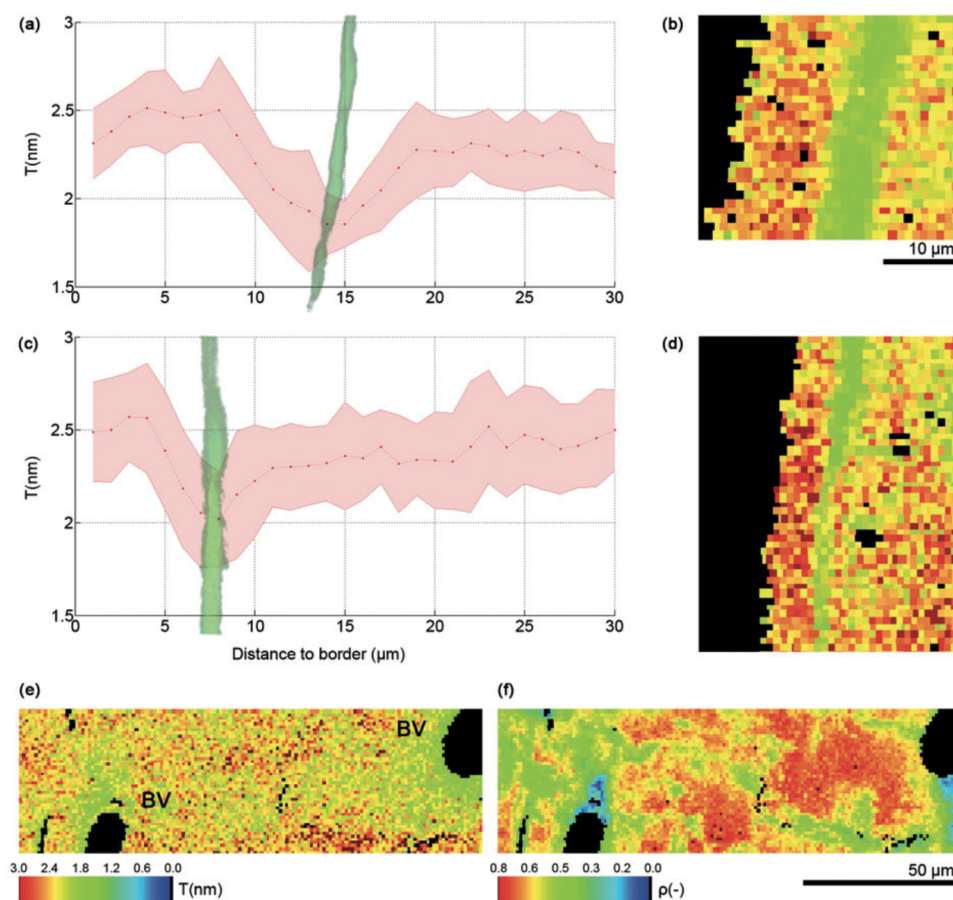


Figure 2. Graph of T parameter profile 30 μm through cortex starting at periosteal border in a loaded tibiae of a (a) 26 week old and (c) 78 week old mouse, the red line in the graphs corresponds to the average T parameter, while the shadowed pink area above and below the red line represents the standard deviation; (b) (d) T parameter map of first 30 μm of the tibial section; Lower T (e) and ρ parameter (f) near blood vessels in control tibia of a 10 week old mouse. BV, blood vessel.

Mineral particles around blood vessels have lower T and ρ parameters

We observed that the regions surrounding blood vessels corresponded to areas with lower mean mineral particle thickness (Figure 2e) and lower degree of alignment of mineral particles (Figure 2f). This pattern could be observed around all the scanned blood vessels and for mice of all ages: 10 (3 blood vessels), 26 (1 blood vessel) and 78 (2 blood vessels) week old mice.

Discussion

Our results showed lower mean mineral thickness and degree of mineral particle alignment where calcein fluorochrome labels were located. A study in rats labeled with different fluorochromes also showed a decrease in the degree of mineral alignment, but not in mean mineral thickness on the border between new and old bone (8). However, an in vitro study showed a direct relationship between alizarin red concentration and apatite like crystal size and growth kinetics (9). Our results indicate that the calcein might not only bind to mineralizing surfaces, but also interfere with mineral growth. The calcein may act in a similar manner as noncollagenous proteins in governing mineral growth. Interestingly, BSE imaging on selected samples did not show different mineral content at the calcein label sites (Figure 1a), but this would need to be studied further using quantitative methods such as quantitative backscattered electron imaging (qBEI) (2). We observed that the regions surrounding blood vessels have thinner and less aligned mineral particles. In contrast to our findings at the calcein label location, the presence of thinner and less aligned mineral particles near the blood vessels was likely mainly related to the age of this tissue rather than alterations in crystal growth.

Due to the high resolution and time restrictions during synchrotron measurements, our sample size was limited and thus further studies examining a larger number of mice are needed to confirm these results. Also, studies examining human bone biopsies have shown different fluorochromes have different binding affinities, so other fluorochromes should be studied, including tetracycline which is used clinically (10).

Conclusion

Using a multi-method approach, we have shown that the mean mineral particle thickness and degree of alignment were lower in the areas where particles were bound with calcein labeling. These findings suggest that fluorochrome labeling might not only bind to mineralizing surfaces, but also alters mineral properties, including stunting their growth, and thus affect bone mineral quality. We also showed that thinner and less aligned particles were present near blood vessels, indicating this tissue is younger.

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Declaration of interest

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