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Deformation regimes of collagen fibrils in cortical bone revealed by *in situ* morphology and elastic modulus observations under mechanical loading

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ABSTRACT

The mechanical properties of the bone play a decisive role in the resistance of the bone to fracture. Clinically, the quantity of the bone in the mineral phase has been considered as the gold-standard indicator for the risk of bone fracture. However, the bone is a complex tissue with a hierarchical-structure consisting of organic matrix, mineral hydroxyapatite, and water. Collagen comprises up to 90% of the organic matrix in the bone, and is vital for its mechanical behavior. To date, the morphological and mechanical responses of collagen fibrils in the bone matrix have been largely overlooked. In the present study, an atomic force microscopy-based imaging and indentation approach is introduced and integrated with a tibia axial loading model. The morphology of mineralized Type I collagen fibrils of the murine cortical tibia is imaged after demineralization, and the *in situ* elastic modulus of the fibrils is quantified at different loading conditions. Results suggested that the mineralized collagen fibrils are stretched in the early phase of bone deformation, characterized by the elongation of the D-periodic spacing. Reorientation of the collagen fibrils is demonstrated in the subsequent phase of bone deformation. The *in situ* radial elastic modulus of the collagen fibrils remained constant under the tested loading conditions. These experimental findings provide evidence in support of the unique deformation regimes of bone tissue from the perspective of alterations of mineralized collagen fibrils. This study allows the understanding of the unique mechanical behavior of the bone at the nanoscale, and reveals the mechanisms of relevant diseases that impair the mechanical properties of the bone.

1. Introduction

The bone is a complex material with extraordinary properties of strength and toughness. These remarkable mechanical properties of bone are maintained by the bone's functional adaptation to mechanical loading during daily life. These mechanical properties also play a decisive role in its innate ability of resist fracture. Clinically, bone mineral density (BMD) has been considered as the gold-standard indicator of bone fracture risk. However, in addition to the mineral phase of bone, there are other parameters that determine bone quality. In fact, it has been shown that both the composition and the hierarchical structure of the bone greatly contribute to its mechanical properties (Tertuliano and Greer, 2016).

Bone tissue has a unique hierarchical-structure. At the macroscopic scale, it is composed of two different types of osseous tissues, namely, cortical and trabecular bone. The cortical and trabecular bone types are normally formed in a lamellar pattern, which is primarily consisted of the mineralized collagen fibril (MCFs) bundles or fibers. At the microscopic scale, MCFs have diameters of the order of 100 nm, are approximately 100 μm long, and are consisted of collagen fibrils and mineral hydroxyapatite (HAP). HAP can either deposit in the space surrounding the fibrils or in the gap regions of the adjacent fibrils. The

Abbreviations: BMD, bone mineral density; HAP, hydroxyapatite; AFM, atomic force microscopy; CDF, cumulative distribution function; K-S test, Kolmogorov-Smirnov test

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typical size of HAP is approximately $3 \times 25 \times 50$ nm^3. Collagen is also one of the essential components of this highly hierarchical structure, composing up to 90% of the organic matrix in a bone, and it is vital to its mechanical behavior (Garnero, 2015). Previous studies have shown that the morphology of collagen in bone matrix is closely related to the mechanical integrity of the bone, and it is altered by pathological changes of bone, such as osteoporosis (Silva et al., 2006) or dysfunction of bone cells (Hammond et al., 2016). Likewise, the maturity of the collagen cross-link also significantly correlates with the fracture toughness of the bone (McNerny et al., 2015).

Since collagen fibrils are the main non-mineral consistent of a bone, they deform in tension, and bear the mechanical load by transferring developed stress to the adjacent fibrils (Gupta et al., 2006). However, it is still challenging to understand the morphological and mechanical responses of the MCFs in bone matrix in response to external loads, and resist tensional stress during load-bearing states (Depalle et al., 2016; Gupta et al., 2006; Nair et al., 2013). Specifically, the mechanical properties of the mineralized collagen in the bone at the fibrillar level are expected to contribute significantly to its overall mechanical behavior of bone. Sophisticated approaches, such as nanoindentation with atomic force microscopy (AFM) (Hang and Barber, 2011) and micro-eletromechanical systems (Shen et al., 2011), or more advanced approaches, such as small-angle X-ray scattering (Gupta et al., 2006), have been adopted to quantify the mechanical properties of single collagen fibril to understand the contribution of collagen to the overall mechanical properties of bone. While the elicited results from these studies have been informative, these techniques have generally either evaluated the mechanical behavior of collagen in the form of isolated fibrils rather than the mechanical response *in situ* or required sophisticated sample preparations.

In this study, we presented a direct experimental quantification of the *in situ* morphology and mechanical properties of the MCFs with macroscopic elastic deformation of the bone. An axial loading device was established to provide physiological-level mechanical loading on the murine tibia bone. After a demineralization process, the morphology of the MCFs in the cortical bone was imaged using AFM during bone deformation. A nanoindentation approach based on AFM is adopted to identify the *in situ* mechanical properties of the MCFs. The possible deformation mechanisms of bone at the nanoscale and the role of the MCFs on the mechanical behavior of bone are discussed.

2. Materials and methods

2.1. Bone sample preparation

Six BALB/c male mice were purchased from the Lab Animal Center of the Fourth Military Medical University (Xi'an, Shaanxi, China) at eight weeks of age, and were allowed to acclimate for two weeks prior to the study. All mice were given standard rodent chow and water *ad libitium*, and were housed with individually at room temperature and in common light/dark conditions. The entire study was approved by the Animal Ethics and Welfare Committee of the Northwestern Polytechnical University. Tibial bones extracted from both extremities from all the studied mice were harvested. The bones were cleaned to remove the adjacent soft tissue for future measurements.

2.2. Axial tibia loading model

The murine axial tibia loading model that was described previously (De Souza et al., 2005), was custom-built while the bone surface was scanned with AFM (Fig. 1A). A piezoelectric actuator (PK2FVF1, Thorlabs, NJ, USA) was used to generate the desired mechanical load on the tibia. A force sensor (L6D21, Zhonghang Electronic Measuring Instruments Co., Ltd., Hanzhong, China) was used to provide a feedback and monitor the load amplitude. The tibia was preloaded with 0.5 N to avoid loosening prior to the AFM scanning and the loading procedure

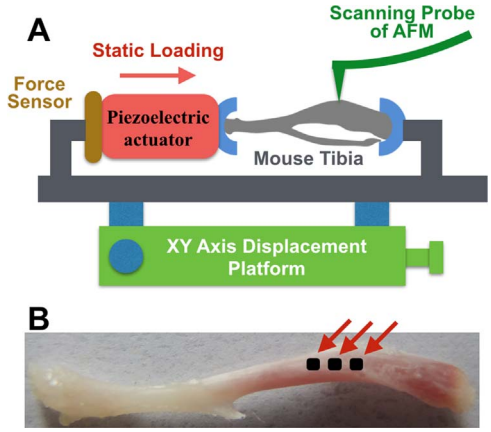

Fig. 1. Schematic representation of the mouse tibia for AFM scanning and nanoindentation. A: Setup of the axial tibia loading model for AFM scanning. B: Approximate locations of three anatomical sites on the tibial bone (indicated by the black dots) used for AFM scans.

(the preloading condition is referred to as the 0 N load state in the following text for the sake of convenience). Three progressive static mechanical loading conditions at 0 N, 3 N and 6 N, were applied to the tibia at each session of the AFM scanning. Strain distribution estimated by previously conducted finite element analyses indicated that the peak strain magnitudes on the antero-medial surfaces of tibia under a loading of 6 N ranged from 1000 to 1500 με (Sugiyama et al., 2012). These findings were also confirmed by the finite element analyses conducted in this study (Supplementary materials).

2.3. AFM image scanning

A flat surface in the antero-medial part of the tibia was chosen for AFM scans (Fig. 1B). The cortical bone at the chosen sites was polished using 7000 grit sand paper, and 2.5 μm diamond suspension and 0.5 μm diamond suspensions, in sequence. Each sample was sonicated in ultrapure water for 5 min to remove the polishing residues and debris. To remove the extra-fibrillar mineral and expose the collage fibrils, the bone was then treated with 0.5 M EDTA at a pH of 8.0 for 15 min at 30 °C, followed by rinsing in ultrapure water for 5 min. The entire demineralization procedure was repeated three times. A mechanical test showed that the adopted demineralization procedure did not significantly affect the mechanical properties of tibia (Supplementary materials).

At each loading condition mentioned above, bone samples (n = 6) were scanned in air immediately after their removal from ultrapure water using the PicoPlus 5500 AFM (Agilent, MI, USA). Images were acquired in tapping mode using a silicon scanning probe (PPP-NCL-20, resonant frequency: 146–236 kHz, force constant: 21–98 N/m, Nanosensors, USA). Images were acquired with field-of-views of 10 μm × 10 μm and 5 μm × 5 μm at a matrix size of 512 × 512 pixels from three sites on each bone sample with an intersample distance of approximately 1 mm along the long axis. The scanning speed was set at 1.2 line/s (Hammond et al., 2016; Wallace et al., 2011). The entire procedure was completed as fast as possible to maintain the hydrated condition of bone samples.

2.4. In situ elastic modulus of the MCFs

AFM was switched to contact mode following the acquisition of the morphological image of the MCFs. At each loading condition, *in situ* radial mechanical properties of the exposed MCFs were measured using the AFM-based nanoindentation approach. The same scanning probe (PPP-NCL-20, Nanosensors, USA) with an inverted pyramidal tip with 20° side angle, and the same image scanning procedure, were used to